The Role of Genetic Variation in Glucokinase and Glucokinase Regulatory Protein in Diabetes and Related Traits

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Memorandum

The work in this thesis is the original work of the author. Experiments were carried out at the Oxford Centre for Diabetes Endocrinology and Metabolism, University of Oxford, under the supervision of Dr Anna Gloyn and Prof Patrik Rorsman. Funding was provided by the Medical Research Council.

The cellular work in this thesis formed a minor part of an existing study aimed at characterising rare glucokinase regulatory protein variants identified in individuals from the on-going ClinSeq program (Biesecker, et al., 2009; Rees, et al., 2010). This work was performed in collaboration with Matthew Rees (University of Oxford, UK) and Prof Francis Collins (National Institutes of Health, Maryland, USA).

Both the work characterising four novel glucokinase mutants (**chapter 3**) and that studying the kinetics of P446L-glucokinase regulatory protein (**chapter 4**) has been published previously, in the Journal of Biological Chemistry and Human Molecular Genetics respectively (Beer, et al., 2009; Beer, et al., 2011).

I hereby state that no part of this thesis has been submitted for any other degree at this or another university. This thesis is approximately 45,000 words.

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The Role of Genetic Variation in Glucokinase and Glucokinase Regulatory Protein in Diabetes and Related Traits

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Abstract

The rising prevalence of type 2 diabetes (T2D) is a global problem, and suggests that we need better therapeutic strategies against this disease. The glycolytic enzyme glucokinase (GCK) catalyses the phosphorylation of glucose, and is a well-established T2D drug target. Rare *GCK* mutations cause monogenic beta-cell dysfunction, whilst common genetic variants within *GCK* are associated with fasting plasma glucose (FPG) levels and T2D risk. Since *GCK* is expressed in both the pancreas and liver, pharmacological GCK activation provides the promise of a two-pronged attack on hyperglycaemia.

In vivo, GCK activity is modulated by the hepatic inhibitor glucokinase regulatory protein (GKRP, gene *GCKR*). GKRP negatively regulates GCK activity competitively with respect to glucose, and is controlled by fructose 6- and fructose 1-phosphate (F6P and F1P), which compete with each other for binding and enhance or diminish GCK inhibition respectively. GKRP also sequesters GCK in the nucleus and paradoxically stabilises the enzyme.

As GCK and its regulatory protein are fundamental to glucose homeostasis, we aimed to investigate the role of genetic variation in both *GCK* and *GCKR* to further our understanding of these important T2D drug targets in a system that would be relevant to man.

I demonstrated that two novel *GCK* mutations (T103S and V389L) identified in patients with hyperinsulinaemic hypoglycaemia were kinetically activating and through structural modelling identified a novel regulatory site for GCK activation by small molecular activators.

Genome-wide association studies (GWAS) identified *GCKR* as a regulator of FPG and triglyceride levels, and showed a role for GKRP in T2D risk. Unlike most GWAS hits, this signal included a non-synonymous variant within *GCKR* (P446L), thus facilitating functional studies. P446L-GKRP was characterised kinetically and at the cellular sequestration-level. This variant showed diminished F6P-mediated modulation, which was proposed to reduce hepatic GCK inhibition, increase glycolytic flux (decreasing FPG), and feed metabolites into liver pathways (elevating triglycerides). As *GCKR* was not expressed at functional levels within human islets, this phenotype was thought to be driven by the liver. Preliminary analysis at the cellular level was inconclusive, with optimisation required to study human P446L-GKRP in this cellular system.

Finally, I showed that mutations within *GCKR* are not a common cause of “GCK-Like” phenotypes in man, despite the regulatory protein directly modulating GCK activity.

These data provide further insight as to the pathogenic consequences of perturbing GCK activity. This must be considered if this enzyme is to be the subject of therapeutic intervention in T2D.

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List of common abbreviations

ATP – Adenosine Triphosphate

ADP – Adenosine Diphosphate

GCK – Glucokinase

G6P – Glucose 6-Phosphate

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

Glucose $S_{0.5}$ – Half Maximal Glucose Concentration

Mg-ATP – Magnesium-Adenosine Triphosphate

GKA – small Molecular Glucokinase Activator

FPG – Fasting Plasma Glucose

OGTT – Oral Glucose Tolerance Test

GSIS – Glucose-Stimulated Insulin Secretion

HH – Hyperinsulinaemic Hypoglycaemia

MODY – Maturity-Onset Diabetes of the Young

PNDM – Permanent Neonatal Diabetes Mellitus

MAF – Minor Allele Frequency

SNP – Single Nucleotide Polymorphism

T2D – Type 2 Diabetes

F1P – Fructose 1-Phosphate

F6P – Fructose 6-Phosphate

GKRP – Glucokinase Regulatory Protein

GWA – Genome Wide Association

PCR – Polymerase Chain Reaction

SDM – Site-Directed Mutagenesis

n_H – Hill Number

K_{cat} – Maximal Specific Enzyme Activity

ATP_{Km} – Half Maximal Adenosine Triphosphate Concentration

Ec_{50} – Half Maximal Stimulatory Effect Concentration

Ic_{50} – Half Maximal Inhibitory Effect Concentration

RAI – Relative Activity Index

Ki – Inhibitor Dissociation Constant

Ki_{app} – Apparent Inhibitor Dissociation Constant

Chapter 1

Introduction

1. Introduction

1.1 Glucokinase (hexokinase IV) preferentially phosphorylates glucose in vivo

The mammalian hexokinases (I-IV) are a group of enzymes which transfer a phosphate group from adenosine triphosphate (ATP) to carbon six of hexose sugars, yielding hexose 6-phosphate and adenosine diphosphate (ADP) (Wilson, 2003). These hexokinases have extensive sequence identity suggesting a common phylogenetic ancestry (Kawai, et al., 2005). However, their differential expression in mammalian tissues allows them to fulfil distinct metabolic roles (Cardenas, et al., 1998). Hexokinase IV is more commonly termed 'glucokinase' (GCK) due to its preferential metabolism of glucose under physiological conditions (Matschinsky, 2002). GCK-mediated glucose phosphorylation yields glucose 6-phosphate (G6P) which is an important metabolic intermediate that feeds into glycolysis, glycogen synthesis, and other metabolic pathways (Lynedjian, 2009). The *GCK* gene is located on chromosome 7p15.3-p15.1, and consists of twelve exons which encode a 465 amino acid protein of 52kDa (Jetton, et al., 1994). GCK is highly conserved, thus suggesting a pivotal metabolic role for this enzyme across organisms. Human GCK shares 98% identity with the rat protein (Tanizawa, et al., 1992), and is 28% identical to the yeast homologue hexokinase A (Stachelek, et al., 1986).

1.2 Glucokinase has unique biochemical properties which allow it to regulate blood glucose levels

GCK has distinctive enzymatic characteristics versus the other hexokinase isoenzymes (Matschinsky, 2002; Matschinsky, et al., 2006; Wilson, 2004). These are, firstly, a low affinity for glucose [concentration needed for half maximal GCK activity (glucose $S_{0.5}$) approximately 7.5mmol/L (Matschinsky, 2002; Matschinsky, et al., 2006; Zelent, et al., 2005)]. GCK is therefore not saturated at physiological levels of this sugar, unlike the other hexokinases whose $S_{0.5}$ values are in the sub-

millimolar range. Consequently, GCK can detect changes in blood glucose levels as may occur postprandially. It also lacks G6P-mediated product inhibition, is saturated by physiological concentrations of its second substrate magnesium (Mg)-ATP (Gloyn, et al., 2004a; Matschinsky, 2002), and displays cooperativity towards glucose despite functioning as a monomer (Lin and Neet, 1990; Meglasson, et al., 1983; Neet, 1980; Niemeyer, et al., 1975; Storer and Cornish-Bowden, 1976). Together, these properties mean GCK is uniquely placed to fulfil a pivotal role in the regulation of glucose metabolism (Matschinsky, 2002; Matschinsky, et al., 2006).

1.3 The glucokinase crystal structure provides further insight into the enzymes monomeric cooperativity

In 2004 Kamata and colleagues successfully obtained the crystal structure of GCK, providing one of the most important findings in the field to date (Kamata, et al., 2004). In this study, GCK was crystallised in the presence and absence of glucose, the former also including a small pharmacological molecular GCK activator (GKA) designated 'compound A'. Briefly, Kamata and colleagues showed that the GCK structure consisted of a large and small domain forming a palm shape, and that these domains were connected by three flexible loops in a hinge-like region. The deep cleft at the interface of these two domains in the glucose-bound (closed) conformation formed the binding site for this substrate, whilst compound A bound to a smaller pocket on the opposite side of the enzyme (figure 1.1) (Kamata, et al., 2004).

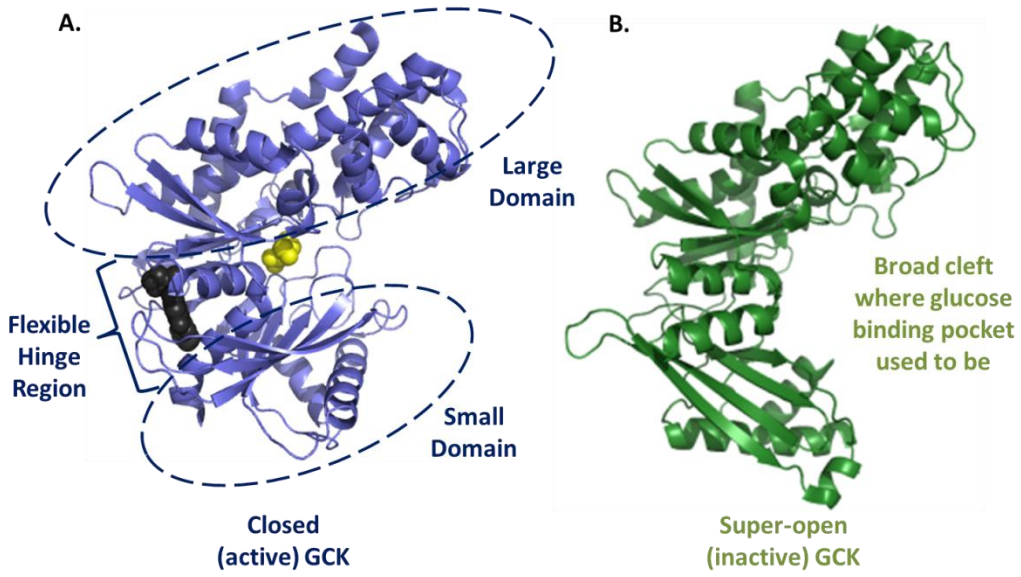


Figure 1.1A and B: Crystal structure of closed (A) and super-open (B) GCK (Kamata, et al., 2004). In the closed conformation, glucose (yellow) binds to the cleft formed between the large and small domains, whilst the small molecular GKA 'compound A' (black) binds in a discrete pocket on the opposite side of the enzyme.

Comparison of the closed structure (figure 1.1A) to that of the unbound apoenzyme which has a broadened glucose-binding cleft and no GKA pocket (figure 1.1B) suggested that transition from super-open to the closed conformation involved global rearrangement and displacement of the C-terminal small domain by ~100^{o} (Kamata, et al., 2004). These two structures were proposed to reflect low- and high-affinity glucose-binding conformations, with broadening of the glucose-binding cleft in the super-open structure displacing substrate-binding residues and reducing the enzymes affinity for this sugar (Kamata, et al., 2004; Storer and Cornish-Bowden, 1976; Storer and Cornish-Bowden, 1977). A third conformation representing the high-affinity unbound enzyme was also proposed to exist (Kamata, et al., 2004). This 'open' structure has yet to be crystallised but is anticipated to be homologous to the C-terminal domain of hexokinase I (Aleshin, et al., 1998; Kamata, et al., 2004). Kamata and colleagues subsequently proposed a three-conformation model to explain the cooperativity of GCK (figure 1.2), with varying rates of conformational change between the three structures (Kamata, et al., 2004).

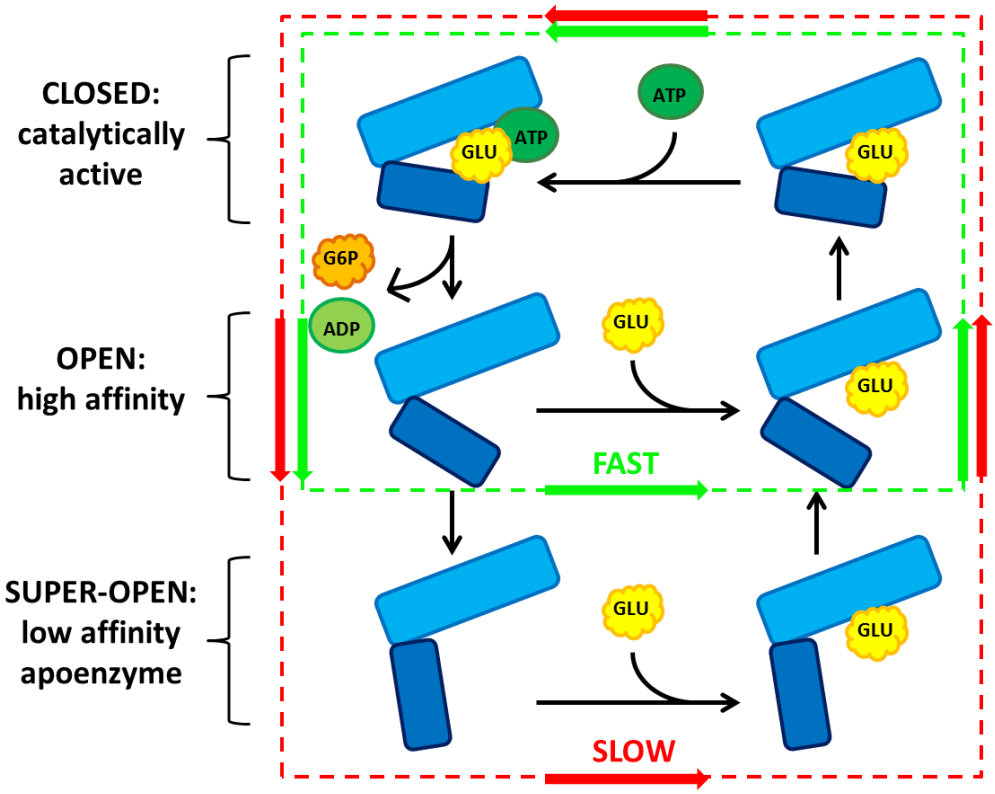


Figure 1.2: Three conformation model of GCK cooperativity. The large and small domains of this enzyme are represented by the light- and dark-blue boxes respectively. Glucose (yellow) is able to bind to both the super-open (low affinity) and open (high affinity) structures. The rate of open to closed conformational change is much quicker than that of super-open to closed, thus accounting for the biphasic kinetics and cooperativity of GCK. High glucose conditions favour the high affinity open and closed structures, whilst the low affinity super-open conformation is predominant when this substrate is absent. Figure adapted from (Kamata, et al., 2004).

1.4 Glucose homeostasis involves several metabolically-important organs and is tightly controlled by hormonal and neuronal regulatory mechanisms

Blood glucose levels are tightly regulated in both the fed and fasted states to ensure they are kept constant and within the 'normoglycaemic' range [fasting plasma glucose (FPG) ≤6.1mmol/L, and ≤7.8mmol/L two hours after an oral glucose tolerance test (OGTT) (Federation (International Diabetes), 1999; Organisation (World Health), 2006)]. This control requires the complex interaction of several key metabolic organs and numerous hormonal and neuronal regulatory mechanisms (figure 1.3) (Cherrington, 1999).

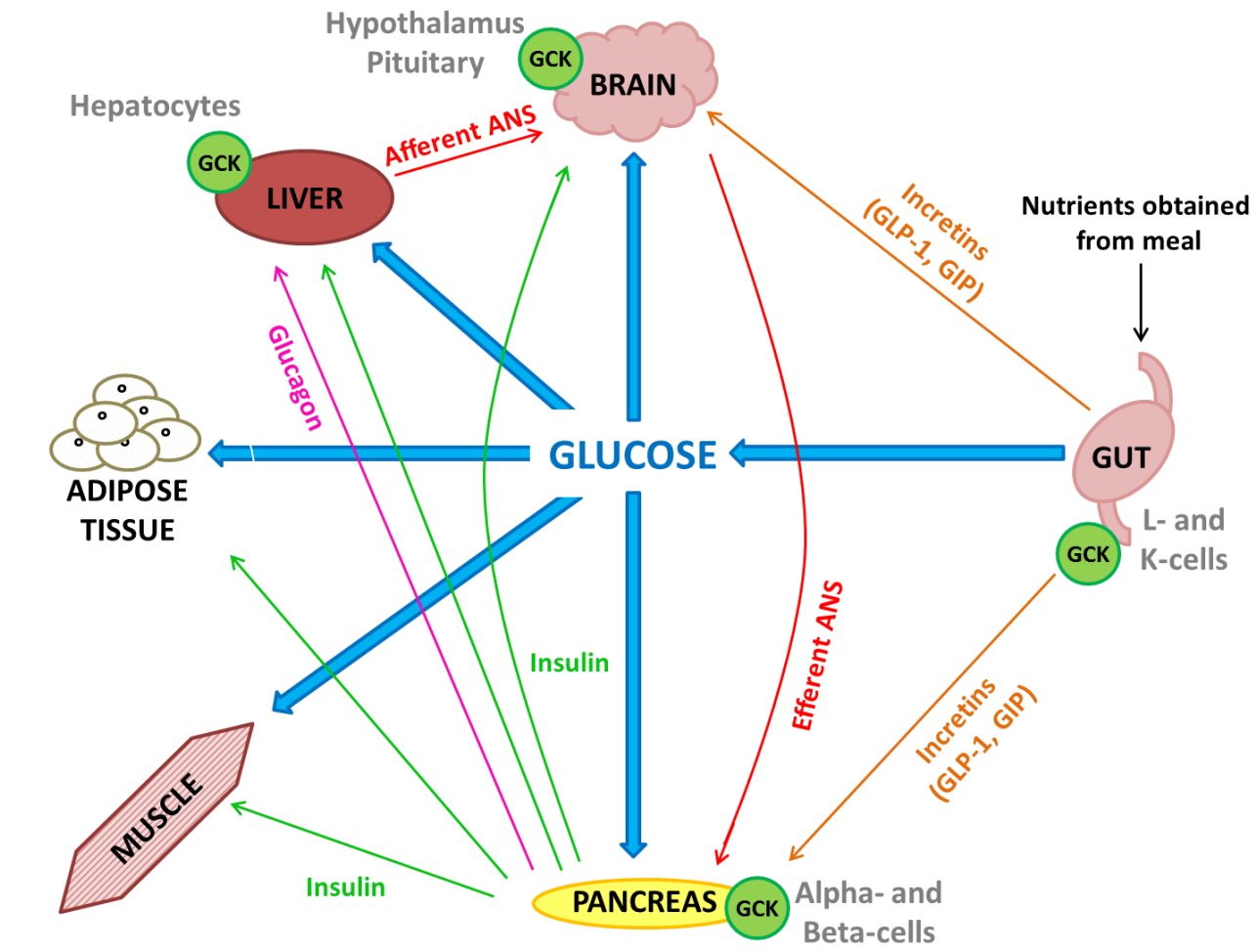


Figure 1.3: The gluco-regulatory network includes many metabolically-important organs. Glucose has a direct effect on the pancreas, liver, brain, gut, skeletal muscle, and adipose tissue. Signalling between the organs within this network can be hormonal (e.g. insulin), or neuronal (e.g. autonomic nervous system [ANS]), and is crucial in order to coordinate whole body glucose homeostasis. Figure taken and adapted from (Matschinsky, 2009; Matschinsky and Porte, 2010).

The rise in blood glucose levels that occurs postprandially or following the administration of an intravenous glucose load serves to both directly and indirectly augment glucose metabolism. Glucose itself serves as a key signalling molecule in the hepatic portal vein which transports blood from the gut and gastrointestinal tract to the liver (Langhans, et al., 2001; Mithieux, et al., 2005; Thorens and Larsen, 2004; Tordoff and Friedman, 1986; Tordoff, et al., 1989). Glucose-sensitive cells here can detect these changes in blood glucose level and signal to the brain via the vagus nerve, thus

modulating appetite and other glucose homeostatic mechanisms accordingly (Adachi, et al., 1984; Schmitt, 1973; Shimizu, et al., 1983). Consequently, hepatic glucose production (gluconeogenesis) is turned off, the liver switches from net breakdown (glycogenolysis) to net synthesis of glycogen, and the expression of crucial metabolic enzymes is up-regulated (Agius, 2008; Cherrington, 1999; Dentin, et al., 2004; Foufelle and Ferre, 2002; Rencurel, et al., 1996).

The elevation of blood glucose also serves to increase insulin and decrease glucagon secretion from pancreatic beta- and alpha-cells respectively (Cherrington, 1999; Davis, et al., 1993; Flattern, et al., 1997); these hormones having further actions on the liver and other metabolically important tissues. Therefore this integrated network including the liver, pancreas, brain, and gut, and the complex signalling between them is critical for the maintenance and regulation of glucose homeostasis (Cherrington, 1999; Lynedjian, 2009).

1.5 Distinct promoters allow tissue-specific transcriptional regulation of glucokinase within the different organs of the gluco-regulatory network

GCK has a distinct expression profile versus the other hexokinase enzymes, with high levels in the pancreatic beta-cell, liver hepatocyte (Lynedjian, et al., 1986), hypothalamus (Jetton, et al., 1994; Lynch, et al., 2000; Roncero, et al., 2004; Roncero, et al., 2000; Zelent, et al., 2006), and L- and K-cells of the gut (Jetton, et al., 1994). Recent evidence also suggests that it may be expressed within the epithelial cells of the respiratory tract and gonado- and thyrotropes of the brain pituitary (Sorenson, et al., 2007). Two distinct promoters (~30kb apart) allow GCK to fulfil tissue-specific roles, producing transcripts with different 5'-untranslated regions and alternative versions of exon 1; a, b, and c. (Bedoya, et al., 1986a; Lynedjian, et al., 1989b; Jetton, et al., 1994; Magnuson, 1990; Magnuson and Shelton, 1989). Consequently, the first 15 amino acids of these transcripts differ, however the remaining exons 2-10 are identical between the two *GCK* isoforms (Lynedjian, 2009). The

neuroendocrine isoform is expressed in the pancreas, gut, and brain, and is transcribed from the upstream promoter which encodes exon 1a. Transcription of hepatic GCK starts from the downstream promoter and includes exons 1b and c (Lynedjian, 1993; Lynedjian, et al., 1986; Lynedjian, et al., 1989b; Jetton, et al., 1994; Magnuson, 1990; Postic, et al., 2001).

Early investigation into the specific regulatory mechanisms governing transcription of hepatic *GCK* revealed a DNA fragment within the rat hepatic *Gck* promoter which stimulated luciferase activity in primary rat hepatocytes and a hepatoma-derived cell line, but not in insulinoma-derived cells (Lynedjian, et al., 1996). These experiments also identified an enhancer element upstream of the transcription start site in the rat gene (in an area homologous to the human sequence) which was only active specifically in primary hepatocytes (Lynedjian, et al., 1996). These data suggested that liver-specific cellular factors were necessary for activation of hepatic *Gck* expression (Lynedjian, 1998; Lynedjian, et al., 1996). Similar experiments looking at expression of neuroendocrine *GCK* identified an upstream promoter element which bound an approximately 50kDa protein. This protein was assumed to be beta-cell specific, as binding was only observed in mouse beta-cell-derived cell lines, and not in those from mouse pituitary, mouse pre-adipocyte, or human cervical carcinoma (Lynedjian, 2009). *In vivo* experiments in transgenic mice also confirmed a similar expression pattern, with this same upstream promoter element causing expression of the reporter gene human growth hormone in pancreas, brain, and gut (Jetton, et al., 1994). Further study showed there were two *cis*-regulatory elements in this promoter fragment, one of which bound a transcription factor important in pancreatic development called Beta2/NeuroD (Moates, et al., 2003; Naya, et al., 1995; Shelton, et al., 1992).

1.6 Hepatic glucokinase expression is responsive to nutritional status

Hepatic *GCK* transcription is different to other 'liver-specific genes' suggesting more complex regulatory mechanisms may be involved in the governance of its transcription (Lynedjian, 1993; Lynedjian, 2009; Sladek and Darnell, 1992). For example, hepatic *GCK* expression is exclusive to the mature differentiated hepatocyte, with *Gck* messenger (m)RNA only present post-weaning in rats (Lynedjian, et al., 1989a; Lynedjian, et al., 1987). Liver *GCK* mRNA also varies in accordance with nutritional status, with insulin shown to stimulate transcription of hepatic *GCK* in rat and human hepatocytes in both the presence and absence of glucose (Lynedjian, 1993; Lynedjian, et al., 1989a; Lynedjian, et al., 1995; Matsuda, et al., 1990). Further data to support this comes from insulin-deficient diabetic rats which lack hepatic *Gck* mRNA. Transcription of this gene can however be restored upon treatment of these rodents with exogenous insulin (Lynedjian, et al., 1988; Lynedjian, 2009). The administration of anti-insulin guinea pig serum to healthy rats fed on a high fat/carbohydrate free diet was also shown to ablate the induction of hepatic *Gck* transcription normally observed upon addition of glucose to their meals (Niemeyer, et al., 1967). This insulin stimulatory effect on hepatic *GCK* transcription means that levels of this enzyme can be increased rapidly in order to enhance glucose uptake by the liver as is needed during the fasted to fed transition (Agius, 2008; Lynedjian, et al., 1989a). Conversely, glucagon elicits dominant opposing effects on hepatic *GCK* transcription (Lynedjian, 1993; Lynedjian, et al., 1989a).

The transcription factors hepatocyte nuclear factor-4-alpha and -1-alpha (HNF4A and HNF1A respectively) and the sterol regulatory binding protein-1c (SREBP1c) are thought to play a role in this insulin-stimulated transcription of hepatic *GCK* (Lynedjian, 2009). Binding sites for HNF4A and -1A have been identified in the liver-specific promoter (Roth, et al., 2004; Roth, et al., 2002), with *Gck* expression increasing in rat hepatocytes in the presence of both transcription factors and the co-

activator protein p300 (Kasper, et al., 2006; Roth, et al., 2004; Vo and Goodman, 2001). The involvement of SREBP1c in insulin-induced hepatic *GCK* expression remains controversial (Lynedjian, 2009), this is despite its own transcription being insulin-responsive (Foretz, et al., 1999b), and the key role it plays in the regulation of other metabolic enzymes (Brown and Goldstein, 1997). Experiments in rat hepatocytes have shown that expression of dominant negative or dominant positive forms of SREBP1c enhance or ablate insulin-induced *Gck* expression respectively (Foretz, et al., 1999a). However, induction of *Gck* transcription is unaffected in SREBP1c knock-out mice (Liang, et al., 2002), and the ability of SREBP1c to physically bind to the hepatic *GCK* promoter remains disputed (Hansmannel, et al., 2006; Lynedjian, 2009; Stoeckman and Towle, 2002).

Epigenetic mechanisms have also been shown to regulate the expression of key metabolic and developmental genes, the most common being 'gene silencing' via promoter methylation (Brons, et al.; Chang, et al.; Jiang, et al., 2011; Jiang, et al., 2008; Maier and Olek, 2002; Park, et al., 2008; Ronn, et al., 2008). Rodent experiments have shown hepatic *Gck* expression to be regulated in this way, with hyper-methylation of the promoter occurring as the age of the rats increased (Jiang, et al., 2008), as well as in response to high fat diet-induced obesity (Jiang, et al., 2011). G6P is also thought to have an inhibitory effect on hepatic *GCK* transcription which is proposed to serve as a negative feedback mechanism on GCK activity as flux through glycolysis proceeds (Agius, 2008; van Dijk, et al., 2001).

1.7 Glucokinase activity is pivotal in the regulation of hepatic glycolytic flux and glycogen synthesis

The liver is crucial in the control of glucose homeostasis as it rapidly clears sugar from the blood in the fed state, metabolising approximately a third of our ingested glucose (Ferrannini, et al., 1985). It also uses gluconeogenesis to produce glucose in the fasted state, as well as regulating hepatic glycogen stores (Agius, 2008; Lynedjian, 2009; Massa, et al., 2011; Radziuk and Pye, 2001). Since its

first identification in the liver back in the 1960s (Sharma, et al., 1964; Sols, et al., 1964; Walker and Rao, 1964) hepatic GCK has been of great interest to metabolic researchers, particularly as the liver has the most GCK in terms of 'total amount per organ' and 'specific activity' (~99%) (Matschinsky, et al., 2006; Weinhouse, 1976). Hepatic glucose uptake is not rate-limited by the bidirectional glucose transporter (GLUT)-2 in the hepatocyte membrane, thus meaning the concentration of this sugar can equilibrate between the blood plasma and hepatocyte cytoplasm (Agius, 2008; Lynedjian, 2009; Williams, et al., 1968). As a result and due to its unique biochemical properties, GCK activity can change in response to plasma glucose levels, thus giving it near total control over glucose uptake by the liver (de la Iglesia, et al., 2000; Lynedjian, 2009; Matschinsky, 2002; Matschinsky, et al., 2006). Accordingly, over-expression of hepatic GCK yields an increase in glycolytic flux, glucose oxidation, and glycogen synthesis (Agius, et al., 1996; Seoane, et al., 1996; Takeuchi, et al., 1996).

1.8 Glucokinase is constitutively expressed within the pancreatic beta-cell

Evidence from rats shows that unlike hepatic *Gck*, the neuroendocrine isoform is constitutively expressed regardless of nutritional state (Lynedjian, 1993; Lynedjian, 2004; Lynedjian, 2009). For example, *Gck* mRNA is virtually depleted in the liver of fasted rats (Lynedjian, et al., 1987; Minderop, et al., 1987; Sharma, et al., 1963), whilst in the beta-cells of freely-fed and fasted rats it is present at comparable levels (Lynedjian, et al., 1989a; Lynedjian, 2009). Additionally, the oral administration of glucose to rats which had been starved for lengthy periods did not induce an increase in beta-cell *Gck* mRNA levels (Lynedjian, et al., 1989a). This view has been challenged in experiments where rat exocrine pancreas tissue was depleted following feeding of a copper-free diet, with the re-introduction of glucose into meals proposed to act as a nutritional stimulus for the induction of pancreatic *Gck* transcription (Tiedge and Lenzen, 1991). However, this phenomenon may have

resulted from trans-differentiation of stem cells/exocrine pancreas into hepatocytes (Lynedjian, 2009) as had been shown in previous copper-free diet experiments (Rao, et al., 1989).

GCK activity and protein levels have been shown to increase marginally in islets undergoing long-term incubation in supra-physiologically high glucose medium, although mRNA levels remain unchanged regardless of culture condition (Liang, et al., 1990; Liang, et al., 1992; Zelent, et al., 2005). Luciferase reporter gene experiments using a mouse insulinoma-cell line showed that this increased GCK activity was not via enhanced transcription (Da Silva Xavier, et al., 2004; da Silva Xavier, et al., 2000). Together these data suggested that glucose or the corresponding rise in insulin served to either enhance translation of *Gck* mRNA, or stabilise the enzyme at the protein level (Bedoya, et al., 1986a; Liang, et al., 1992; Lynedjian, 2004; Lynedjian, 2009).

Additionally, it has been shown that *GCK* expression in beta-cells is heterogeneous (Heimberg, et al., 1993; Jetton and Magnuson, 1992; Kiekens, et al., 1992; Pipeleers, et al., 1994; Van Schravendijk, et al., 1992), with this thought to explain the functional differences noted between beta-cells (Dean and Matthews, 1970; Orci, 1986; Schuit, et al., 1988). This suggests that beta-cells form sub-populations with different glucose sensitivity (Dean and Matthews, 1970; Jetton and Magnuson, 1992; Schuit, et al., 1988), a phenomenon that has been demonstrated during *in vivo* studies where groups of rat beta-cells vary in function following sulphonylurea treatment (Ling, et al., 2006; Martens, et al., 2010). Beta-cells with the greatest glucose sensitivity are also less sensitive to oxidative damage (Martens, et al., 2005; Pipeleers and Van de Winkel, 1986).

1.9 Beta-cell glucokinase activity couples fasting plasma glucose levels to insulin secretion

Early experiments suggested that insulin secretion from the pancreas rapidly increased in response to rising glucose levels (Grodsky, et al., 1967), but the mechanism behind this process remained

undetermined. It was subsequently shown that islet glucose concentration equilibrated with that of blood, and that the amount of G6P within islets increased in correlation with blood/cellular glucose concentration. This lead to the hypothesis that GCK within islets, more specifically beta-cells, was responsible for coupling extracellular glucose levels to insulin secretion (Bedoya, et al., 1986b; Matschinsky and Ellerman, 1968; Matschinsky, et al., 2006). Beta-cell GCK elicits this function by increasing the intracellular ATP:ADP ratio, which closes the ATP-sensitive potassium channel (K_{ATP}) causing membrane depolarisation, Ca^{2+} influx, and exocytosis of insulin-containing secretory granules (Henquin, 2000; Henquin, et al., 2003; Meglasson and Matschinsky, 1984; Meglasson and Matschinsky, 1986) (figure 1.4).

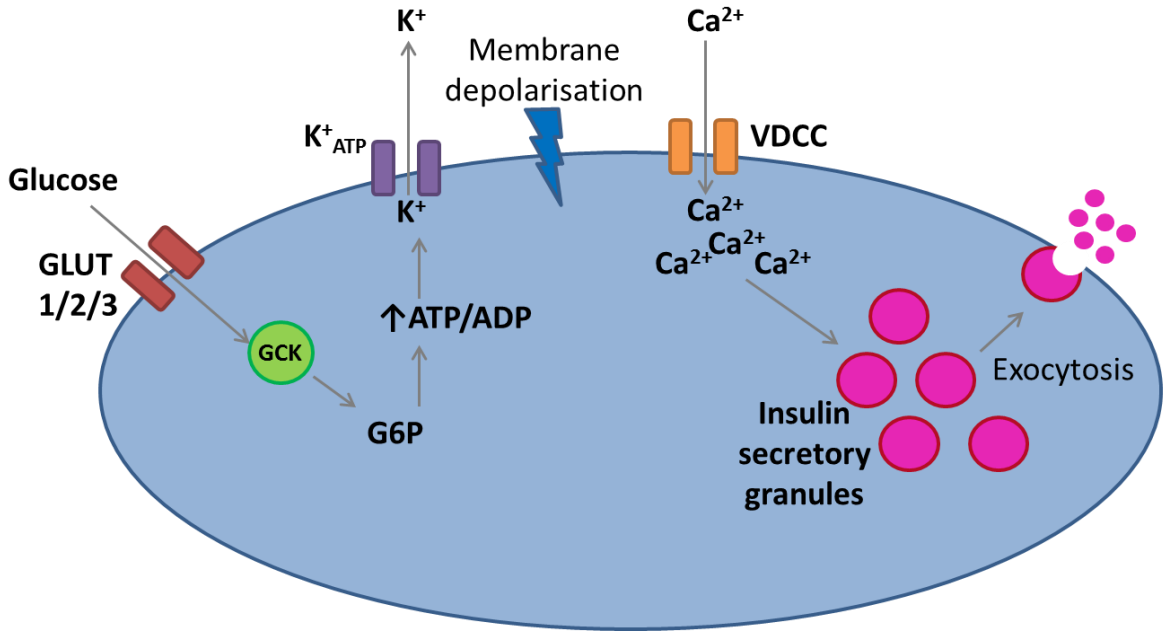


Figure 1.4: Minimal model for human beta-cell glucose-stimulated insulin secretion (GSIS). Glucose uptake is not limited by glucose transport meaning GCK activity dictates glycolytic flux in the beta-cell. VDCC is the voltage-dependent calcium channel. Other abbreviations are as defined in main text.

Experiments in insulinoma cell lines and mice showed that GCK is the key regulator of beta-cell glycolysis, with flux through this pathway serving to channel metabolites into glucose oxidation, thus providing a glucose-dependent signalling cascade which increases the intracellular ATP:ADP ratio (Berman and Newgard, 1998; Sakura, et al., 1998; Wang and Lynedjian, 1997a; Wang and Lynedjian, 1997b). Therefore, it is widely accepted that beta-cell GCK acts as the body's glucose sensor which is capable of responding to changes in blood glucose, determining the threshold for glucose-stimulated insulin secretion (GSIS), and maintaining FPG levels within a tight boundary (Gloyn, et al., 2004a; Matschinsky, 2002; Matschinsky, et al., 2006). Under normal GCK activity the GSIS threshold is ~5mmol/L, however mutations within *GCK* cause either a left- or rightward shift of the sigmoidal glucose response curve with activating and inactivating substitutions respectively (figure 1.1) (Gloyn, et al., 2004a; Matschinsky, 2002; Matschinsky, et al., 2006).

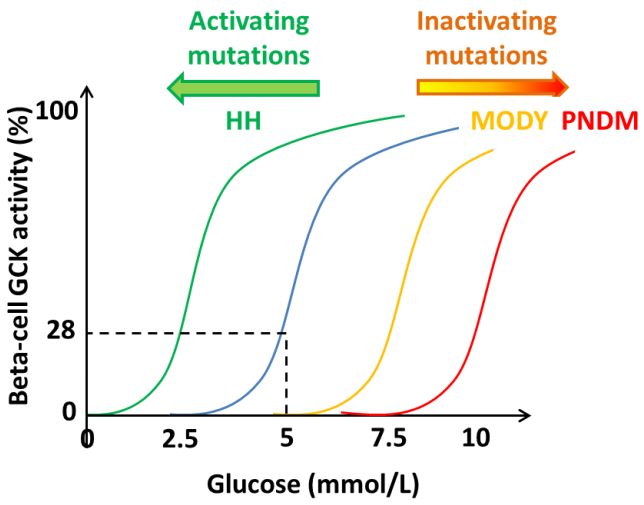


Figure 1.1: GCK glucose response curve. Wild-type GCK (blue) is cooperative with respect to the substrate glucose, and thus it exhibits a sigmoidal glucose response curve. The GSIS threshold is tightly maintained at ~5mmol/L, this corresponding to GCK operating at ~28% of its overall capacity in the beta-cell. Mutations can activate (green) or inactivate (orange and red) GCK, thus shifting the curve to the left or right respectively (Gloyn, et al., 2004a; Matschinsky, 2002; Matschinsky, 2009; Matschinsky, et al., 2006). HH stands for hypoglycaemic hyperinsulinaemia, MODY for maturity-onset diabetes of the young, and PNDM for permanent neonatal diabetes mellitus.

A minimal mathematical model has also been derived which explains the relationship between the enzymes activity index which includes all measurable kinetic parameters and the GSIS threshold, thus allowing prediction of the insulin secretion set-point in patients where *GCK* is mutated (Gloyn, et al., 2004a; Matschinsky, et al., 2006).

1.10 Glucokinase and beta-cell mass

The role of GCK in beta-cell mass remains controversial, however there is some evidence to support the involvement of this enzyme in beta-cell replication, as *Gck* haploinsufficient mice are incapable of beta-cell hyperplasia in response to diet-induced insulin resistance (Terauchi, et al., 2007). However, *Irs2* was also down-regulated in these rodents suggesting that either altered GCK and/or Irs-2 levels may have caused this inadequate beta-cell response (Terauchi, et al., 2007). Studies using Fatty Liver Shionogi (FLS) mice also challenge this theory, as these mice spontaneously recover from diabetes via increased beta-cell hyperplasia without changes to *Gck* or *Irs2* mRNA levels (Oze-Fukai, et al., 2009). There is also evidence that GCK activity my directly influence beta-cell mass in humans, as it has been reported that patients with *GCK* activating mutations have increased islet size and beta-cell area (Cuesta-Munoz, et al., 2004; Kassem, et al., 2010). These data therefore suggest that glycolytic flux through GCK acts as a homeostatic control mechanism to regulate beta-cell mass in accordance with metabolic requirements (Cuesta-Munoz, et al., 2004; Kassem, et al., 2010; Porat, et al., 2011). However it has conversely been suggested that human beta-cells are long-lived, with significant amounts of turnover limited to the first 20-30 years of life (Cnop, et al., 2010; Cobo-Vuilleumier and Gauthier, 2010; Perl, et al., 2010).

Data from experiments using pharmacological GKAs have also shown that treatment with these drugs shortens the time it takes for newly-replicated beta-cells to re-enter the cell division cycle (Salpeter, et al.), and protects against chronic hyperglycaemia-induced apoptosis (Wei, et al., 2009). One study

does however point out that the reduction in blood glucose levels upon administration of GKAs means that this increase in beta-cell mass/replication may not occur *in vivo* (Nakamura, et al., 2009).

1.11 The role of GCK in the brain and gut remains ill-defined

The brain - *GCK* mRNA (Jetton, et al., 1994; Yang, et al., 1999) and protein (Roncero, et al., 2000) have been identified in various parts of the hypothalamus including both glucose-excitatory and –inhibitory neurones (Kang, et al., 2004; Liu, et al., 1999), and in cells that synthesise hormones important to appetite and food intake (Dunn-Meynell, et al., 2002; Zhou, et al., 2011). However, one study proposes that although *GCK* mRNA is present in the pituitary, alternative spicing causes a reading frame shift which results in an inactive protein (Liang, et al., 1991). Additionally, brain glucose concentrations peak at 1.5mmol/L, a level much lower than that at which GCK operates within the hepatocyte and beta-cell, although the pituitary may be exposed to higher glucose concentrations as it is outside the blood-brain-barrier. Consequently, the precise role of GCK in the brain and pituitary remains disputed (Lynedjian, 2009; Parton, et al., 2007; Wolfgang and Lane, 2006).

The gut - As for the brain, the role of GCK in the gut remains controversial. L- and K-cells secrete glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP) respectively. These incretin hormones potentiate insulin secretion from the beta-cell, and thus suggest an indirect role of gut-GCK activity in the regulation of beta-cell insulin secretion (Cheung, et al., 2000; Han, et al., 2007; Reimann and Gribble, 2002). However, one study has observed GIP and GLP-1 secretion in patients with heterozygous *GCK* inactivating mutations, and showed no difference in incretin secretion versus controls during an OGTT. From this it was concluded that GCK was not the main glucose-sensor within the gut (Murphy, et al., 2009).

1.12 Glucokinase mutations cause disorders of aberrant glucose homeostasis

Mutations within *GCK* cause a spectrum of glycaemic disorders, the resultant phenotype dependent upon whether these mutations activate or inactivate the enzyme (Osbak, et al., 2009). Heterozygous activating mutations cause hyperinsulinaemic hypoglycaemia (HH) (Glaser, et al., 1998), heterozygous inactivating mutations cause maturity-onset diabetes of the young (MODY) (Froguel, et al., 1992; Hattersley, et al., 1992), and homozygous/compound heterozygous inactivating mutations cause permanent neonatal diabetes mellitus (PNDM) (Njolstad, et al., 2001).

Hyperinsulinaemic Hypoglycaemia (HH) - HH is a rare yet severe condition in which there is inappropriate insulin secretion despite hypoglycaemia (Flanagan, et al., 2011; Glaser, et al., 1998). It is a heterogeneous disorder, with studies into the prevalence of this condition estimating *GCK* mutations to account for only 1.2% of cases (Christesen, et al., 2008). GCK-HH differs from similar syndromes caused by mutations within the genes encoding the K_{ATP} channel (*ABCC8* and *KCNJ11*), as the hypoglycaemia experienced in these patients is often milder and more stable (Christesen, et al., 2002; Glaser, et al., 1998; Gloyn, et al., 2003a; Meissner, et al., 2009; Osbak, et al., 2009; Sayed, et al., 2009; Wabitsch, et al., 2007). This is because activating *GCK* mutations merely re-set the threshold for GSIS at a lower blood glucose concentration, whilst there is uncontrolled insulin secretion in patients with mutations in the K_{ATP} channel genes. As such, patients with GCK-HH are on the whole responsive to treatment with the K_{ATP} channel opener diazoxide (Christesen, et al., 2002; Christesen, et al., 2008; Glaser, et al., 1998; Gloyn, et al., 2003a; Meissner, et al., 2009; Osbak, et al., 2009; Sayed, et al., 2009; Touati, et al., 1998; Wabitsch, et al., 2007), and it has also been suggested that some may even be treated without pharmacological intervention, but solely with a well-managed eating regime (Gloyn, et al., 2003a; Wabitsch, et al., 2007). Despite this, a lack of compensation by GCK encoded by the wild-type allele (Liang, et al., 1992) results in a range of phenotype severity, dictating

not only response to treatment (Cuesta-Munoz, et al., 2004; Sayed, et al., 2009), but potentially also islet size and morphology, although this remains controversial (Cuesta-Munoz, et al., 2004; Gloyn, et al., 2003a; Kassem, et al., 2010; Wabitsch, et al., 2007). One study aimed at investigating the relationship between mutation severity and clinical phenotype showed that patients who responded poorly/not at all to diazoxide therapy had *GCK* mutations which yielded the greatest increase in activity versus wild-type enzyme (Sayed, et al., 2009).

Maturity-Onset Diabetes of the Young (MODY) - GCK-MODY is a dominantly-inherited condition that is characterised by mild fasting hyperglycaemia (5.5-8.0mmol/L), and only a small increment in blood glucose levels (<3.0mmol/L) following a two hour OGTT. It is present from birth, but is often only diagnosed following routine testing as blood glucose levels are only marginally elevated in these patients (Ellard, et al., 2008; Murphy, et al., 2008; Stride, et al., 2002). Accurate diagnosis of these patients is crucial, because GCK-MODY differs from other MODY subtypes in that there is a minimal decline in beta-cell function with age, this in-line with that observed within the normal population (Frayling, et al., 2001). The beta-cell GSIS-threshold is just 're-set' at a higher blood glucose concentration in these patients as the inactivating *GCK* mutation right-shifts the glucose-response curve (Byrne, et al., 1994; Gloyn, et al., 2004a). As the resultant elevation in blood glucose levels is only mild, this means that diabetes-associated complications are rare in these patients (Ellard, et al., 2008; Velho, et al., 1997). However, they do have reduced liver glycogen content (Velho, et al., 1996a) and decreased hyperglycaemia-mediated suppression of gluconeogenesis (Tappy, et al., 1997), both resulting from altered hepatic metabolism as a consequent of defective GCK function. Conversely, patients with *HNF1A* mutations (the other most common cause of MODY) experience a progressive decline in beta-cell function leading to diabetes, and require oral anti-diabetic agent or insulin treatment (Ellard, et al., 2008; Frayling, et al., 2001; Pearson, et al., 2001).

GCK-MODY patients can often be treated by diet alone, with studies suggesting pharmacological intervention with oral anti-diabetic agents has little effect on glycosylated haemoglobin (HbA1C) levels (Gill-Carey, et al., 2007; Stride, et al., 2002), as does exogenous insulin administered to children incorrectly diagnosed as having type 1 diabetes (Schnyder, et al., 2005). The presence of a *GCK* mutation also means that there is a counter-regulatory response to hypoglycaemia in the beta-cells of these patients, and so 'over-medicating' can actually have pathogenic consequences (Guenat, et al., 2001). Accurate diagnosis during pregnancy is also crucial, as mother and foetus genotype have been shown to effect birth weight (Colom and Corcoy, 2010; Hattersley, et al., 1998; Shields, et al., 2008; Singh, et al., 2007). For example, if the mutation is in the mother only, baby will be large for gestational age (Hattersley, et al., 1998; Singh, et al., 2007) and thus the pregnancy must be managed accordingly (Kjos, et al., 2001; Spyer, et al., 2001).

GCK-MODY patients who exhibit symptoms are often misdiagnosed as having type 1, type 2 (Ellard, et al., 2008), or gestational diabetes, as FPG levels are routinely tested during pregnancy (Ellard, et al., 2000). Correspondingly, estimates of GCK-MODY prevalence are difficult (Osbak, et al., 2009). Studies into the prevalence of GCK-MODY in European Caucasian populations have yielded markedly different figures (Barrio, et al., 2002; Costa, et al., 2000; Estalella, et al., 2007; Froguel, et al., 1993; Johansen, et al., 2005; Mantovani, et al., 2003; Massa, et al., 2001; Osbak, et al., 2009; Pruhova, et al., 2003; Sagen, et al., 2008; Thomson, et al., 2003), although this has been attributed to differences in patient recruitment strategy and age. For example the study of paediatric (Mantovani, et al., 2003; Massa, et al., 2001) versus adult patients (Sagen, et al., 2008) yields much higher prevalence estimates. However despite these difficulties, *GCK* mutations have been shown to be a common cause of childhood hyperglycaemia (Pruhova, et al., 2003), whilst a rare cause of adult mild fasting hyperglycaemia (Gloyn, et al., 2009).

Permanent Neonatal Diabetes Mellitus (PNDM) - Homozygous/compound heterozygous inactivating *GCK* mutations cause the severe diabetic phenotype of permanent neonatal diabetes mellitus (PNDM) (Njolstad, et al., 2001). PNDM is an extremely rare condition diagnosed within the first six months of life, and with an estimated incidence of 1 in 260,000 live births (Edghill, et al., 2006; Slingerland, et al., 2009). *GCK* mutations are an infrequent cause of PNDM (Gloyn, et al., 2002; Vaxillaire, et al., 2002), with this disease more commonly caused by mutations within the K_{ATP} channel genes *ABCC8* (Babenko, et al., 2006) and *KCNJ11* (Gloyn, et al., 2004b), as well as by mutations within the insulin gene (*INS*) (Stoy, et al., 2007). There are only 16 reported cases of GCK-PNDM to date (Bennett, et al., 2010; Bennett, et al., 2011; Njolstad, et al., 2003; Njolstad, et al., 2001; Porter, et al., 2005; Rubio-Cabezas, et al., 2008; Turkkahraman, et al., 2008), the majority of cases in the offspring of consanguineous parents (Bennett, et al., 2010; Njolstad, et al., 2003; Porter, et al., 2005; Rubio-Cabezas, et al., 2008; Turkkahraman, et al., 2008). Therefore, although *GCK* mutations are a rare cause of this disorder, this gene should be submitted to mutational screening if any of the first-degree relatives have MODY or there is a history of glucose intolerance, particularly if the parents are consanguineous (Gloyn, et al., 2002; Vaxillaire, et al., 2002).

GCK-PNDM patients are insulin-dependent from birth (Bennett, et al., 2010; Njolstad, et al., 2003; Njolstad, et al., 2001; Porter, et al., 2005; Rubio-Cabezas, et al., 2008; Turkkahraman, et al., 2008), although studies suggest there may be a dose-dependent beneficial effect of sulphonylurea-treatment (Bennett, et al., 2010; Turkkahraman, et al., 2008). The total GCK-deficiency in these patients makes them ideal candidates for treatment with small molecular activators, as these would serve to lower the threshold for GSIS, thus reinstating normoglycaemia (Matschinsky, 2009; Matschinsky and Porte).

1.13 There are no mutational hotspots within the glucokinase gene

Over 600 mutations have been identified within *GCK*, these spreading throughout the coding sequence of this gene. Only six mutations have been reported in exon 1a (neuroendocrine isoform), although this could be artefactually low as some diagnostic laboratories only analyse exons 2-10 (Ellard, et al., 2000; Gloyn, et al., 2002; Thomson, et al., 2003).The recent identification of the -71G>C mutation within the neuroendocrine promoter in six MODY families (Gasperikova, et al., 2009) has prompted review of how much of the *GCK* gene is screened for diagnostic purposes.

The *GCK* mutations identified to date include non-synonymous, nonsense, and splice site mutations (Osbak, et al., 2009). CpG dinucleotides are methylated by methyltransferases as a way of regulating gene expression, and deamination of these methylcytosines (CG>TG) is a common mutational mechanism within human genes (Berg, et al., 2002; Cooper, et al., 1995). However, only 5% of *GCK* mutations (versus 30% in other genes) occur at CpG dinucleotides, thus meaning they are not a mutational hotspot in this gene (Cooper, et al., 1995; Osbak, et al., 2009). Also, gene duplication is not a common cause of GCK-HH (van de Bunt, et al., 2008), although gene deletions have been shown to cause GCK-MODY (Ellard, et al., 2007; Garin, et al., 2008).

1.14 Common glucokinase variation is associated with fasting plasma glucose and type 2 diabetes

In addition to *GCK* mutations causing monogenic disorders of glucose homeostasis, common variation in this gene has subsequently been shown to associate with FPG and birth weight (Weedon, et al., 2006; Weedon, et al., 2005). The authors of these studies demonstrated that the risk allele at the -30 position in the neuroendocrine *GCK* promoter (-30G>A; minor allele frequency [MAF] 30%) was associated with a 0.06mmol/L increase in FPG (Weedon, et al., 2005). Further studies identified another non-coding single nucleotide polymorphism (SNP) in high linkage-disequilibrium with the

-30G>A variant which also affected FPG (Prokopenko, et al., 2009). *GCK* SNPs are also associated with increased type 2 diabetes (T2D) risk (Dupuis, et al., 2010).

Despite this genetic association (Dupuis, et al., 2010), the activity/expression status of GCK in T2D remains to be definitively established (Agius, 1998; Caro, et al., 1995; Matschinsky, 2009; Matschinsky and Porte, 2010; Willms, et al., 1970). In the hepatocyte, patients with mild disease have an unexpected increase in GCK activity, whilst those who are severely obese/have advanced disease exhibit decreased activity of this enzyme (Caro, et al., 1995; Willms, et al., 1970). Obese diabetic Zucker rats also have reduced liver GCK activity (Torres, et al., 2009), as have streptozotocin-induced diabetic rats (Lynedjian, et al., 1988). This reduction in enzyme activity could, in part, be due to the regulation of hepatic GCK by insulin which is either reduced in these patients and/or they are insulin-resistant (Matschinsky, 2009; Matschinsky and Porte, 2010). The picture in the pancreas is much less clear, however data obtained from rodent diabetes models (Bedoya, et al., 1987; Liang, et al., 1994) and findings in human T2D islets (Deng, et al., 2004) suggest that GCK activity may be similar to controls in mild/early T2D, but decreased in correlation with disease progression (Matschinsky, 2009). This reduced GCK in advanced T2D may be an indirect result of reduced beta-cell mass or function (Matschinsky and Porte, 2010).

1.15 Rodent models confirm a pivotal role for glucokinase in glucose homeostasis

Rodent models allow manipulation of the *Gck* gene to be studied at the whole organism and tissue-specific level. These models have helped shed light on the physiological role of GCK, and further support a key role for this enzyme in glucose metabolism.

Global Gck knock-out mice - Grupe and colleagues generated hetero- and homozygous global *Gck* knock-out mice via targeted disruption of *Gck* exons 3-5 with the neomycin-resistance gene (Grupe,

et al., 1995). Homozygous mice suffered extreme hyperglycaemia (blood glucose levels of 24.0 versus 2.9mmol/L for wild-type littermates) and died within the perinatal period. Heterozygous littermates had marginally elevated FPG levels, but were still able to develop normally. Despite hyperglycaemia they also exhibited reduced insulin release versus wild-type mice, with altered first- and second-phase insulin secretion following pancreatic glucose perfusion (Grupe, et al., 1995). Study of a second global *Gck* knock-out mouse showed similar results (Postic, et al., 1999). These homo- and heterozygous global knock-out mice mimic the phenotypes of human GCK-PNDM and GCK-MODY respectively, and thus have helped with understanding the pathogenesis behind these monogenic disorders.

Beta-cell-specific Gck knock-out mice - *T*issue-specific *Gck* knock-out mice allow the role of this enzyme in an organ of interest to be observed. This provides a different insight versus the study of patients with monogenic *GCK* mutations, as human mutations within exons two-ten of this gene will affect both the hepatic and neuroendocrine *GCK* isoforms (Lynedjian, 2009). Beta-cell specific *Gck* knockout mice have been generated using numerous techniques, including the expression of Cre recombinase from the *Ins* promoter (Postic, et al., 1999), and targeted disruption of *Gck* exon 1a which is specific to the neuroendocrine isoform (Terauchi, et al., 1995). These mice exhibited defective insulin secretion and suffered from severe, promptly lethal, hyperglycaemia (Postic, et al., 1999; Terauchi, et al., 1995). This was particularly interesting in the case of the beta-cell knock-out mice expressing Cre recombinase from the *Ins* promoter, as these rodents still expressed *Gck* in the hypothalamus and L-/K-cells (Lynedjian, 1993; Lynedjian, et al., 1986; Lynedjian, et al., 1989b; Jetton, et al., 1994; Lynedjian, 2009; Magnuson, 1990). Therefore, it was proposed that *Gck* expression in the liver, brain, and gut, was not enough to compensate for the defective glucose-sensing in these animals (Postic, et al., 1999). Additionally, the similar phenotype between the global *Gck* knock-out mice (Grupe, et al., 1995; Postic, et al., 1999) and the beta-cell-specific knock-outs suggests that this

lethal hyperglycaemia is largely driven by beta-cell dysfunction, and thus beta-cell GCK alone is predominantly responsible for the determination of FPG levels in mammals (Grupe, et al., 1995; Postic, et al., 1999; Terauchi, et al., 1995).

Hepatocyte-specific Gck knock-out/over-expression mice - Mice in which *Gck* was ablated solely in the liver (Postic, et al., 1999) displayed a markedly different phenotype compared to both global- and beta-cell-specific *Gck* knock-outs (Grupe, et al., 1995; Postic, et al., 1999; Terauchi, et al., 1995). These mice did not die perinatally, and exhibited mild hyperglycaemia and defective glycogen synthesis. These mice also had plasma insulin levels twice that of control mice suggesting they were insulin resistant. Therefore, the defective hepatic glucose utilisation and disposal in these rodents was thought to reflect both decreased liver GCK activity, as well as reduced insulin-stimulated hepatic gene expression (Postic, et al., 1999). Finally, the unexpected impaired insulin secretion in these rodents was thought to result from the sustained elevation in blood glucose levels (Postic, et al., 1999), a result supported by rat models in which mild, yet chronic, hyperglycaemia causes beta-cell dysfunction (Leahy, et al., 1986; Leahy, et al., 1987; Leahy and Weir, 1988; Trent, et al., 1984).

Conversely, rodents in which hepatic *Gck* was over-expressed had improved glucose tolerance and mild hypoglycaemia which did not result from increased insulin secretion, as well as increased liver glycogen stores (Ferre, et al., 1996b; Hariharan, et al., 1997; Jackerott, et al., 2002; O'Doherty, et al., 1999). This was the same as mice with elevated hepatic GCK activity through increased gene copy number (Niswender, et al., 1997a; Niswender, et al., 1997b). Hepatic *Gck* over-expression was also found to prevent the hyperglycaemia or diabetes inducible by high-fat diets and streptozotocin (Desai, et al., 2001; Ferre, et al., 1996a; Konrad, et al., 2001; Shiota, et al., 2001), as well as increasing the survival rate and reducing blood glucose levels of insulin receptor-deficient mice which suffer from severe diabetes, liver steatosis and perinatal death (Jackerott, et al., 2002). Unfortunately some

rodents did have elevated triglycerides, but this was dependent on the level of hepatic *Gck* over-expression (Ferre, et al., 1996a; Ferre, et al., 1996b; Jackerott, et al., 2002; O'Doherty, et al., 1999).

Together these data suggest that GCK activity in the liver is crucial for the regulation of hepatic glucose metabolism and glucose clearance by this organ, with perturbations/loss of this activity serving to elevate blood glucose levels with subsequent pathological effects.

Other Gck rodent models - Rodent models in which *Gck* is mutated as opposed to un-physiologically abolished or over-expressed provide a more relevant model for studying human monogenic *GCK* conditions. To date ENU-mutagenesis has produced 14 mice with inactivating mutations in *Gck* which cause elevated FPG (Aigner, et al., 2008; Inoue, et al., 2004; Pino, et al., 2007; Toye, et al., 2004), and of these six have been identified in MODY or PNDM patients (Aigner, et al., 2008), thus allowing these mutations from human monogenic disorders to be studied at the whole organism level (Osbak, et al., 2009). A mouse with an activating *Gck* mutation previously identified in a HH patient has also been generated via gene targeting (Christesen, et al., 2002; Pino, et al., 2007), highlighting another approach for the generation of rodent models of human disease (Osbak, et al., 2009).

1.16 The central role of glucokinase in glucose homeostasis makes it an attractive therapeutic target

The global rise in the prevalence of T2D not only reflects changes in lifestyle, diet, and behaviour (Zimmet, et al., 2001), but also suggests that our current therapeutic strategies against this disease are ineffective. This leaves an unmet medical need for better treatments that are capable of lowering glycaemia to appropriate levels safely as well as maintaining their efficacy over many years (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010). The crucial role of GCK in both the beta-cell and hepatocyte (Matschinsky, 2002; Matschinsky, et al., 2006) provides the promise of a

two-pronged attack on hyperglycaemia by pharmacological GKAs, by both lowering the threshold for GSIS and increasing hepatic glucose uptake and utilisation (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010).

The first GKA to be identified was the Hoffman-La Roche compound RO0280450, with further analysis revealing that only the R-enantiomer RO0281675 was active (Grimsby, et al., 2003). *In vitro* studies showed this compound enhanced GCK activity by increasing its affinity for glucose (reduced $S_{0.5}$) and turnover rate (K_{cat}), with subsequent tests in isolated rat and mouse islets lowering the GSIS-threshold and enhancing maximal insulin secretion from these cells. At the systemic level, large doses of this GKA caused HH in obese and normal weight mice (Grimsby, et al., 2003); a phenotype consistent with GCK activation in both the beta-cell and hepatocyte (Glaser, et al., 1998; Osbak, et al., 2009). Further investigation via hyperinsulinaemic clamp also showed RO0281675 suppressed hepatic glucose production and improved glucose tolerance after an OGTT (Grimsby, et al., 2003). The test of GKA compounds from other pharmaceutical companies showed similar beneficial effects, with GKAs enhancing insulin secretion and reducing hepatic glucose output (Brocklehurst, et al., 2004b; Castelhano, et al., 2005; Coope, et al., 2006; Efanov, et al., 2005; Fyfe, et al., 2007; Grimsby, et al., 2003; Pal, 2009).

Preliminary clinical trials in humans were equally as promising, with the Hoffman-La Roche compound Piraliatin (RO4289620) showing a dose-dependent lowering of glycaemia in both T2D and control subjects during clinical trials (Bonadonna, et al., 2010). However, a significantly greater number of rescue glucose infusions had to be given to patients on the highest Piragliatin dose in order to prevent hypoglycaemia (Bonadonna, et al., 2010), thus highlighting one of the main potential problems of GKA-mediated diabetes treatment. Nevertheless, pharmaceutical companies are continuing to invest in bringing GKAs to market (Pal, 2009; Sarabu and Grimsby, 2005).

All GKA compounds identified to date bind within the hinge region of GCK in a cavity lined with hydrophobic residues. GKAs only bind in the presence of glucose as their binding pocket is only present in the closed structure (Ralph, et al., 2008), and serve to enhance GCK activity by sterically-hindering enzyme transition back to the super-open structure (Agius, 2008; Kamata, et al., 2004). This hinge region maps next to the allosteric activator site within GCK, which is a location where all bar one of the 13 activating mutations reported to date cluster. GKAs therefore aim to mimic the structural and conformational effects of these naturally occurring activating mutations (Heredia, et al., 2006; Kamata, et al., 2004; Osbak, et al., 2009; Sayed, et al., 2009). However, GCK-HH, GCK-MODY, and GCK-PNDM, have shown that slight perturbations to GCK activity in either direction can have profound effects on glucose metabolism and FPG (Osbak, et al., 2009). As GKAs seek to pharmacologically enhance GCK activity in patients with T2D, this potential therapy must be approached with caution so as not to induce the acute hypoglycaemia seen in GCK-HH, and thus requires careful drug design and clinical management of patients treated with these drugs (Matschinsky, 2009; Osbak, et al., 2009). This proposed-pitfall has already been demonstrated in clinical trials of Piragliatin where subjects on a higher dose needed more 'rescue glucose infusions' to avoid hypoglycaemic episodes (Bonadonna, et al., 2010). GCK is also highly expressed in tissues other than pancreatic beta-cells, and so the consequence of stimulating GCK activity here must also be considered. For example, *Gck* over-expression in rat livers has been shown to elevate triglyceride levels (Ferre, et al., 1996a; Ferre, et al., 1996b; Jackerott, et al., 2002; O'Doherty, et al., 1999), as has diminished inhibition of hepatic GCK in humans (Orho-Melander, et al., 2008; Saxena, et al., 2007; Sparso, et al., 2008; Vaxillaire, et al., 2008). Additionally, our current understanding of the roles of GCK in the hypothalamus and gut is limited, as is the interaction of this enzyme with cellular binding partners and regulators. Therefore, the effects of GKAs in these tissues/on whole body glucose

homeostasis must also be considered (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010).

1.17 Future directions for small molecular glucokinase activators as an anti-diabetes treatment

Tests in both rodents and humans have shown GKAs to potentiate insulin secretion in a dose-dependent manner (Bonadonna, et al., 2010; Bonadonna, et al., 2008; Castelhano, et al., 2005; Coghlan and Leighton, 2008; Coope, et al., 2006; Efanov, et al., 2005; Fyfe, et al., 2007; Grimsby, et al., 2003; Pal, 2009; Zhi, et al., 2008), predominantly by increasing the affinity of GCK for glucose by stabilising the closed conformation of the enzyme and preventing its relaxation to the super-open low affinity state (Coghlan and Leighton, 2008; Grimsby, et al., 2003; Kamata, et al., 2004; Matschinsky, 2009; Matschinsky and Porte, 2010). The insulinotropic effects of GKAs would be of little use therapeutically in type 1 diabetes, as these patients have no (or few) insulin-producing beta-cells due to their selective autoimmune destruction (Atkinson and Eisenbarth, 2001; Walker, et al., 2011). However, studies into the GCK status in T2D suggests that GCK activity decreases but is not absent as this disease progresses (Agius, 1998; Bedoya, et al., 1987; Caro, et al., 1995; Deng, et al., 2004; Liang, et al., 1994; Matschinsky, 2009; Matschinsky and Porte, 2010; Torres, et al., 2009; Willms, et al., 1970), with GKA clinical trials in humans with T2D promisingly showing the dose-dependent lowering of blood glucose (Bonadonna, et al., 2010). Additionally, the suggestion that enhancing GCK activity may increase beta-cell mass (Kassem, et al., 2010; Maiztegui, et al., 2009; Salpeter, et al.; Takamoto, et al., 2008; Wei, et al., 2009) means GKAs may help counteract the decline in beta-cell numbers/mass that is proposed to contribute to T2D pathogenesis (Cnop, et al., 2010; Perl, et al., 2010). GKAs would also be ideal for the treatment of GCK-PNDM (Njolstad, et al., 2001), as these drugs may reverse the effect of inactivating *GCK* mutations in these patients (Osbak, et al., 2009).

1.18 The next generation of drug design will require further investigation into the cellular regulatory mechanisms controlling glucokinase activity

GCK activity is tightly regulated at the posttranslational level via both covalent modification and protein-protein interaction mechanisms (Agius, 2008).

Covalent modification - Auto-phosphorylation has been shown to occur with both mammalian and yeast hexokinases *in vitro*, however the physiological relevance of this for GCK remains uncertain (Agius, 2008). Analysis of the GCK sequence does show that this enzyme contains several phosphorylation motifs for protein kinase A (PKA), although the eligibility of these motifs as *in vivo* PKA substrates remains disputed (Hanks, et al., 1988; Kemp and Pearson, 1990). To date PKA-mediated phosphorylation and subsequent inactivation of GCK has only been shown *in vitro* (Agius, 2008; Ekman and Nilsson, 1988; Munoz-Alonso, et al., 2000).

GCK also contains a large number of cysteine residues (Tanizawa, et al., 1992), these amino acids having sulfhydryl groups that are sensitive to both oxidation and nitrosylation (Foster, et al., 2003; Gow, et al., 2002; Hess, et al., 2005; Stamler, et al., 2001). Early attempts at GCK protein purification showed that sulfhydryl reducing agents were needed to retain enzyme activity (Grossman, et al., 1974; Holroyde, et al., 1976a; Holroyde, et al., 1976b; Parry and Walker, 1966; Pilkis, 1972; Salas, et al., 1965). Treatment with the oxidising agent alloxan was also shown to inactivate GCK, and yielded an apparent lower molecular weight band upon gel electrophoresis thought to represent a more compact enzyme locked together by excessive intramolecular disulphide-bonds (Lenzen and Munday, 1991; Tiedge, et al., 1997; Tiedge, et al., 2000). Alloxan is consequently the most potent chemical inhibitor of GCK (Lenzen and Panten, 1988; Lenzen, et al., 1987), and is subsequently capable of inducing diabetes (Lenzen, 2008). Conversely, S-nitrosylation by neuronal nitric oxide synthase indirectly enhances intracellular GCK activity by facilitating dissociation of the enzyme from insulin

secretory granules (Rizzo, et al., 2002; Rizzo and Piston, 2003; Stubbs, et al., 2000; Toyoda, et al., 1999).

Protein-protein interactions – the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase) – Peptide phage screens aimed at identifying novel GCK intracellular binding partners showed that the 'SLKVWT' amino acid motif within the bisphosphatase domain of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase) bound GCK with great affinity (Baltrusch, et al., 2001; Okar, et al., 2001; Pilkis, et al., 1995). This bifunctional enzyme has both a kinase and bisphosphatase domain which catalyse the formation and degradation of fructose-2,6-bisphosphate. In the liver, fructose-2,6-bisphosphate activates 6-phosphofructo-1-kinase thus channelling metabolites into hepatic glycolysis (Hers and Van Schaftingen, 1982; Hue and Rider, 1987; Pilkis, et al., 1995), although this effect can be counter-acted by glucagon which stimulates phosphorylation of a critical serine within the bifunctional enzyme causing its inactivation (Guigas, et al., 2006; Mukhtar, et al., 2008; Payne, et al., 2005; Rosa, et al., 1993). In the beta-cell, PFK-2/FBPase is proposed to directly enhance GCK activity (Massa, et al., 2004), however it is unclear whether this 'apparent activation' is purely a result of enzyme stabilisation (Agius 2008; Smith, et al., 2007).

Protein-protein interactions –B-cell lymphoma 2 (Bcl2)-associated death promoter (BAD) - The B-cell lymphoma 2 (Bcl2) protein family includes both pro- and anti-apoptotic proteins, one example of which is the Bcl2-associated death promoter (BAD) (Kelekar and Thompson, 1998). BAD activates proteins involved in the permeabilisation of the outer mitochondrial membrane, thus leading to programmed cell death (Wang, 2001; Wei, et al., 2001). Some of the kinases responsible for regulating BAD function localise to the mitochondrial membrane where they form large multi-protein complexes which tightly control the activity of this pro-apoptotic protein (Datta, et al., 2000; Harada, et al., 2001; Harada, et al., 1999; Zha, et al., 1996). It has since been shown in mouse hepatocytes

that BAD formed part of- and was essential for- the assembly of this complex, which also included GCK (Arden, et al., 2006; Danial, et al., 2003). The BAD-GCK complex therefore uniquely links apoptosis and glycolysis, with hepatocytes in which BAD is absent exhibiting reduced GCK activity and mitochondrial respiration (Danial, et al., 2003), and mice expressing mutant BAD showing defects in glucose homeostasis (Datta, et al., 2002). However, it should be noted that the amount of GCK in complex with BAD is only a small proportion of the total GCK content of the hepatocyte (Danial, et al., 2003) and another study in fact suggests that GCK does not associate with liver mitochondria at all (Bustamante, et al., 2005; Lynedjian, 2009).

Studies into the role of BAD within the beta-cells show that mice lacking this protein experienced beta-cell dysfunction and defective insulin secretion as a result of reduced glucose-stimulated mitochondrial respiration (Danial, et al., 2008). These *Bad*$^{-/-}$ islets also exhibited lower GCK activity and decreased glucose sensing (Danial, et al., 2008), and were absent for the mitochondria-associated protein complex which had been seen previously in mouse hepatocytes (Danial, et al., 2003) and in the wild-type islets from this study (Danial, et al., 2008). A further role for BAD in the modulation of beta-cell mass was also proposed following the observation that *Bad*$^{-/-}$ mice were resistant to high fat diet-induced hyperglycaemia. It was postulated that despite reduced GSIS in these animals, the absence of pro-apoptotic BAD meant that overall insulin levels were increased as beta-cell mass expanded (Danial, et al., 2008).

Other protein-protein interactions - There are a number of other proteins which have been shown to bind GCK, although the physiological relevance of these remains disputed (Agius, 2008; Lynedjian, 2009; Matschinsky, 2009). For example, a yeast two-hybrid screen identified the propionyl-CoA carboxylase beta-subunit as a novel binding partner for GCK, and showed it increased the enzymes activity (Shiraishi, et al., 2001). However, this activation only occurred when propionyl-CoA

carboxylase beta-subunit was present in 50-100molar excess (Shiraishi, et al., 2001), and so its physiological role in the regulation of GCK activity remains debatable (Agius, 2008).

Non-protein regulators - Other cellular macromolecules are reported to modulate GCK activity, including penta-ubiquitin (Bjorkhaug, et al., 2007) and long-chain acyl-CoAs (Dawson and Hales, 1969; Tippett and Neet, 1982a; Tippett and Neet, 1982b). Penta-ubiquitin chains were found to activate recombinant GCK *in vitro* (Bjorkhaug, et al., 2007), however as ubiquitination often targets proteins for degradation in the cell it is unclear how this mechanism may be important *in vivo* (Agius, 2008). Conversely, long-chain acyl-CoAs are potent inhibitors of GCK activity (Dawson and Hales, 1969; Tippett and Neet, 1982a; Tippett and Neet, 1982b), with palmitate inhibiting glycolysis in isolated hepatocytes (Hue, et al., 1988). It is thought that long-chain acyl-CoAs induce a conformational change which favours and stabilises the inactive enzyme structure (Agius, 2008; Kim, et al., 2007). Initial investigations into the pharmacological activation of GCK aimed to identify a molecule capable of binding these fatty acids, however the role of acyl-CoA intermediates in other metabolic pathways bought into question the safety of this approach (Matschinsky, 2009).

1.19 The glucokinase regulatory protein is an endogenous hepatic glucokinase inhibitor

Early experiments in isolated hepatocytes investigating the regulation of hepatic GCK showed that GCK activity and affinity for glucose was increased in response to micromolar fructose concentrations (Clark, et al., 1979) and medium containing high potassium levels (Bontemps, et al., 1978), although subsequent studies revealed that this potassium effect was a result of simultaneous increases in intracellular chloride ion (Cl^-) levels (Vandercammen and Van Schaftingen, 1991). Further investigations into the stimulatory effect of fructose on GCK activity showed that it was reversible and in fact mediated by fructose 1-phosphate (F1P), as tagatose and sorbitol showed comparable stimulatory properties (Van Schaftingen and Vandercammen, 1989). Follow-up revealed that this

F1P-mediated effect was not apparent in partially purified rat liver GCK preparations, with further purification yielding an endogenous protein regulator of hepatic GCK. Fructose 6-phosphate (F6P) potentiated the inhibitory effect of this regulatory protein on GCK activity, whilst F1P reversed its inhibition. This protein was thus deemed the 'glucokinase regulatory protein' (GKRP) (Detheux, et al., 1991; Van Schaftingen, 1989; Vandercammen and Van Schaftingen, 1990; Vandercammen and Van Schaftingen, 1991).

1.20 Glucokinase regulatory protein competitively inhibits glucokinase activity

The gene encoding glucokinase regulatory protein (*GCKR*) is located on chromosome 2p23 and corresponds to a 2.2kB transcript encoding a 626 amino acid ~68kDa protein (Warner, et al., 1995). It is expressed in the liver prior to birth compared to hepatic GCK which appears much later (after weaning in rats) (Agius, 2008; Vandercammen and Van Schaftingen, 1993). GKRP inhibits GCK competitively with respect to glucose by binding directly to the enzyme (Baltrusch, et al., 2001; Brocklehurst, et al., 2004b; Mookhtiar, et al., 1996; Vandercammen and Van Schaftingen, 1990), and does not covalently modify GCK or catalyse the formation of a smaller molecular weight inhibitor (Van Schaftingen, 1989; Vandercammen and Van Schaftingen, 1990; Vandercammen and Van Schaftingen, 1991). Maximal binding between GKRP and GCK occurs at 5mM glucose (de la Iglesia, et al., 2000).

The mechanism of GKRP-mediated GCK regulation has been highly conserved throughout evolution, with many different vertebrate species exhibiting hepatic co-expression of *GCK* and *GCKR* (Vandercammen and Van Schaftingen, 1993). Additionally, regulatory proteins obtained from human, rat and the frog *Xenopus laevis* are able to inhibit the enzyme from the other three species (Brocklehurst, et al., 2004a; Vandercammen and Van Schaftingen, 1993), even though the *X. laevis* GKRP is insensitive to phosphate ester-mediated modulation (Agius, 2008; Vandercammen and Van

Schaftingen, 1993). This suggests that the GKRP-binding site within GCK is well conserved, as is the majority of the regulatory protein structure (Detheux, et al., 1993; Veiga-da-Cunha, et al., 1996a; Veiga-Da-Cunha, et al., 1994; Warner, et al., 1995).

Early mutagenesis experiments showed that mutations affecting GCK substrate affinity altered regulatory protein binding to a different extent, thus suggesting that glucose and GKRP bind at different sites within GCK (Gidh-Jain, et al., 1993; Pilkis, et al., 1994; van Schaftingen, et al., 1997; Veiga-da-Cunha, et al., 1996a; Veiga-da-Cunha, et al., 1996b). Hepatic and neuroendocrine GCK were shown to have equal GKRP-affinity (Magnuson and Shelton, 1989; Veiga-da-Cunha, et al., 1996b), thus it was concluded that the 15 N-terminal residues that differ between the two isoforms were not involved in regulatory protein binding (Veiga-da-Cunha, et al., 1996b). Instead, mutations altering GKRP affinity were largely located in the hinge-region of GCK (Moukil, et al., 2000; van Schaftingen, et al., 1997; Veiga-da-Cunha, et al., 1996a; Veiga-da-Cunha, et al., 1996b). Subsequent studies also showed that GCK binds a particular GKRP amino acid motif (LSAXXVAG, where 'X' is any amino acid), thus proving a direct binding interaction between the two proteins (Baltrusch, et al., 2001). Further experiments showed that the GKRP-binding site within GCK involves leucine/asparagine motifs, specifically L58/N204 and L355/N350. Subsequent mutagenesis revealed that the first of these motifs confers the ability to bind GKRP, whilst the latter affects binding affinity (Baltrusch, et al., 2005).

The glucose analogues mannoheptulose and N-acetylglucosamine have differing effects on GKRP-mediated GCK inhibition, the latter unable to out-compete the regulatory protein for binding to the enzyme (Niculescu, et al., 1997; Vandercammen and Van Schaftingen, 1991). As these molecules bind to the closed and super-open GCK conformations respectively, it was postulated that GKRP binding must stabilise the super-open GCK structure (Agius, 2008; van Schaftingen, et al., 1997). Further evidence to support this comes from co-immunoprecipitation assays which show GKRP:GCK binding

to be reduced in conditions favouring the closed enzyme conformation (Futamura, et al., 2006), and that the binding of GKRP and GKAs to GCK is mutually exclusive (Anderka, et al., 2008).

1.21 Phosphate esters produced during metabolism modulate glucokinase regulatory protein-mediated inhibition of hepatic glucokinase activity

The phosphate esters F6P and F1P enhance and diminish the ability of GKRP to complex with GCK respectively, with both phosphate esters shown to bind directly to the regulatory protein (Vandercammen, et al., 1992; Vandercammen and Van Schaftingen, 1990). This short-term regulatory mechanism fits with the metabolic requirements for GCK-mediated glucose phosphorylation in the liver. F6P is a glycolytic intermediate which acts via feedback inhibition on GKRP to ensure that liver GCK activity and hepatic flux through glycolysis is tightly controlled (Van Schaftingen, et al., 1994). F1P is produced in numerous metabolic pathways, including the phosphorylation of fructose by hepatic fructokinase (Staub and Vestling, 1951; Van Schaftingen, et al., 1994). Fructose is a monosaccharide found in common dietary di/polysaccharides, thus it makes sense that upon ingestion of such foods, relief of GKRP-mediated GCK inhibition in the liver would be beneficial (Clark, et al., 1979; Davies, et al., 1990; Van Schaftingen and Vandercammen, 1989; van Schaftingen, et al., 1992).

Sorbitol and D-glyceraldehyde have also been shown to stimulate GCK activity via their conversion to F1P (Agius, 2008; Davies, et al., 1990; Van Schaftingen and Vandercammen, 1989). F1P (and sorbitol) are more potent inhibitors of GKRP:GCK complex formation than glucose, and also act through a much quicker mechanism as the conformational changes caused by glucose binding to GCK are slower than F1P-mediated GKRP release (Agius, 1994; Agius, 2008; Agius and Peak, 1993). Other physiological mediators have also been shown to modulate GKRP:GCK binding both by direct and indirect mechanisms, and these include the pancreatic hormones insulin and glucagon (Agius and

Peak, 1993). Whether GKRP is a substrate for 5' adenosine monophosphate-activated protein kinase (AMPK) remains controversial as *in vitro* and *vivo* results differ, however this suggests that intracellular mechanisms independent of F1P formation may have a physiological role in the governance of GKRP binding (Agius, 2008; Mukhtar, et al., 2008).

1.22 Glucokinase regulatory protein expression studies in rodent islets remain inconclusive

Controversy remains in the literature as to whether GKRP also regulates GCK activity in pancreatic beta-cells (Alvarez, et al., 2002; Grimsby, et al., 2000; Zawalich, et al., 1977). The majority of studies state that *Gckr* is not expressed in rodent beta-cells (Grimsby, et al., 2000; Vandercammen and Van Schaftingen, 1993; Zawalich, et al., 1977), although Alvarez and colleagues provided evidence to suggest there was an alternatively spliced *Gckr* variant expressed in the beta-cells of rodents, and that this represented the major isoform in this tissue (Alvarez, et al., 2002). *Gckr* mRNA has also been found in islets (Becker, et al., 1996), although this alleged-expression was not reproducible at the protein level. Further evidence in support of pancreatic GKRP comes from the observation that F1P stimulates GCK activity in beta-cell extracts, as does D-glyceraldehyde (via metabolism to F1P) (Malaisse, et al., 1990). Interestingly, fructose has no effect on beta-cell GCK, although the low fructokinase activity in these cells may explain this (Malaisse, et al., 1989). Other studies suggest that there is a protein within rodent beta-cells which fulfills a similar role to GKRP, but this was shown to have different properties to the hepatic inhibitor (Tiedge, et al., 1999). However despite all of this investigation, no studies have been reported which investigate *GCKR* expression in human islets.

Phosphate ester-responsive GKRP has been observed in hypothalamic neurones, suggesting that GCK activity and its modulation by the regulatory protein is important in glucose-sensing within the central nervous system (Alvarez, et al., 2002). There is also some evidence that *GCKR* may be very

lowly expressed in the lungs (Detheux, et al., 1993; Tiedge, et al., 1999), although the physiological importance of this remains unclear.

1.23 Glucokinase regulatory protein has a paradoxical glucokinase stabilisation role

Despite negatively regulating hepatic glycolytic flux, GKRP also has a paradoxical role in which GCK is stabilised at the protein level, thus indirectly enhancing the total activity of this enzyme in the liver. This is most elegantly demonstrated in cellular and rodent models (Farrelly, et al., 1999; Grimsby, et al., 2000; Postic, et al., 2001; Slosberg, et al., 2001).

Gckr over-expression in high fat diet-induced diabetic mice and a human liver cell line - Slosberg and colleagues administered adenovirus containing the human *GCKR* coding sequence into high fat-diet induced diabetic mice (Slosberg, et al., 2001). Despite the well-established inhibitory role of GKRP on GCK activity, the diabetic mice over-expressing *GCKR* unexpectedly showed significantly improved glucose tolerance. They also had lower FPG levels, reduced circulating insulin (meaning increased insulin sensitivity), and lower bodyweight than controls (Slosberg, et al., 2001). However, Western blot analysis of liver lysates showed no increase in GCK protein levels in the *GCKR*-adenovirus transfected animals, nor was there an increase in GCK activity upon liver lysate assay. This contradicted the *in vivo* findings which showed administration of the *GCKR*-adenovirus corrected the high fat diet-induced diabetic phenotype (Slosberg, et al., 2001). To further investigate this paradox, the cells from the human hepatocellular carcinoma cell-line HepG2 were transfected with adenovirus containing either human *GCKR* or *GCK* coding sequence. Unsurprisingly there was increased GCK activity in cells transfected with adenovirus-*GCK*, however the highest GCK activity was observed in cells in which both adenoviruses were co-transfected. These data supported the *in vivo* findings which showed *GCKR* over-expression in the livers of high fat diet-induced diabetic mice to lower blood glucose levels, suggesting GKRP 'stabilised' GCK (Slosberg, et al., 2001). The discrepancy with

the Western and assay data obtained from liver lysates was thought to reflect post-transcriptional down-regulation of hepatic GCK due to decreased circulating levels of insulin (Lynedjian, et al., 1988; Lynedjian, et al., 1989a; Slosberg, et al., 2001).

Homo- and heterozygous Gckr knockout mice - Mice in which 1.7kb of *Gckr* was ablated via homologous recombination were bred to produce hetero- and homozygous knock-outs (Farrelly, et al., 1999). Western blot analyses of liver extracts showed decreased GCK levels in heterozygous knock-outs versus wild-type, and even lower levels again in the homozygous mutant mice. Assay of liver extracts also reflected this trend, with wild-type mice yielding the greatest GCK activity, followed by the heterozygotes, and then the homozygous knock-outs. Despite these differences, mRNA levels remained similar between the three mice groups, supporting the proposal that GKRP plays a positive post-transcriptional regulatory role in maintaining GCK levels within the hepatocyte (Farrelly, et al., 1999).

Gckr knock-out mice were also generated by Grimsby and colleagues using a replacement-type gene targeting vector which replaced exons 4 and 5 of *Gckr* with a neomycin gene cassette (Grimsby, et al., 2000). Again, *Gck* expression levels were lowest in the hetero- and then homozygous *Gckr* knockout mice. Upon assay at low (5mM) glucose, GCK activity was comparable between the three mice groups, however when glucose levels were increased to saturating concentrations (50mM), hepatic *Gck* activity was reduced in both the hetero- and homozygous *Gckr* knockouts versus wild-type mice. This was particularly interesting considering there was no difference in plasma glucose levels between the three genotype groups, although the homozygous *Gckr* knockout did perform worse in an intraperitoneal glucose tolerance test versus the other two sets of mice (Grimsby, et al., 2000). These data agree with that observed in liver-specific *Gck* knockout mice which are only mildly hyperglycaemic, but show defective hepatic glucose utilisation upon challenge with a hyperglycaemic

clamp (Postic, et al., 2001). Additionally, hepatic GCK activity has been shown to be particularly low in cats, an animal which lacks endogenous GKRP (Hiskett, et al., 2009). This further supports the proposal that GKRP serves to stabilise GCK in the liver.

1.24 Glucokinase regulatory protein acts as a nuclear anchor for glucokinase

GCK and GKRP localisation were observed via immunocytochemistry in isolated hepatocytes from wild-type, heterozygous, and homozygous *Gckr* knock-outs (Farrelly, et al., 1999). These studies showed reduced *Gckr* and *Gck* expression in heterozygotes versus wild-type, with the homozygous knock-outs having the lowest *Gck* expression and no GKRP at all (table 1.1) (Farrelly, et al., 1999).

	Low glucose (5.5mM)	**High glucose (27.5mM)**	**Low glucose (5.5mM) plus low fructose (0.5mM)**
***Gckr* (+/+)**	GKRP nuclear GCK nuclear	GKRP nuclear GCK cytoplasmic	GKRP nuclear GCK cytoplasmic
***Gckr* (+/-)**	GKRP nuclear GCK predominantly nuclear, some cytoplasmic	GKRP nuclear GCK cytoplasmic	GKRP nuclear GCK cytoplasmic
***Gckr* (-/-)**	No GKRP GCK cytoplasmic	No GKRP GCK still cytoplasmic	No GKRP GCK still cytoplasmic

Table 1.1: Summary of GCK and GKRP localisation in isolated mice hepatocytes cultured in different glucose and fructose conditions. *Gckr* (+/+) denotes wild-type mice, *Gckr* (+/-) is for heterozygotes knock-outs, and *Gckr* (-/-) represents homozygous knock-out mice.

A glucose concentration of 5.5mM lies within the normal fasting plasma glucose range in humans and mice (Farrelly, et al., 1999; Federation (International Diabetes), 1999; Organisation (World Health), 2006). Postprandial increases in glucose are reflected by the 27.5mM glucose culture medium (Farrelly, et al., 1999). Observing the localisation of GCK and GKRP in the presence of fructose shows that nuclear retention of GCK is mediated by GKRP, as this sequestration is alleviated upon fructose metabolism which yields F1P, an antagonist of GKRP:GCK binding (Van Schaftingen, 1989; Van Schaftingen and Vandercammen, 1989; van Schaftingen, et al., 1992). Interestingly, the effect of

fructose on GCK translocation has been shown to be more potent than that induced by conditions of high glucose, suggesting GCK compartmentalisation is mediated by metabolites/metabolic pathways in addition to absolute glucose levels (Brown, et al., 1997; Farrelly, et al., 1999; Grimsby, et al., 2000).

Immunohistochemical analysis of livers extracted from the second set of *Gckr* knock-out mice (Grimsby, et al., 2000) also agreed with this finding; with GKRP and GCK also localising to the hepatocyte nucleus in non-fasted wild-type and heterozygous *Gckr* knockout mice. However, GCK could not be detected in the homozygous *Gckr* knockouts, not even in the cytoplasm. As enzyme activity and protein levels were drastically reduced in these animals versus their wild-type littermates (41 and 75% less respectively), this could reflect an inability of the anti-GCK antibody to detect such small GCK protein levels (Grimsby, et al., 2000). Studies on isolated rat hepatocytes also showed co-localisation of GCK and GKRP in the nucleus when cultured in low glucose, with GCK alone trafficking to the cytoplasm upon addition of high glucose or low glucose plus low fructose to the medium (Brown, et al., 1997). The data from both *Gckr* knock-out mice therefore proposed that GKRP maintains a nuclear GCK reserve under quiescent metabolic conditions, which can be released upon increasing plasma glucose or low fructose levels. By acting as a nuclear anchor GKRP stabilises GCK so that adequate protein levels are maintained which can respond to changes in hepatic glucose metabolism (Brown, et al., 1997; Farrelly, et al., 1999; Grimsby, et al., 2000). Additionally, GCK has to be actively transported into the hepatocyte nucleus as its molecular weight of 50kilo-Daltons (kDa) means it is too large to diffuse through the nuclear pore complex (Dworetzky and Feldherr, 1988; Shiota, et al., 1999). However, as it lacks a nuclear localisation sequence (Shiota, et al., 1999), results obtained from these rodent models suggest that GKRP facilitates GCK entry into the nucleus by a presumed 'piggy-back' mechanism (Shiota, et al., 1999). This is corroborated by the results of transfected cell-line experiments which showed that three mutant *X. laevis* GCK enzymes which had a reduced affinity for GKRP were incapable of entering the nucleus even when medium glucose

concentrations were low (de la Iglesia, et al., 1999). Conversely, another experiment in which cells were transfected with plasmids encoding *Gckr* and a mutant *Gck* which had a high affinity for the regulatory protein saw the mutant enzyme 'stuck' in the nucleus (Baltrusch, et al., 2005). However, despite the lack of a nuclear localisation sequence, GCK is believed to contain a nuclear export signal between residues 300-310 in a leucine-rich region, which allows it to exit the nucleus independently of GKRP (Agius, 2008; Shiota, et al., 1999).

1.25 Both glucokinase and glucokinase regulatory protein shuttle between the nucleus and cytoplasm

The use of sensitive confocal microscopy and quantitative imaging techniques in cell-based experiments have shown that GKRP also translocates to the cytoplasm upon metabolic stimulation, although the percentage of the overall GKRP pool leaving the nucleus is much less than is observed with GCK (Mukhtar, et al., 1999). Other experiments also support GKRP translocation, with leptomycin B-treatment of HEK-293T cells expressing transfected *GCKR* (not *GCK*) seeing the regulatory protein accumulate in both the nucleus and the cytoplasm (Bosco, et al., 2000). This suggested that the regulatory protein was capable of intracellular 'shuttling' between the two cellular compartments (Bosco, et al., 2000). Another study did however show that leptomycin-B did not affect nuclear export of either GCK or GKRP (Mukhtar, et al., 1999). *In situ* perfusion of male Wistar rat livers with 20mM glucose for 10 minutes also showed an increase in the cytoplasmic:nuclear GKRP ratio, further supporting the intracellular GKRP shuttling hypothesis (Toyoda, et al., 1995).

1.26 Glucokinase translocation is responsive to nutrient- and metabolic signals in vivo

GKRP-mediated retention of GCK in the hepatocyte nucleus is a mechanism of post-transcriptionally maintaining adequate levels of this enzyme for effective postprandial glucose clearance (Brown, et

al., 1997; Farrelly, et al., 1999; Grimsby, et al., 2000). Modulation of GKRP-binding and release of GCK into the cytoplasm is regulated by numerous nutrient and hormonal stimuli (Agius and Peak, 1993), coupling hepatic GCK activity to the metabolic milieu. Metabolites which can be converted to F1P (or analogous compounds) stimulate GCK dissociation from GKRP and hepatic glucose phosphorylation, and include; fructose, D-glyceraldehyde, sorbitol and tagatose (Clark, et al., 1979; Van Schaftingen, 1989; Van Schaftingen and Davies, 1991; Van Schaftingen and Vandercammen, 1989; van Schaftingen, et al., 1992). Conversely, the glycolytic intermediate F6P enhances GKRP-mediated inhibition of hepatic GCK activity, thus providing a level of negative feedback regulation of glycolytic flux (Van Schaftingen, 1989; van Schaftingen, et al., 1992).

A study investigating GCK and GKRP localisation in the livers of Wistar rats in different nutritional states demonstrated that this GCK release and intracellular trafficking are dynamic processes that occur *in vivo* (Fernandez-Novell, et al., 1999). Immunohistochemical staining was carried out on liver sections obtained from fasted (24hours) and re-fed (one, two, or four hours) rats. Both GKRP and GCK localised to the hepatocyte nucleus in the fasted rodents, whilst the cytoplasmic:nuclear GCK ratio increased after one, and further still upon two hours after feeding [although this translocation was not as marked as occurred in previous isolated hepatocyte experiments (Brown, et al., 1997)]. However, four hours postprandial most GCK had returned to the nucleus. GKRP remained predominantly nuclear-bound regardless of nutritional state (Fernandez-Novell, et al., 1999). *In vivo* translocation of hepatic GCK has also been seen in response to intraduodenal glucose infusions in conscious rats (Chu, et al., 2004). Fructose also increases hepatic glucose uptake in conscious dogs [via both intraportal (Shiota, et al., 1998) and intraduodenal (Shiota, et al., 2002) administration], and stimulates hepatic glycogen synthesis in healthy humans (Petersen, et al., 2001). Both studies provide further evidence for the alleviation of GKRP-mediated GCK inhibition directly affecting hepatic physiology.

Numerous rodents with defective nutrient-responsive GCK translocation have been reported, including the Otsuka Long-Evans Tokushima Fatty (OLETF)- (Kawano, et al., 1994) and Goto-Kakizaki (GK) rats (Tsuura, et al., 1993), both of which are models of T2D (Toyoda, et al., 2000). It was proposed that the hyperglycaemia in both models resulted from reduced GCK translocation from the nucleus to cytoplasm in isolated hepatocytes incubated in high glucose/fructose medium. The GK rat also exhibited a lower glucose phosphorylation rate versus controls, further suggesting these rodents were less capable of hepatic glucose uptake and utilisation (Toyoda, et al., 2000). Defective GCK translocation is also seen in the Zucker diabetic fatty rat (Fujimoto, et al., 2004; Fujimoto, et al., 2006; Shin, et al., 2007). As Zucker rats have defects in the leptin receptor (Kurtz, et al., 1989), this suggests that factors other than GKRP may be affecting hepatic GCK translocation (Agius, 2008). The role of PFK-2/FBPase has also been considered as fructose-2,6-bisphosphate is elevated in Zucker rats (Payne, et al., 2007; Payne, et al., 2005). Site-directed mutagenesis experiments have also shown that constitutive inactivation of PFK-2/FBPase may counteract the effects of glucagon on GCK translocation, thus enhancing enzyme trafficking (Agius, 2008; Baltrusch, et al., 2001; Payne, et al., 2005). However, these mechanisms require further investigation before a definite physiological role can be firmly established.

1.27 The association of glucokinase regulatory protein gene with metabolic disorders and relevant quantitative traits highlights the importance of hepatic glucokinase regulation

Given the importance of GKRP in regulating hepatic GCK activity and glucose utilisation, it is perhaps unsurprising that genome wide association (GWA) studies have shown *GCKR* to be associated with T2D risk (Dupuis, et al., 2010). Interestingly, an early GWA looking for T2D susceptibility loci found an intronic SNP on chromosome 2 that associated with the unusual phenotype of lowered FPG and raised triglycerides (Saxena, et al., 2007). This was surprising as elevated triglycerides normally

accompany insulin resistance and hyperglycaemia as occurs in T2D (Reaven, 1988). Fine mapping showed the genetic signal came from a common non-synonymous SNP within *GCKR* (rs1260326, c.1403C>T, p.P446L, MAF 34%) (Orho-Melander, et al., 2008; Saxena, et al., 2007). Further evidence to support the association of this biologically plausible gene with the unusual FPG/triglyceride phenotype came from a study by O'Doherty and colleagues, who showed that *in vivo* over-expression of *Gck* in rat hepatocytes led to the expected drop in FPG levels, but was also accompanied by an increase in circulating triglycerides (O'Doherty, et al., 1999)

The *GCKR* locus has also since been shown to associate with other metabolic traits, including; fasting insulin, homeostatic model assessment of insulin resistance (HOMA-IR) (Dupuis, et al., 2010), C-peptide, insulin sensitivity (Ingelsson, et al., 2010), two hour glucose (Saxena, et al., 2010), total cholesterol (Teslovich, et al., 2010), serum uric acid (Kolz, et al., 2009), c-reactive protein (Ridker, et al., 2008), creatinine (Kottgen, et al., 2010), albumin (Kamatani, et al., 2010), plasmalogen (phosphatidylcholine) (Illig, et al., 2010), protein C (Tang, et al., 2010), factor VII (Smith, et al., 2010), and Crohns disease (Franke, et al., 2010). Given the broad spectrum of associations it is extremely possible that GKRP-mediated regulation of GCK activity effects many more metabolic pathways than previously appreciated.

1.28 Aims of thesis

1 – To functionally characterise GCK mutations which occur at the same amino acid residue, but which cause opposite clinical phenotypes

Pharmacological GKAs are currently under-development as a potential new therapeutic strategy against T2D. These drugs seek to mimic naturally occurring *GCK* activating mutations in order to increase the activity of this key glycolytic enzyme. Through collaboration with Prof Sian Ellard (Peninisula Medical School, University of Exeter, UK), Dr Paul Arundel (Sheffield Childrens' NHS Foundation Trust, UK), and Prof Constance Chik (University of Edmonton, Canada), we have identified two novel *GCK* mutations in HH patients. Importantly, these mutations are at residues where GCK-MODY mutations had previously been reported (Osbak, et al., 2009) suggesting they are of great regulatory importance. I aim to characterise these mutations with regards to their intrinsic biochemical properties, as well as their response to physiological (GKRP) and pharmacological (GKA) regulators. It is hoped that this will provide further insight into critical sites of activity/regulation in this important drug target.

2 – To kinetically characterise the P446L GCKR variant associated with FPG and triglyceride levels to establish whether it is as capable of inhibiting GCK as wild-type regulatory protein

The difficulty in characterising the mutational mechanisms behind most GWA genetic signals is that they often fall within non-coding regions of the genome, thus it is hard to connect the association with nearby genes and thereby attribute function (McCarthy, et al., 2008; McCarthy and Hirschhorn, 2008a; McCarthy and Hirschhorn, 2008b). The association of the non-synonymous P446L *GCKR* variant with FPG and triglyceride levels (Orho-Melander, et al., 2008; Saxena, et al., 2007; Sparso, et al., 2008; Vaxillaire, et al., 2008) is an exciting find as it facilitates functional studies which will

decipher the mutational mechanism behind this mutated protein. Previous kinetic characterisation using rat recombinant P446L-GKRP showed that the variant inhibited GCK no differently to wild-type regulatory protein, thus failing to explain the initial genetic association (Veiga-da-Cunha, et al., 2003). However, as there are intrinsic differences between rat and human GKRP (Brocklehurst, et al., 2004a), I aim to re-characterise P446L-GKRP at the kinetic level using recombinant human proteins. Additionally, *GCKR* expression in human islets remains to be definitively established. Therefore, I also aim to quantitate *GCKR* mRNA levels in human islets and observe if/whether they are present at functionally-relevant levels versus *GCK*. This would explain whether the effect of P446L-*GCKR* on FPG was driven by the just the liver, or also the beta-cell as well.

3 – To observe whether the nuclear sequestration of GCK is altered with the P446L-GKRP variant

GKRP is essential for GCK localisation to the nucleus; the regulatory protein acting as a chaperone due to the lack of a nuclear localisation signal within the enzyme (Agius, 2008; Shiota, et al., 1999). I aim to investigate whether P446L-GKRP has an altered ability to bind GCK and sequester it in the cell nucleus using fluorescent fusion proteins and confocal microscopy, as defective GCK localisation and trafficking could impact upon glycolytic flux through this enzyme.

4 – To determine whether GCKR mutations cause monogenic disorders of glucose homeostasis

It is well-established that *GCK* mutations cause disorders in which glucose homeostasis is perturbed (Osbak, et al., 2009). However, not only does GKRP has a pivotal role in acutely regulating hepatic GCK activity (Van Schaftingen, 1989; Van Schaftingen and Vandercammen, 1989), but a genome-wide linkage scan has also showed linkage between an area on chromosome 2p (to which *GCKR* maps) and MODY of unknown genetic aetiology (Lindgren, et al., 2007). Together this suggests that *GCKR* mutations could indirectly perturb glucose metabolism enough to have pathogenic effects on

glycaemia. I therefore aim to screen *GCKR* in patient collections from Prof Sian Ellard and Dr Anna Gloyn, including; 'GCK-like MODY' patients (have a similar phenotype to GCK-MODY but no mutations within *GCK*), 'GCK-like HH' patients (again similar phenotype to GCK-HH but absent for *GCK* mutations), and normoglycaemic controls.

Chapter 2

Materials & Methods

2. Materials & Methods

2.1 Polymerase chain reaction

Polymerase chain reaction (PCR) reagent mix was set-up as described in table 2.1. Forward and reverse primers were designed using Primer3 and OligoCalc web-based software (http://frodo.wi.mit.edu/ and http://www.basic.northwestern.edu/biotools/oligocalc.html respectively) using gene sequences obtained from the University of California Santa Cruz Genome Bioinformatics or National Center for Biotechnology Information websites (http://genome.ucsc.edu/ and http://www.ncbi.nlm.nih.gov/ respectively). Details of all primers are included in the methods sections of the relevant experimental chapters. All PCR primers were obtained from Eurofins MWG Operon (Ebersberg, Germany) and had to have a melting temperature between 55-65^{o}C, a length of 18-25 nucleotides, and have a GC content of 40-60%. Universal M13 sequences were added onto the primers for ease of subsequent sequencing; forward $^{5'}$TGTAAAACGACGGCCAGT$^{3'}$, reverse $^{5'}$CAGGAAACAGCTATGACC$^{3'}$.

Reagent	**Concentration in reaction (mM)**
AmpliTaq Gold Buffer II (10x)	n/a
$MgCl_2$	1.5
dNTPs	0.1
Forward Primer	0.0002
Reverse Primer	0.0002
Distilled Water	n/a
	Concentration in reaction (U)
Amplitaq Gold	4.0

Table 2.1: PCR reagents for n=1 reaction. Amplitaq Gold Buffer II, Amplitaq Gold, magnesium chloride ($MgCl_2$), and deoxynucleoside triphosphates (dNTPs) all Applied Biosystems (Warrington, UK). One enzyme unit (U) catalysed the conversion of one µmole substrate per minute under standard reaction conditions.

Twenty three microlitres of PCR reagent mix was added to each reaction, plus 40ng (2µl of 20ng/µl) DNA sample. Following brief mixing via centrifugation, samples were amplified on a PTC-225 Peltier Thermal-Cycler (MJ Research, Waltham, Massachusetts, USA) using the conditions below**.**

Programme section	Number of cycles	Temperature (^{o}C)	Time (mins)
Polymerase activation	1	95	12.0
DNA melting	35	94	0.5
Primer annealing		58	0.5
Synthesis and extension		72	1.0
Final extension	1	72	10.0

Table 2.2: PCR thermal-cycler reaction conditions.

2.2 DNA visualisation using agarose gels and ethidium bromide staining

Two percent agarose gels were made from 3g agarose (Sigma Aldrich Company Ltd, Gillingham, UK) dissolved in 150ml Tris/Borate/Ethylenediaminetetraacetic acid (TBE) buffer (NBS Biologicals, Huntingdon, UK); this solution then heated in an 800W microwave for 2 minutes to aid dissolution. Once cooled below 50^{o}C, 3µl ethidium bromide (to give a 0.002% solution) was added to the agarose-TBE, with this mixture then poured into a MultiSUB midi gel electrophoresis tank containing appropriately spaced well combs (Appleton Woods Ltd, Birmingham, UK). This gel was then allowed to set at room temperature for 30 minutes. Once the gel had solidified, the tank was filled with 500ml TBE buffer. Five microlitres of PCR product were then mixed with 5µl orange-G loading buffer (Sigma Aldrich Company Ltd), which was then vortexed briefly. PCR product-orange-G samples were then loaded onto the gel, as was either 3.5µl of a 100bp or 1kb DNA ladder (New England Biolabs UK Ltd, Hitchin, UK and Promega UK Ltd, Southampton, UK respectively). The ladder used depended on the size of the PCR product to be resolved. DNA electrophoresis was carried out by passing 120V across the gel, the duration of electrophoresis again dependent upon the size of the PCR product to be

resolved (usually 1 hour). The electrophoresed gel was then viewed with a Gel Doc 2000 using Quantity One v4.3.1 software (both Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

2.3 DNA sequencing

The sequence of all PCR-amplified products was obtained via a modified version of the chain-termination (Sanger) sequencing method using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). This kit included fluorescently-labelled dideoxynucleotide triphosphates (ddNTPs); each of the four ddNTPs types having a different fluorescence emission spectrum. These ddNTPs were included in sequencing reactions which also contained PCR product template, PCR product-specific primers, un-labeled dNTPs, and DNA polymerase. The ddNTPs became incorporated into the elongating DNA strand during the sequencing reaction, however their lack of a 3'-hydroxyl group meant they were unable to form a phosphodiester bond with the next nucleotide in the sequence and so there was premature chain termination. This produced sequencing products of varying length which ended with a fluorescently-labelled ddNTP tag. Analysis of these fragments via capillary electrophoresis (which separated these fragments based on charge/length) and fluorescence detection was then carried out, and thus allowed the sequence of the original PCR products to be determined (Rosenblum, et al., 1997; Sanger and Coulson, 1975; Sanger, et al., 1977; Smith, et al., 1986; Tracy and Mulcahy, 1991).

In detail, these sequencing reactions involved the following three distinct steps.

2.3.1 Clean-up of PCR products

Exonuclease (EXO) (New England Biolabs UK Ltd) and shrimp alkaline phosphatase (SAP) (Promega UK Ltd) were added together in a 1:1 volume ratio, which was equivalent to 20 and 1 unit of each enzyme respectively per microliter. One microlitre of EXO-SAP mix was then added to 1µl of PCR

product and mixed briefly. These samples were then incubated at 37°C for 30 minutes, followed by heating at 80°C for 15 minutes to inactivate the enzymes.

2.3.2 Sequencing reactions

The constituents of the sequencing mastermix are given in table 2.3. Eight microlitres of this mastermix was then added to 2µl PCR product-EXOSAP reaction and mixed briefly before submission to the sequencing reaction thermal-cycler conditions given in table 2.4.

Reagent	Volume added to reaction (µl)
BigDye terminator v1.1 mix	0.25
BigDye reaction buffer	1.88
M13 Sequencing primer (2.5µM)	2.00
Distilled water	3.88

Table 2.3: DNA sequencing reagents for n=1 reaction. BigDye reaction mix and buffer were supplied as part of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems catalogue #4337450). Either M13 forward or reverse primers were used to amplify PCR products which had been produced using M13-tagged PCR primers (both Eurofins MWG Operon).

Programme section	Number of cycles	Temperature (°C)	Time (secs)
Denaturation and activation	1	95	10
Primer annealing and extension	25	50	5
		60	240

Table 2.4: Thermal-cycler reaction conditions for DNA sequencing.

2.3.3 Sequencing product clean-up

Five microlitres of distilled water was added to each sequencing reaction to make a total sample volume of 15µl. Unused sequencing reaction reagents were removed from the final product using 96well Performa DTR V3 short clean-up plates (VH Bio, Gateshead, UK) according to the

manufacturer's guidelines. Briefly, Performa plates were centrifuged at 1600xg for 3 minutes on a Heraeus Multifuge 3S-R centrifuge (DJB Labcare Ltd, Newport Pagnell, UK) to remove residual buffer. This plate was then placed on top of an empty 96well non-skirted PCR plate (ThermoScientific, Cramlington, UK) and 15µl sequencing reaction-water mix transferred to its individual wells. This was then centrifuged at 1600xg for 5minutes, at the end of which the 'clean' sequencing products had eluted into the 96 well plate. Residual water was then removed from the sequencing products via evaporation by heating the 96 well plate on a thermal-cycler for 90 minutes at 70^{o}C. Samples were sequenced via capillary electrophoresis by Lynne Richardson at the University's core sequencing facility within the Department of Zoology using an ABI3730xl DNA Analyser (standard run module, 15 seconds injection, v1.1 dye set and analysis module). DNA sequences and corresponding electropherograms were viewed using the BioEdit Sequence Alignment Editor (Ibis Therapeutics, California, USA) and Mutation Surveyor v3.24 (SoftGenetics, Cambridge, UK), and compared to appropriate reference sequences obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/nuccore/).

2.4 Site-directed mutagenesis

Site-directed mutagenesis (SDM) was carried out using the QuikChange Site-Directed Mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer's guidelines. A series of reactions with a range of template DNA amounts were routinely set up, as mutagenesis efficiency differed depending on template/mutation to be introduced. All reaction mixes contained the reagents listed in table 2.5, and SDM and template amplification was carried out using the thermal-cycler conditions given in table 2.6.

Reagent	Volume added to reaction (µl)
10x Buffer	5.0
dNTP mix	1.0
Forward mutagenesis primer (125nM)	1.0
Reverse mutagenesis primer (125nM)	1.0
Pfu polymerase (2.5U/µl)	1.0
DNA template (0-50ng)	As appropriate
Distilled water	Volume made up to 50µl

Table 2.5: SDM reagents for n=1 reaction. The 10x buffer, dNTP mix, and *Pfu* polymerase were supplied as part of the QuikChange Site-Directed Mutagenesis Kit (Stratagene Europe). Details of site-directed mutagenesis primers are given in the appropriate experimental chapters, and all were obtained from Eurofins MWG Operon.

Programme section	Number of cycles	Temperature (^{o}C)	Time (mins)
Polymerase activation	1	95	0.5
DNA melting	35	95	0.5
Primer annealing		55	1.0
Primer extension		68	12.0

Table 2.6: SDM thermal-cycler reaction conditions.

SDM reaction products were then cooled on ice for 2 minutes, before being submitted to *DpnI*-mediated digestion to remove non-mutagenised template DNA.

2.5 DpnI-mediated removal of template DNA

Ten microlitres of the 50µl SDM reaction product were removed and placed in a clean 0.2ml PCR tube (Alpha Laboratories Ltd, Eastleigh, UK), this designated the 'undigested SDM product'. To the remaining 40µl was added 1µl (20U) *DpnI* restriction endonuclease (Stratagene Europe), and contents mixed briefly. The *DpnI*-containing sample was then placed on a thermal-cycler and heated at 37^{o}C

for 90 minutes, before enzyme inactivation at 65°C for 20 minutes. Digested and undigested SDM samples were then viewed on a 2% agarose gel to check that i) the SDM reaction had worked, and ii) that *DpnI*-digestion had not destroyed all of the mutagenesis product. This was confirmed by the presence of appropriately-sized DNA bands on the gel for both digested and undigested samples.

2.6 Preparation of agar-antibiotic culture plates

Seventeen and a half grams of agar (Sigma Aldrich Company Ltd) were dissolved in 500ml distilled water in a sterile 1L glass conical flask (Fisher Scientific Ltd, Loughborough, UK), and mixed using a magnetic stirrer and stirring block (Jencons Scientific Ltd, East Grinstead, UK). Following dissolution, the mouth of the flask was covered with tin foil and autoclave tape (Fisher Scientific Ltd). Flasks containing the agar solution were then sterilised by heating at 120°C for 20 minutes, and then 120-60°C for one hour in a steam autoclave (Priorclave Ltd, London, UK). Upon completion of the autoclave cycle, the flasks were cooled at room temperature. Once below 35°C, 30mM ampicillin or kanamycin (for pGEX-3X and pFLAG-CTC/pmCherry-N1/pEGFP-C3 vectors respectively) was added to the sterilised agar. The agar-antibiotic solution was then mixed by briefly 'swirling' the contents of each flask, before being poured into sterile plastic culture plates (Appleton Woods Ltd) where it was allowed to set as a ~1cm deep gel at room temperature. Once solid, the plates were sealed with parafilm (Fisher Scientific Ltd) and stored at 4°C.

2.7 Preparation of lysogeny broth medium for bacterial culture

This protocol was as for the preparation of agar-antibiotic culture plates, but with the following modifications. Ten grams lysogeny broth (LB) medium (Sigma Aldrich Company Ltd) was dissolved in 500ml distilled water. After sterilisation and addition of 30mM antibiotic, the solution was stored at 4°C until further use.

2.8 Transformation of competent Escherichia coli

Ten nanograms of plasmid DNA (0.5µl of a 20ng/µl working stock) were added to 0.5µl 1.42M beta-mercaptoethanol and 30µl supercompetent *E.coli* (Stratagene Europe). The reaction reagents were mixed gently by flicking the tube with the index finger, and incubated on ice for one hour. Following this incubation, the *E.coli* were transformed via 'heat-shock' at 42^{o}C for 30 seconds in a pre-heated water bath. The reactions were then immediately transferred back onto ice and left to recover for two minutes. Five hundred microlitres of super optimal broth with catabolite repression (SOC) medium (Sigma Aldrich Company Ltd) was added to each reaction, followed by incubation at 37^{o}C, 250rpm, for 90 minutes. Transformation reactions were then removed from the incubator, and 100µl added to and spread across an agar-ampicillin plate. This culture plate was then incubated at 37^{o}C overnight, and the following day wrapped in parafilm before storage at 4^{o}C.

2.9 DNA extraction from transformed Escherichia coli

SCS1 Escherichia (E) coli are a commercially-available strain of bacteria (Stratagene Europe) that are routinely used to amplify plasmid DNA. This is because they are both endonuclease- and recombination-deficient, thus meaning less degradation of extracted DNA and increased stability of the transformed plasmid respectively. Transformed SCS1 colonies were picked from agar-antibiotic plates and used to inoculate 5ml LB-antibiotic medium and cultured overnight at 37^{o}C, 250rpm. The next day, bacteria were pelleted via centrifugation at 14,000xg for 10minutes in a force 1618 desktop microcentrifuge (Labnet International, Rutland, UK). Following centrifugation the supernatant was removed and discarded, and DNA extracted according to the QIAprep Spin Miniprep Kit manufacturer's guidelines with elution in 50µl distilled water (QIAgen Ltd, Crawley, UK).

2.10 DNA quantification

The Nanodrop 2000 (ThermoScientific) was used to quantitate DNA concentration by measuring sample absorbance at 260nm. It was also able to determine sample purity by recording the absorbance of proteins and phenol/other organic compounds at 280 and 230nm respectively. DNA concentration and purity were determined from 1µl of sample using the 'nucleic acids' and 'DNA50' application programmes following blanking of the machine using distilled water (or whichever buffer the DNA sample was dissolved in).

2.11 Restriction digest of DNA

All restriction digests contained the same basic components given in table 2.7. Reactions were incubated at 37^{o}C for 2hours (or another temperature/time suggested by enzyme manufacturer), and then inactivated as appropriate. Restriction enzymes were sourced from New England Biolabs UK Ltd or Fermentas Life Sciences (Sheriff Hutton, UK).

Reagent	Volume added to reaction (µl)
Template DNA	5.0
Restriction enzyme	1.0
Restriction enzyme buffer (10x)	2.0
Distilled water	To make volume up to 20µl

Table 2.7: Restriction digest reagents for n=1 reaction. Details of the specific restriction enzymes used given in the appropriate experimental chapters.

2.12 Recombinant human glucokinase production

The restriction enzymes *Hind*III and *Bam*HI were used to clone the human glucokinase (*GCK*) neuroendocrine coding sequence NM_000162 into the pGEX-3X vector (Sigma Aldrich Company Ltd).

This construct was a gift from Prof Mark Magnuson of Vanderbilt University (Nashville, Tennessee, USA). Recombinant human GCK in the form of a fusion protein with glutathione s-transferase (GST) was purified following heterologous expression in BL21 supercompetent *E.coli* (Stratgene Europe) and affinity chromatography via a glutathione-agarose column (Sigma Aldrich Company Ltd).

In detail, a single transformed BL21 colony was picked following culture on an agar-ampicillin plate and used to inoculate 5ml LB-ampicillin medium. This was then cultured overnight at 37^{o}C, 250rpm. The following day, this 5ml culture was added to 500ml LB-ampicillin medium, which was then also cultured at 37^{o}C, 250rpm. Protein production was induced by addition of 0.2M isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma Aldrich Company Ltd) once the optical density (OD) at 600nm had reached 0.6. Following addition of IPTG, the culture flask was incubated overnight at 22^{o}C, 150rpm. The following day, the bacterial cells were pelleted via centrifugation at 3,500xg, 4^{o}C, for 10 minutes. The pellet was resuspended in ice-cold phosphate-buffered saline-dithiothreitol (PBS-DTT; Sigma Aldrich Company Ltd), and the bacterial cells lysed using a VibraCell sonicator (amplitude 20, 40% max power [Jencons Scientific Ltd]). Ten percent Triton-X (Sigma Aldrich Company Ltd) was then added to the lysed cells, and the solution mixed gently via inversion. The sample was then centrifuged at 5,000xg, 4^{o}C, for 8 minutes, so as to pellet cell debris yet leave the cytoplasmic proteins in suspension. Glutathione-agarose dissolved in PBS-DTT was then added to this cytoplasmic protein-supernatant, and the resulting solution mixed by gentle inversion for 2 minutes to allow GST-tag-glutathione binding.

Following mixing the solution was taken into the 4^{o}C cold room where it was poured into a PolyPrep chromatography column (Bio-Rad Laboratories Ltd). This allowed the slow elution of buffer/unwanted cellular proteins whilst retaining the recombinant glucokinase bound to the glutathione agarose slurry in the body of the column. Once the column contents had been washed

adequately, bound recombinant GCK was eluted by addition of glutathione elution buffer. Eluent fractions were collected in 1.5ml micro test tubes (Alpha Laboratories Ltd) and quickly stored on ice. The amount of protein in each eluent fraction was determined using fivefold-diluted Bradford reagent (Bio-Rad Laboratories Ltd) and measuring sample absorbance at 595nm. The highest protein-yielding eluent fractions were then pooled together and the total volume noted. Storage buffer was added to the pooled eluents in a ~2:3 ratio before snap-freezing on dry ice and storage at -80^{o}C (shelf life ~2-4months).

2.12.1 Glutathione elution buffer

The elution buffer contained; 6µM glutathione (GE Healthcare Biosciences, Amersham, UK), 0.8M potassium chloride (KCl), 0.2M pH6.8 tris(hydroxymethyl)aminomethane-hydrochloric acid (Tris-HCl), and 0.02M DTT (all Sigma Aldrich Company Ltd). The solution was mixed by vortexing and stored at 4^{o}C away from light.

2.12.3 Glucokinase storage buffer

The storage buffer contained; 5.5mM glucose, 0.25M KCl, 0.06M pH8.0 Tris-HCl, and 60ml glycerol (all Sigma Aldrich Company Ltd). Before addition to eluted glucokinase, a working stock was made which contained; 10ml storage buffer and 5mM DTT. The storage buffer for glucokinase regulatory protein (GKRP) was exactly the same as that for GCK, but minus the glucose.

2.13 Recombinant human glucokinase regulatory protein production

The restriction enzymes *Nde*I and *Sal*I were used to clone the human GKRP sequence NM_001486 into the pFLAG-CTC vector (Sigma Aldrich Company Ltd). This construct was a gift from Katy Brocklehurst (AstraZeneca, Alderley Park, Cheshire, UK) (Brocklehurst, et al., 2004a). Recombinant human GKRP in the form of a fusion protein with the FLAG octa-peptide was purified following

heterologous expression in BL21 *E.coli* and affinity chromatography via an anti-FLAG-agarose column (Sigma Aldrich Company Ltd).

The GKRP purification process was as for human GCK, but with the following modifications. Twelve one litre cultures of LB-ampicillin medium were prepared from transformed *E.coli* colonies, with IPTG-induced protein production as before. The sonicated cell lysates were centrifuged twice at 5000xg for 8 minutes at 4^{o}C to pellet the substantial amount of cell debris, which was subsequently discarded. Sodium chloride was added to the retained supernatant to make a final concentration of 0.2M, and pH was adjusted 7-8 [to mimic intracellular conditions (Graslund, et al., 2008)]. Anti-FLAG M2 affinity resin was added to the protein sample in the 4^{o}C cold room, before incubation and gentle mixing upon the belly-dancer laboratory shaker (Fisher Scientific Ltd) for 2 hours. Following incubation, the resin-recombinant GKRP were pelleted via centrifugation at 1000xg, 4^{o}C, for 5 minutes. Recombinant GKRP was eluted by addition of FLAG peptide dissolved in PBS (Sigma Aldrich Company Ltd), and the sample concentration determined using Bradford reagent as for GCK. Storage buffer minus glucose was then added to this protein before storage at -80^{o}C.

2.14 Assessment of recombinant protein purity

Recombinant protein purity was assessed using the Agilent protein 230 kit (Agilent Technologies UK Ltd, Stockport, UK) according to manufacturer's guidelines. The cut-off for acceptable purity was 95%. Briefly, protein samples and ladder were denatured in a denaturation solution by heating at 95^{o}C for five minutes. These samples were then added to a 12 well protein chip along with a protein dye-gel matrix. This loaded chip was then placed into an Agilent 2100 bioanalyser, and the electrodes inserted into each well on the chip, before electrophoresis of the samples. Electrophoretic analysis was then able to identify the number of different proteins in each sample tested, and their molecular mass.

2.15 Quantification of recombinant protein concentration

The concentration of all recombinant protein preparations was determined using Bradford reagent (Bio-Rad Laboratories Ltd) and colourimetric assays. The colours of unbound and protein-bound Bradford reagent differ, the latter absorbing light at 595nm, thus the protein concentration within each of our samples could be determined. Ten 0-2mg/ml protein standards were made to produce a standard curve for these experiments, and these contained; bovine serum albumin (BSA; Sigma Aldrich Company Ltd), distilled water, and Bradford reagent. Each sample was also diluted in distilled water and Bradford reagent, with numerous dilutions needed to ensure that the sample concentration lay within the middle of the standard curve. The protein standards and samples were in triplicate within a 96well spectrophotometer plate, and absorbance at 595nm recorded using a Versamax ELISA microplate reader and Softmax pro v5.2 software (both Molecular Devices Ltd, Wokingham, UK).

2.16 Glucokinase regulatory protein-mediated competitive inhibition assays

GKRP-mediated inhibition of GCK activity was determined spectrophotometrically using glucose 6-phosphate dehydrogenase-linked assays based on published protocols (Brocklehurst, et al., 2004a; Liang, et al., 1995; Veiga-da-Cunha, et al., 1996b). One milli-unit (m-unit) GCK was defined as that which converted one nmole substrate per minute. One GKRP unit inhibited 10m-units GCK by 50% under standard assay conditions (5mM glucose, no phosphate esters) (Brocklehurst, et al., 2004a). One unit of regulatory protein was used per equal activity assay in the presence of 10m-units wild-type GCK.

GKRP competitive inhibition assays were carried out at, 37^{o}C, pH7.1, and at 5mM glucose (**chapter 3**) or over a 0-100mM glucose concentration range (**chapter 4**). Two separate reaction mixes were made on ice; the first containing GCK (A), the second with GKRP and glucose (B). Both mixes contained

25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 0.5mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 1mM adenosine triphosphate (ATP), 2mM $MgCl_2$, 25mM KCl, 1mM DTT, 3.8µM BSA, 8.6 or 13.2ml distilled water (mix 'A' and 'B' respectively). Mix B also contained 5 or 0-100mM glucose. All reagents were from Sigma Aldrich Company Ltd, except ATP and $NADP^+$ which were from Roche Diagnostics Ltd (Burgess Hill, UK). The pH of both reaction mixes was checked to ensure it was 7.1 before addition of 4units glucose 6-phosphate dehydrogenase (Sigma Aldrich Company Ltd), as pH influenced reaction rate (Dixon, 1953b).

Ten m-units wild-type GCK was then added to eight glass test tubes (Fisher Scientific Ltd) containing 1ml reaction mix A, and mixed briefly by vortex. GKRP was then added to 12 glass test tubes containing 1ml mix B and glucose. Two 96 well spectrophotometer plates (Appleton Woods Ltd) were then set up, plate one containing GCK in mix A, plate 2 containing GKRP and glucose in mix B. An eight well mechanical multi-channel pipette (Biohit Ltd, Torquay, UK) was then used to transfer the contents of plate two to the corresponding well in plate one. Reaction rate was then measured using a Versamax ELISA microplate reader and Softmax Pro v5.2, which recorded the rate of NADPH formation based on absorbance at 340nm. Data was represented as 'percentage GCK activity', which was normalised to that obtained with no GKRP. The Ic_{50} values (half maximal inhibitory concentration) for GKRP-mediated inhibition was also calculated from this data. Final result graphs were plotted using Sigmaplot v11.0 (Systat Software Inc., Hounslow, UK).

2.17 Glucokinase glucose-dependent assays

The reaction mix was prepared on ice and contained; 7.5mM $MgCl_2$, 187.5mM KCl, 125mM HEPES buffer, 1.25mM $NADP^+$, 6.25mM ATP, 2.5mM DTT, 0.125% BSA, and 6.45ml distilled water. The pH of both reaction mixes was checked to ensure it was 7.4 before addition of 66units of glucose 6-phosphate dehydrogenase (Sigma Aldrich Company Ltd). This reaction mix was used to make 1ml

0.2M glucose solution (Sigma Aldrich Company Ltd), before the remainder of the mix was diluted using 3.5ml distilled water. This diluted reaction mix was then used to serially dilute the 0.2M glucose stock to produce the following concentration range (mM); 100.00, 50.00, 25.00, 12.50, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.00. Ten m-units GCK were then added to eight glass test tubes containing 1ml diluted reaction mix A, which was mixed then mixed briefly via vortex. Two 96 well spectrophotometer plates were then set up, plate one containing the diluted glucose samples, and plate two containing the diluted GCK enzymes to be tested. The contents of plate two were then transferred to the corresponding well in plate one, and reaction rate measured as for section 2.16. Kaleidagraph v3.5 (Synergy Software, Reading, Philadelphia, USA) was used to plot all assay data, with subsequent fitting of the Hill equation (Hill, 1910; Kesavan, et al., 1997; Matschinsky, et al., 1998; Storer and Cornish-Bowden, 1976) allowing the accurate calculation of the half maximal glucose concentration ($S_{0.5}$) and Hill number (n_H).

(2.1) $$v = (S^{nH} \times V_{max}) / (S^{nH} \times S_{0.5}{}^{nH})$$

Equation 2.1: The Hill equation (Hill, 1910; Kesavan, et al., 1997; Matschinsky, et al., 1998; Storer and Cornish-Bowden, 1976). 'v' is reaction rate, 'S' represents glucose concentration, and '$S_{0.5}$' the half maximal glucose concentration. To fit the assay reaction data (v) over the whole glucose concentration range (S), Kaleidagraph was provided with estimates of each of the equation parameters. The estimated values used were V_{max} = 10, $S_{0.5}$ = 7.5, and n_H = 1.5.

The maximal reaction rate (V_{max}) and total enzyme concentration (E_t) were then used to calculate the overall turnover rate of the enzyme (K_{cat}; equation 2.4), which was derived from a rearrangement of the Michaelis-Menten equation [equation 2.2; (Lineweaver and Burk, 1934; Michaelis and Menten, 1913; Roskoski, 1995)].

(2.2) $v = (V_{max} \times S) / (K_m + S)$

(2.3) $V_{max} = K_{cat} \times E_t$

(2.4) $K_{cat} = [V_{max} \times (K_m + S)]/(E_t \times S)$

Equations 2.2, 2.3, and 2.4: Michealis-Menten equation (Lineweaver and Burk, 1934; Michaelis and Menten, 1913; Roskoski, 1995) is shown in equation 2.2, where K_m corresponds to glucose $S_{0.5}$ and ATP_{Km} in the glucose- and ATP-dependent assays respectively. As K_{cat} is the maximal turnover rate of the enzyme, in our assays v is equivalent to V_{max}, and S is 100mM glucose.

2.18 Glucokinase adenosine triphosphate-dependent assays

The reaction mix was prepared on ice and was the same as that for the glucose-dependent assays but instead contained 18.75mM glucose and no ATP. This reaction mix was then used to make a 10mM ATP-$MgCl_2$ solution, which was then serially-diluted to make the following ATP-$MgCl_2$ concentration range (mM); 5.00, 4.00, 3.00, 2.50, 2.00, 1.50, 1.00, 0.75, 0.50, 0.25, 0.10, 0.00. One millilitre of reaction mix was then added to eight test tubes on ice and 10m-units GCK added to these tubes as for the glucose-dependent assays. Two 96 well spectrophotometer plates were then set up, plate one containing the diluted ATP-$MgCl_2$, and plate two containing 10m-units of the GCK enzymes to be tested. An eight well multichannel pipette was then used to transfer the contents of plate two to plate one, and rate of reaction recorded as before. The Michaelis-Menten calculation (equation 2.2) was used to fit assay data and determine the half maximal ATP concentration (ATP_{Km}) for each enzyme.

2.19 Small molecular glucokinase activator-mediated activation assays

These assays were carried out at 30^{o}C in the presence of saturating GKA (0-60µM) and glucose (0-100mM) concentrations. They were set up as for glucose-dependent assays, but with the following modifications. The GKA used in this study was RO0281675 (Hoffman La-Roche Inc., Nutley, New

Jersey, USA) which was resuspended in dimethyl sulfoxide (DMSO) and diluted to produce the following concentration range (µM); 60.0, 27.0, 9.0, 3.0, 1.0, 0.3, 0.0. A DMSO control was also set-up to ensure this chemical did not influence the reaction rate. Ten m-units GCK was then added to each GKA/DMSO tube (only one GCK enzyme tested per assay), before gentle mixing via vortex. Two 96 well spectrophotometer plates were then set up; plate one containing the glucose concentration range, and plate two containing the GKA-GCK mix. An eight well multichannel pipette was then used to transfer the contents of plate two to plate one, and rate of reaction recorded as described previously. Data was fit to the Hill and Michaelis-Menten calculations (equations 2.1-4) to determine V_{max}, $S_{0.5}$, n_H, and K_{cat} values. Data representing GKA-stimulated GCK activity was expressed in terms of 'fold activation' which described the ratio between the $K_{cat}/S_{0.5}$ determined in the presence and absence of saturating levels of RO0281675 (60µM). The GKA half maximal effect concentration (EC_{50}) was calculated by plotting log GKA concentration versus percentage GCK stimulation, where maximal stimulation by 60µM GKA equalled 100%.

Chapter 3

Discovery of a novel site regulating glucokinase activity following the characterisation of a new mutation causing hyperinsulinaemic hypoglycaemia in humans[1]

[1] This research was originally published in The Journal of Biological Chemistry. Beer NL, van de Bunt M, Colclough K, Lukacs C, Arundel P, Chik CL, Grimsby J, Ellard S, Gloyn AL. Discovery of a novel site regulating glucokinase activity following the characterisation of a new mutation causing hyperinsulinaemic hypoglycaemia in humans. *JBC*. 2011; 286 (21); 19118-26. © the American Society for Biochemistry and Molecular Biology.

3.1 Introduction

3.1.1 Current unmet medical need for more effective therapeutic strategies against diabetes

The global rise in the prevalence of type 2 diabetes (T2D) not only reflects changes in lifestyle, diet, and behaviour (Zimmet, et al., 2001), but also perhaps suggests that our current therapeutic strategies against this disease are ineffective (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010). At present clinicians have a number of therapies available including; metformin which antagonises the insulin signalling enzyme AMP kinase to increase glucose uptake and suppress hepatic glucose production (Abbud, et al., 2000; Kirpichnikov, et al., 2002; Zhou, et al., 2001), sulphonylureas which close the beta-cell ATP-sensitive potassium channel causing membrane depolarisation and sustained insulin secretion(Downie, et al., 1956; Group, 1998), and thiazolidinediones (TZDs) which are agonists of the transcription factor peroxisome proliferator-activated receptor (PPAR)-γ and act to decrease insulin resistance in adipose tissue and muscle (Aronoff, et al., 2000; Scherbaum and Goke, 2002; Spiegelman, 1998).

However, despite the success of these medications in lowering diabetic-hyperglycaemia, the use of these compounds is not without controversy or proposed pitfalls. For example, it has been suggested that prolonged use of sulphonylureas can cause beta-cell exhaustion (Alvarsson, et al., 2003; Maedler, et al., 2005), although there remains controversy in the literature regarding this matter (Nyback-Nakell, et al., 2010). Additionally, the decline in glycaemic control observed during the progression of T2D means these drugs are often used as part of a combination therapy. The potential increased risk of pharmacologically-induced hypoglycaemia and its associated pathological consequences following the use of such intensive therapeutic strategies has been the subject of much research (Bonds, et al., 2010; Evans, et al., 2006; Fisman, et al., 2001; Gerstein, et al., 2008; Olsson, et al., 2000; Skyler, et al., 2009; Svensson, et al., 2005; Zoungas, et al., 2010), although there

remains controversy in the field regarding this matter (Gulliford and Latinovic, 2004; Johnson, et al., 2002; Johnson, et al., 2005; Kosiborod, et al., 2009; Patel, et al., 2008; Sillars, et al., 2010; Turnbull, et al., 2009). Therefore, there remains an unmet medical need for better treatments; ones that are capable of both lowering glycaemia to appropriate levels safely as well as maintaining their efficacy over many years (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010).

3.1.2 Monogenic mutations highlight a critical role for glucokinase in glucose homeostasis

One drug target that has generated much interest over recent years is the glycolytic enzyme glucokinase (GCK) (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010). Mutations within the gene encoding this enzyme cause a spectrum of glycaemic phenotypes (Osbak, et al., 2009).

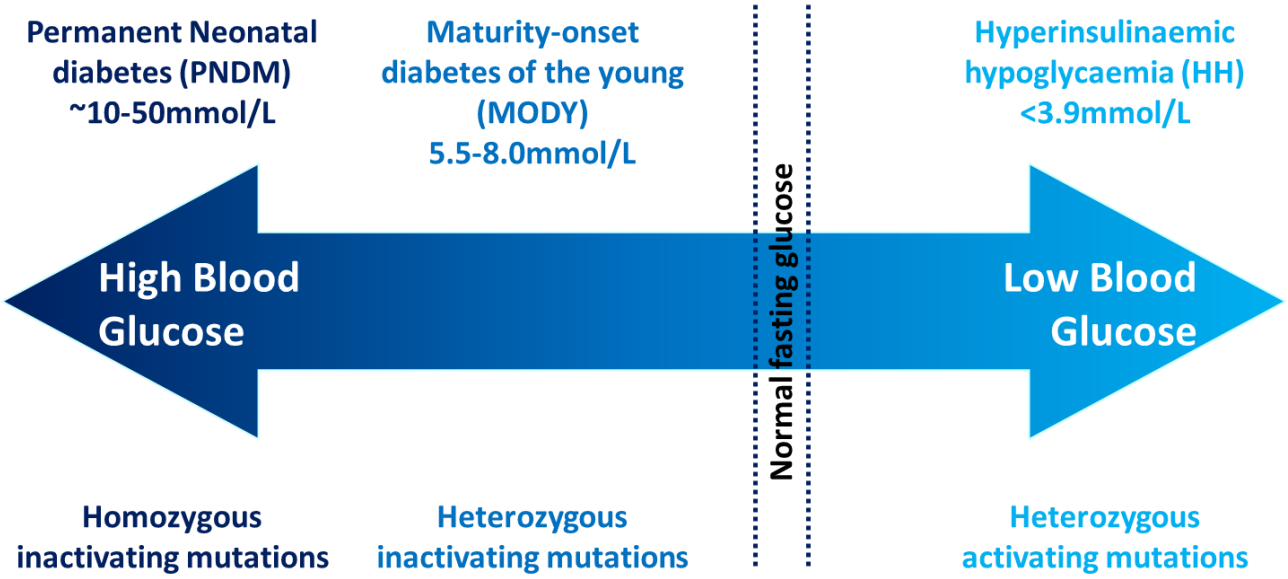


Figure 3.1 Naturally occurring *GCK* mutations cause monogenic disorders beta-cell dysfunction (Froguel, et al., 1992; Glaser, et al., 1998; Njolstad, et al., 2001).

Heterozygous *GCK* activating mutations cause hyperinsulinaemic hypoglycaemia (HH), a rare yet severe condition in which there is inappropriate insulin secretion despite hypoglycaemia (Glaser, et

al., 1998). Conversely, heterozygous *GCK* inactivating mutations cause the opposite hyperglycaemic phenotype of maturity-onset diabetes of the young (MODY) (Froguel, et al., 1992; Hattersley, et al., 1992), a condition characterised by mild fasting hyperglycaemia (5.5-8.0mmol/L) and only a marginal increment in blood glucose levels following a two hour oral glucose tolerance test (<3.0mmol/L) (Stride, et al., 2002). Despite being present from birth, the mild stable phenotype of this condition means it is often only diagnosed upon routine blood glucose testing (Ellard, et al., 2008; Murphy, et al., 2008; Stride, et al., 2002). Finally, *GCK* homozygous/compound heterozygous inactivating mutations cause the severe diabetic phenotype of permanent neonatal diabetes mellitus (PNDM) (Njolstad, et al., 2001).

Common variation within *GCK* has also been shown to associate with fasting plasma glucose (FPG) levels (Prokopenko, et al., 2009; Weedon, et al., 2006; Weedon, et al., 2005), birth weight (Weedon, et al., 2006; Weedon, et al., 2005), and T2D risk (Dupuis, et al., 2010). This provides further evidence to suggest that GCK is a key regulator of glucose homeostasis (Matschinsky, 2002; Matschinsky, et al., 2006).

3.1.3 Small molecular activators could modulate glucokinase activity in the pancreatic beta-cell and liver hepatocyte

GCK has a distinct expression profile versus the other hexokinase enzymes, with highest levels in the pancreatic beta-cell and liver hepatocyte (Lynedjian, et al., 1986), although it is also expressed in the L- and K-cells of the gut (Jetton, et al., 1994) and the hypothalamus (Lynch, et al., 2000; Roncero, et al., 2004; Roncero, et al., 2000). Two distinct promoters allow GCK to fulfil tissue-specific roles, with the neuroendocrine and hepatic *GCK* isoforms transcribed from the up- and downstream promoters respectively (Lynedjian, et al., 1986; Lynedjian, et al., 1989b; Magnuson, 1992; Magnuson and Shelton, 1989). GCK has a crucial glucose homeostatic role in the beta-cell, as here the activity of this

enzyme is directly coupled to glucose-stimulated insulin secretion (GSIS) (Matschinsky, 2002; Matschinsky, et al., 2006; Postic, et al., 2001). Observations from beta-cell-specific *Gck* knock-outs show these mice exhibit defective insulin secretion and suffer from severe hyperglycaemia which is promptly lethal (Grupe, et al., 1995; Postic, et al., 1999; Terauchi, et al., 1995). In the hepatocyte GCK acts as a key regulator of hepatic glucose metabolism, facilitating glucose uptake by the liver, thus elevating levels of other metabolites which are subsequently channelled into both glyco- and lipogenesis. GCK activity also influences expression of other key hepatic enzymes such as pyruvate kinase and fatty acid synthase (Agius, 2008; Matschinsky, 1990; Matschinsky, et al., 1998). Again rodent models support this role, with liver-specific *Gck* knock-out mice exhibiting defective hepatic glucose clearance and utilisation (Postic, et al., 1999). Conversely, rodents in which *Gck* is over-expressed cell-specifically in hepatocytes exhibit hypoglycaemia which cannot be attributed to increased insulin secretion (Jackerott, et al., 2002; Niswender, et al., 1997b).

Therefore, the crucial role of GCK in both the beta-cell and hepatocyte provides the promise of a two-pronged attack on hyperglycaemia by small molecular GCK activators (GKAs), by both lowering the threshold for GSIS and increasing hepatic glucose uptake and utilisation (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010). This builds upon current therapeutic strategies where a combination of anti-diabetic treatments is commonly prescribed to target both defects in insulin secretion and action (Distefano and Watanabe, 2010; Nyenwe, et al., 2010).

The first published GKA test results were obtained using the Hoffman-La Roche compound RO0281675, and suggested that this class of drugs could produce anti-hyperglycaemic effects. For example, *in vitro* tests showed the compound to increase the turnover rate (K_{cat}) and affinity for glucose ($S_{0.5}$) of the enzyme, the GSIS-threshold was lowered and maximal insulin secretion increased in isolated rodent islets, and hyperinsulinaemic hypoglycaemia induced upon administration of the drug to both diabetic and non-diabetic mice (Grimsby, et al., 2003). Other early GKA tests in isolated

rat hepatocytes and beta-cells (Brocklehurst, et al., 2004b; Efanov, et al., 2005; Fyfe, et al., 2007) as well as those in rodent models (Castelhano, et al., 2005; Coope, et al., 2006; Fyfe, et al., 2007; Grimsby, et al., 2003) were also promising, with GKAs again enhancing insulin secretion and hepatic glucose metabolism (Pal, 2009). Accordingly, several small molecular GKAs have been patented and are in clinical trials (AstraZeneca, 2007; BioPharma, 2009; Bonadonna, et al., 2010; Bonadonna, et al., 2008; McKerrecher, et al., 2005; McKerrecher, et al., 2006; Roche and Daniewski, 2007; Roche., 2003; Zhai, et al., 2008; Zhi, et al., 2008).

Preliminary human trials have suggested equally as promising results as the early rodent studies, with the Hoffman-La Roche compound Piragliatin (RO4389620) yielding a dose-dependent lowering of glycaemia in patients with T2D (Bonadonna, et al., 2010; Castelhano, et al., 2005; Roche and Daniewski, 2007). However, it was also noted that a significantly greater number of 'rescue glucose infusions' had to be given to patients on the highest Piragliatin dose in order to prevent hypoglycaemia (Bonadonna, et al., 2010), thus highlighting one of the main potential problems of GKA-mediated diabetes treatment. Nevertheless, pharmaceutical companies are continuing to invest in the research and development needed to bring GKAs to market (Pal, 2009; Sarabu and Grimsby, 2005).

3.1.4 Elucidation of the glucokinase crystal structure has provided a more detailed understanding of key regulatory regions within this important drug target

In 2004 Kamata and colleagues successfully obtained the crystal structure of GCK, providing one of the most important findings in the field to date (Kamata, et al., 2004). As well as explaining some of the fundamental biochemical properties of GCK such as its cooperativity with respect to glucose despite functioning as a monomer (Niemeyer, et al., 1975; Storer and Cornish-Bowden, 1976), the elucidation of the GCK crystal structure has allowed us to study naturally occurring mutations in

much greater molecular detail. For example, structural modelling shows us that the >600 inactivating mutations reported in the literature spread throughout the entire molecule, with no mutational hotspots (Kamata, et al., 2004; Osbak, et al., 2009). Conversely, of the 13 activating mutations found to date (Barbetti, et al., 2009; Kassem, et al., 2010; Osbak, et al., 2009), all bar one cluster at a discrete structural location deemed the 'allosteric activator site', which is interestingly the location at which Kamata and colleagues noted compound A also bound (Kamata, et al., 2004; Osbak, et al., 2009). However, additional scrutiny has allowed the location of these mutations to be categorised further, with Pal and Miller suggesting there were three clear groups; group 1 including residues within the allosteric activator site that formed direct contacts with GKAs, group 2 also including residues within the allosteric activator site but that did not interact with GKAs, and group 3 including residues whose side-chains were greater than 15Å from the allosteric activator site (Pal and Miller, 2009). Interestingly, only one activating mutation has been identified in group three to date; M197I (Pal and Miller, 2009; Sayed, et al., 2009).

As pharmacological interest in GCK aims to increase the activity of this enzyme in patients with T2D, characterisation of these activating mutations is crucial, with their investigation providing further information about sites where manipulation of the enzyme serves to enhance its activity.

3.1.5 Identification of two novel hyperinsulinaemic hypoglycaemia mutations at residues where maturity-onset diabetes of the young mutations have also been reported

Despite the low number of activating mutations currently reported in the literature, two novel mutations were found in two HH families; V389L (c.1165C>G; p.Val389Leu) and T103S (c.308C>G; p.Thr103Ser). These mutations were identified in the Molecular Genetics Laboratory of Prof Sian Ellard (Peninsula Medical School, University of Exeter, UK) following patient referral from Dr Paul Arundel (Sheffield Childrens' NHS Foundation Trust, UK) and Prof Constance Chik (Division of

Endocrinology & Metabolism, University of Alberta, Canada). Importantly, these mutations occurred at residues where GCK-MODY mutations had previously been reported; V389D (c.1166T>A; p.Val389Asp) and T103I (c.308C>T; p.Thr103lle) respectively (Osbak, et al., 2009). This suggests that these sites are of great regulatory importance, as has been shown with previous studies investigating contiguous mutations causing opposite phenotypes (Barbetti, et al., 2009). I aim to characterise these mutations with regards to the intrinsic biochemical properties of GCK by measuring; K_{cat}, glucose $S_{0.5}$, affinity for the second substrate adenosine triphosphate (ATP_{Km}), and cooperativity (n_H; Hill number), to elucidate the mutational mechanisms behind these four mutants and gain further insight into critical regulatory sites within this important drug target. Additionally, as the pathogenesis of *GCK* mutations may not always lie at the level of altered enzyme kinetics (Arden, et al., 2007; Burke, et al., 1999; Gloyn, et al., 2004a; Gloyn, et al., 2005; Heredia, et al., 2006; Miller, et al., 1999; Sagen, et al., 2006), I also aim to observe the effect of both physiological and pharmacological moderators of GCK activity on these four mutants. I hypothesise that the structural position and physiochemical properties of the substituted residues will influence GCK activity, and consequently the clinical phenotype observed.

3.2 Materials and Methods

3.2.1 Subjects Studied

The male proband of family 1 was Caucasian (UK) and born at 38 weeks gestation by caesarean section weighing 5.4kg. There were postnatal records of low capillary blood glucose levels but no action was taken. He presented with seizures at 2.0 years of age, and was diagnosed at 3.6 years with hyperinsulinaemia (39.8pM) and hypoglycaemia (fpg 2.9mM). Following diagnosis the proband was treated with diazoxide (5.0mg/kg/day divided into 3 doses) and chlorothiazide (7.0mg/kg/day in 2 doses). Within 3 days this regime had to be increased to 7.5 and then 10.0mg/kg/day in order to adequately control his blood glucose levels. The patient was then discharged from hospital a week after his initial diagnosis with stable sugars. However, over the following month the dose of diazoxide was increased further to 15.0mg/kg/day. At the age of 5.2 years the diazoxide dose per kg was reduced to 10.0mg/kg/day, but within a month had to be increased back up to 14.0 and then 15.0mg/mg/day in response to periods of increased frequency of (typically morning) hypoglycaemic episodes with symptoms (not seizures). Chlorothiazide was reduced to 3.5mg/kg/day a fortnight after initial diagnosis, although it was quickly raised back up to 7.0mg/kg/day a week later. At the age of 4.3 years the chlorothiazide dose was gradually reduced and has now been discontinued. The proband now only suffers a handful of fasting hypoglycaemic episodes per month (consumption of carbohydrate supplement restoring normoglycaemia promptly). Unfortunately the proband has marked hypertrichosis as a side effect of the diazoxide treatment.

The proband's father also carries the mutation. Following long periods without food he feels "lightheaded" and "weak", and has capillary glucose levels of approximately 2.9mM. Referring clinicians were Dr Paul Arundel, Dr Mars Skae (Central Manchester University Hospital's NHS Foundation Trust, UK) and Consultant Molecular Geneticist Prof Sian Ellard.

The female proband of family 2 is from Canada and is Caucasian with Metis ancestory (her paternal grandmother is of mixed European and First Nations parentage [Metis]). She was born at 38 weeks gestation weighing 3.2kg. There was no history of neonatal hypoglycaemia but in retrospect she had an episode of symptomatic hypoglycaemia at age 15 after a long hike. She presented at age 22 with frequent episodes of fatigue, weakness and unexplained sweating which could be prevented by frequent snacks in addition to her regular meals, and correspondingly was diagnosed with hyperinsulinaemia (36.1pM) and hypoglycaemia (2.8mM). Triglyceride levels were in the normal range (1.4mM), and she was normal weight (BMI of 22.0kg/M^2). Investigations including endoscopic ultrasound, computed tomography (CT) and magnetic resonance imaging (MRI) of abdomen, and an exploratory laparotomy, were negative for an insulioma. While the proband was in hospital, her 27 year old sister was found to have hypoglycaemia and subsequent investigations confirmed hyperinsulinaemia (143.1pM) with hypoglycaemia (2.5mM). She was born at 42 weeks gestation weighing 4.1kg. With both sisters having symptomatic hypoglycaemia when glucose readings are lower than 3mM, they are both currently managed with frequent small meals, diazoxide 50 to 75mg twice daily and hydrochlorothiazide 12.5 to 25mg once a day.

Screening of the family revealed that the proband inherited the mutation from her 56 year old father who also has two affected male siblings, and her 78 year old paternal grandmother who was diagnosed with T2D at age 75. The father's fasting glucose values are between 3.1 and 4mM with corresponding insulin levels of 51.4 and 56.3pM. Although he does not have symptomatic fasting hypoglycaemia, he does have occasional hypoglycaemic symptoms post exercise. Referring clinicians were Dr Constance Chik and Prof Sian Ellard.

3.2.2 Screening of the glucokinase gene and identification of mutations

All patient screening and mutation identification was carried out by Dr Kevin Colclough (Department of Molecular Genetics, Royal Devon & Exeter NHS Foundation, UK). Genomic DNA was extracted from peripheral leukocytes and the 10 exons of the pancreatic beta-cell isoform of the *GCK* gene amplified via polymerase chain reaction (PCR). Following amplification, products were observed on a 2% agarose gel using ethidium bromide staining (Sigma Aldrich Company Ltd, Gillingham, UK). Unidirectional sequencing was performed using either forward or reverse universal M13-tailed primers (forward: $^{5'}$TGTAAAACGACGGCCAGT$^{3'}$, reverse: $^{5'}$CAGGAAACAGCTATGACC$^{3'}$, both obtained from Eurofins MWG Operon, Ebersberg, Germany) and the Big Dye Terminator Cycler Sequencing Kit v3.1 (Applied Biosystems, Warrington, UK). Reactions were analysed on an ABI 3730 capillary sequencer (Applied Biosystems), and sequences were compared with the GenBank reference sequence NM_000162 (http://www.ncbi.nlm.nih.gov/nuccore/) using Mutation Surveyor v3.24 (SoftGenetics, Cambridge, UK). Mutation testing was undertaken in family members to establish co-segregation and in 400 normal chromosomes to establish mutation frequency.

3.2.3 Site-directed mutagenesis

Site-directed mutagenesis (SDM) was used to introduce the mutations of interest into the human neuroendocrine *GCK* coding sequence within the pGEX-3X vector (Sigma Aldrich Company Ltd). This construct was a gift from Prof Mark Magnuson (Vanderbilt University, Nashville, Tennessee, USA). SDM was carried out using the QuikChange SDM kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer's guidelines and using the primers listed in tables 3.1 (naturally occurring mutations) and 3.2 (additional mutations).

Primer	Sequence (5'- 3')	Primer	Sequence (5'- 3')
T103I	F- CAGTGGAGCGTGAAGATCAAACACCAGATGTA	V389D	F – CTGGCGGGCGACATCAACCGC
	R - GTACATCTGGTGTTTGATCTTCACGCTCCACT		R – GCGGTTGATGTCGCCCGCCAG
T103S	F – GCGTGAAGAGCAAACACCAGA	V389L	F – GCTGGCGGGCCTCATCAACCG
	R – TCTGGTGTTTGCTCTTCACGC		R – CGGTTGATGAGGCCCGCCAGC
T103N	F - AGCGTGAAGAACAAACACCAG		
	R - CTGGTGTTTGTTCTTCACGCT		

Table 3.1: SDM primers for naturally occurring GCK mutations (Eurofins MWG Operon). Mismatched bases are underlined, and 'F' denotes forward primer whilst 'R' denotes reverse.

Following mutagenesis, samples were submitted to *DpnI* -mediated digestion to remove template DNA (New England Biolabs Ltd, Hitchin, UK), and used to transfect SCS1 super-competent *Escherichia (E) coli* (Stratagene Europe) in order to amplify the mutagenised samples. Following overnight culture at 37^{o}C on agar-ampicillin plates, single colonies were picked and used to create 10ml lysogeny broth (LB) medium –ampicillin cultures (both Sigma Aldrich Company Ltd), which were subsequently incubated overnight at 37^{o}C, 250rpm. The bacterial cells were then pelleted via centrifugation at 14,000xg for 10 minutes at room temperature, and DNA extracted using the QIAprep Spin Miniprep Kit (Qiagen Ltd, Crawley, UK). Extracted DNA quality and quantity was determined using the Nanodrop 2000 (ThermoScientific, Basingstoke, UK). The GCK coding sequence was then amplified via PCR using the primers listed in table 3.3 (Eurofins MWG Operon), and the products viewed on a 2% agarose gel using ethidium bromide staining (Sigma Aldrich Company Ltd). These were then submitted to bidirectional capillary electrophoresis sequencing using M13 primers (Eurofins MWG Operon), this work carried out by Lynne Richardson at the University's core sequencing facility within the Department of Zoology.

Primer	Sequence (5'- 3')	Primer	Sequence (5'- 3')
T103P	F – CAGTGGAGCGTGAAGCCCAAACACCAGATGT	V389P	F – GCGGGGCTGGCGGGCCCCATCAACCGCATGC
	R – ACATCTGGTGTTTGGGCTTCACGCTCCACTG		R - GCATGCGGTTGATGGGGCCCGCCAGCCCCGC
T103K	F - CAGTGGAGCGTGAAGAAAAAACACCAGATGTAC	V389C	F - GCGGGGCTGGCGGGCTGCATCAACCGCATGC
	R - GTACATCTGGTGTTTTTTCTTCACGCTCCACTG		R - GCATGCGGTTGATGCAGCCCGCCAGCCCCGC
T103Y	F - CAGTGGAGCGTGAAGTACAAACACCAGATGTAC	V389S	F - GCGGGGCTGGCGGGCTCCATCAACCGCATGC
	R - GTACATCTGGTGTTTGTACTTCACGCTCCACTG		R - GCATGCGGTTGATGGAGCCCGCCAGCCCCGC
T103C	F - CAGTGGAGCGTGAAGTGCAAACACCAGATGTAC	V389R	F - GCGGGGCTGGCGGGCCGCATCAACCGCATGC
	R - GTACATCTGGTGTTTGCACTTCACGCTCCACTG		R - GCATGCGGTTGATGCGGCCCGCCAGCCCCGC

Table 3.2: SDM primers the extra GCK mutants to be included in this study. Mismatched bases are underlined, 'F' denotes forward primer and 'R' reverse.

Primer	Sequence (5'- 3')	Primer	Sequence (5'- 3')
GCK Frag1	F - GGCTGGCAAGCCACGTTTGGTG	GCK Frag4	F - CGAAGACCATCAGTGCGAGGTC
	R - TTGGTCTTCACGCTCCACTGCC		R - GCAGCTTGTACACGGAGCCATC
GCK Frag2	F - GGAGACCCATGAAGAGGCCAGT	GCK Frag5	F - TCGACCACCGACTGCGACATCG
	R - CCCACGACATTGTTCCCTTCTG		R - TCCGGGAGCTGCATGTGTCAGA
GCK Frag3	F – CATGACCGGCACTGCTGAGATG		
	R - CCACTCGGTATTGACGCACATG		

Table 3.3: GCK coding sequence primers (Eurofins MWG Operon). All primers had M13-tail attachments for ease of sequencing. 'F' denotes forward primers, and 'R' reverse. Primers 'GCK Frag 1 F' and 'GCK Frag 5 R' were designed against the pGEX-3X vector sequence.

3.2.4 Protein extraction

Two preparations of each mutant glutathione S-transferase (GST)-tagged human pancreatic glucokinase were prepared as described previously following heterologous expression in *E.coli* (Liang, et al., 1995). Briefly, following confirmation that only the correct mutation had been introduced into the *GCK* coding sequence, the mutated GCK-pGEX-3X plasmid was used to transform BL21 super-competent *E. coli* (Stratagene Europe). Single colonies were picked the following day and used to inoculate LB medium-ampicillin cultures which were incubated at 37^{o}C, 250rpm. Once the optical density (OD) at 600nm had reached 0.6, protein production was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma Aldrich Company Ltd) and heating overnight at 22^{o}C, 150rpm. The following day, the recombinant GCK-GST fusion protein was then extracted using 50% glutathione-agarose slurry (Sigma Aldrich Company Ltd), before addition of a glycerol-rich storage buffer in a 1:1 ratio and storage at -80^{o}C. For further detail on this protocol, please refer to **chapter 2 section 2.12**.

Recombinant FLAG-tagged human glucokinase regulatory protein (GKRP, gene *GCKR*) was also prepared using a previously published protocol, but without the use of a DEAE-sepharose exchange resin (Brocklehurst, et al., 2004a). This protocol was similar to that used to obtain GCK, but recombinant GKRP-FLAG was instead extracted using anti-FLAG M2 affinity resin (Sigma Aldrich Company Ltd) before addition of storage buffer and -80^{oC} storage. More information on this protocol is supplied in **chapter 2 section 2.13**. The original pFLAG-CTC-*GCKR* construct was kindly supplied by Katy Brocklehurst (AstraZeneca, Alderley Park, UK).

Extracted enzyme purities were determined using the Agilent 230 Protein Kit (Agilent Technologies UK Ltd, Stockport, UK), with a cut-off for purity of ≥95%. Protein concentration was calculated using the Bio-Rad Bradford reagent assay (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

3.2.5 Kinetic assays

Glucose-dependent assays

GCK activity was determined spectrophotometrically using glucose 6-phosphate dehydrogenase (G6PDH)-linked assays as described previously (Liang, et al., 1995). In these assays, GCK activity was observed at 30^{o}C, pH 7.4, over a 0-100mM glucose concentration range with ATP in excess. As the mutants V389D, V389R and V389P had greatly reduced affinity for glucose versus wild-type enzyme, the glucose $S_{0.5}$ were instead determined from assays using 0-600mM glucose. Briefly, the reaction mix was prepared on ice and contained; 7.5mM magnesium chloride ($MgCl_2$), 187.5mM potassium chloride (KCl), 125mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1.25mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 2.5mM dithiothreitol (DTT), 6.25mM ATP, 0.13% bovine serum albumin (BSA), and 66units G6PDH. The activity of 10m-units GCK was observed over the following glucose concentration range (mM) for 5 minutes; 100.00, 50.00, 25.00, 12.50, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.00. One m-unit GCK was defined as that which converted 1nmole substrate per minute. Further information on this protocol can be found in **chapter 2 section 2.17**.

ATP-dependent assays

These assays were conducted as for the glucose-dependent assays, although GCK activity was instead measured over a 0-5mM ATP concentration range with glucose in excess. The altered reaction mix contained 18.75mM glucose and no ATP. Ten m-units GCK were assayed in the presence of 5.00, 4.00, 3.00, 2.50, 2.00, 1.50, 1.00, 0.75, 0.50, 0.25, 0.10, and 0.00mM ATP.

Glucose $S_{0.5}$, n_H, and K_{cat} values were calculated using the Hill equation, whilst ATP_{Km} was derived from the Michaelis-Menten equation (**chapter 2 section 2.17**). All data fits were performed using Kaleidagraph v3.5 (Synergy Software, Reading, Philadelphia, USA).

GKRP-mediated inhibition assays

GKRP-mediated inhibition of GCK activity was determined spectrophotometrically using G6PDH-linked assays based on published protocols (Brocklehurst, et al., 2004a). The assays were carried out 5mM glucose, 37^{o}C, pH7.1.

The number of GKRP units used per assay was 0.5, 1.0, and 1.5, with one GKRP unit inhibiting 10m-units GCK by 50% under standard assay conditions. The reaction mix contained 25mM HEPES buffer, 0.5mM $NADP^{+}$, 1mM ATP, 2mM $MgCl_2$, 25mM KCl, 1mM DTT, 3.8µM BSA, and 4units G6PDH. The reaction rate was recorded over five minutes as before. Data was presented as 'percentage GCK activity' which was normalised to that obtained with no GKRP, and IC_{50} values (half maximal inhibitory concentration) also calculated from this data.

GKA-mediated activation assays

These assays were performed as described previously in the presence of 10m-units GCK, and saturating GKA (0-60µM) and glucose (0-100mM) concentrations (Liang, et al., 1995). The reaction mix was as for the glucose-dependent assays, but also contained RO0281675 re-suspended in dimethyl sulfoxide (DMSO) at the following concentrations (µM); 60.00, 27.00, 9.00, 3.00, 1.00, 0.30, 0.00. Data was expressed as 'fold activation' which described the ratio between the $K_{cat}/S_{0.5}$ determined in the presence and absence of saturating levels of RO0281675 (60µM). The GKA half maximal effect concentration (EC_{50}) was also calculated, and described the drug concentration that gave GCK activity 50% between the baseline and maximal response.

3.2.6 Calculating relative activity index and predicted glucose-stimulated insulin secretion threshold

Activity indices (IA), more specifically relative activity indices (RAI) allow the direct comparison of mutant versus wild-type GCK, and take into account all the intrinsic kinetic parameters that may be

altered upon enzyme mutation; $S_{0.5}$, ATP_{Km}, n_H, and K_{cat}. The formulae for each are given in equations 3.1 and 3.2.

(3.1) $IA = (K_{cat} / S_{0.5}^{nH}) \times (ATP / (ATP \times ATP_{Km}))$

(3.2) RAI = Mutant IA / Wild-type IA

Equations 3.1 and 3.2: Formulae for the calculation of mutant IA and RAI versus wild-type GCK (Gloyn, et al., 2004a). ATP corresponds to intracellular ATP concentration which is 2.5mM under normal conditions.

Additionally, as GCK activity is directly linked to GSIS in the pancreatic beta-cell (Matschinsky, 2002; Matschinsky, et al., 2006), a mathematical model has been devised which uses the kinetic parameters of a mutant GCK and some basic assumptions to calculate the predicted blood glucose threshold for GSIS in patients with *GCK* mutations (Gloyn, et al., 2004a; Matschinsky, 2002). These assumptions are that GCK is the beta-cell glucose sensor, and that the GSIS threshold for wild-type GCK is 5mmol/L (approximately 28% of GCK phosphorylating capacity is needed to stimulate beta-cell insulin secretion). As the glucose- and ATP-dependencies of GCK obey the Hill and Michaelis-Menten equations respectively, combining these two equations describes the two substrate kinetics of this enzyme. Finally, taking into account the altered GCK expression that may occur in response to changes in blood glucose (equation 3.3) (Liang, et al., 1992; Matschinsky, 2002; Sturis, et al., 1994), the mathematical to predict the GSIS threshold can be derived (equation 3.4) (Gloyn, et al., 2003a; Gloyn, et al., 2004a; Matschinsky, 2002)).

(3.3) $E = (G^{nH} \times 2) / (G^{nH} + S_{0.5}^{nH})$

Equation 33: Formula to describe GCK expression relative to blood glucose levels. E is the GCK expression coefficient and G is the glucose concentration. The number two is included in the numerator of this equation, as half maximal induction occurs at glucose $S_{0.5}$ (Gloyn, et al., 2003a; Gloyn, et al., 2004a; Matschinsky, 2002).

(3.4) $$28\% = ([0.5G^{nHwt}] / [S_{0.5wt}{}^{nHwt}]) \times (2.5 / [2.5 + ATP_{Kmwt}]) \times (E^{wt} / 0.66) + ([0.5G^{nHmut}] / [G^{nHmut} + S_{0.5mut}{}^{nHmut}]) \times (2.5 / [2.5 + ATP_{Kmmut}]) \times (K_{catmut} / K_{catwt}) \times (E^{mut} / 0.66)$$

Equation 3.4: Equation to predict the blood glucose concentration at GSIS threshold in patients heterozygous for a particular GCK mutation (as occurs in both HH and MODY probands in this study) (Gloyn, et al., 2003a; Gloyn, et al., 2004a; Matschinsky, 2002). 'Wt' and 'mut' corresponds to values for wild-type and mutant GCK enzymes respectively. Therefore, when glucose phosphorylating capacity equals 28%, 'G' corresponds to blood glucose concentration at which the GSIS-threshold occurs.

3.2.7 Structural analysis

Crystal structures for the closed and super-open conformations of GCK (Kamata, et al., 2004) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.pdb.org/pdb/home/home.do). Molecular modelling was carried out using PyMol v.0.99 (DeLano Scientific LLC, Palo Alto, California, USA).

3.3 Results

3.3.1 Identification of novel glucokinase mutations in two families with hyperinsulinaemic hypoglycaemia

Figure 3.2 A. and B show the pedigrees for the HH probands identified in the UK and Canada (family 1 and 2 respectively). Direct sequencing of *GCK* in the proband of family 1 identified a novel heterozygous mutation; V389L (c.1165C>G, p.Val389Leu). Family studies established that the mutation was inherited from the proband's affected father.

The proband from family 2 was also heterozygous for a novel mutation; T103S (c.308C>G, p.Thr103Ser). Family studies established that the mutation was also present in the affected sister, father, two paternal uncles and paternal grandmother, all bar the latter exhibiting fasting hypoglycaemia. The paternal grandmother was diagnosed with T2D aged 75 years, although notably she is of Metis descent [a First Nation Canadian population at high risk of developing diabetes (Bruce, 2000)].

Neither variant was identified in >400 normal chromosomes. Mutations in other known HH genes (*ABCC8, KCNJ11* and *HNF4A*) were also excluded.

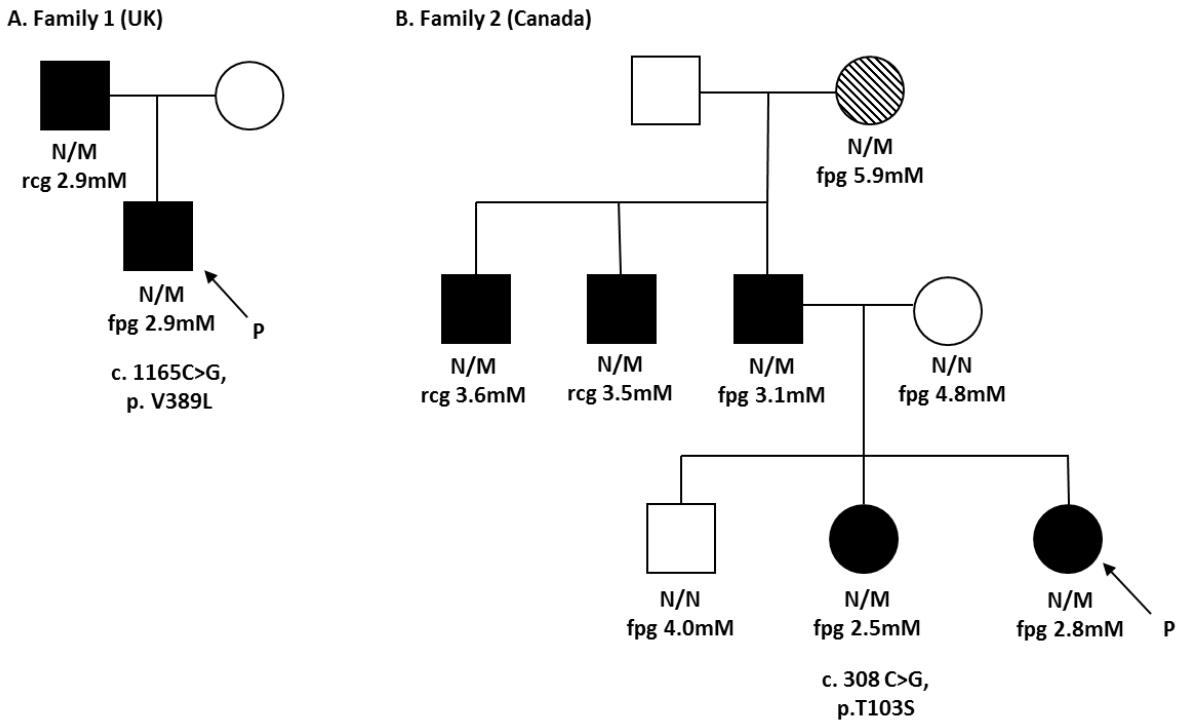


Figure 3.2A and B: Pedigrees for HH families 1 and 2 (1A and 1B respectively). Males are shown as squares and females as circles. Filled symbols show individuals with hypoglycaemia, open symbols normoglycaemic individuals, and hatched symbols T2D. Fasting plasma glucose (fpg) or random capillary glucose (rcg) levels and mutation status for each tested individual are shown under symbol (N/M shows family members heterozygous for mutation, N/N denotes those who do not carry the mutation).

3.3.2 Kinetic analyses of recombinant human wild-type and mutant glucokinase

Table 3.4 shows the kinetic characteristics of wild-type, V389L, V389D, T103S and T103I GCK as determined using *in vitro* G6PDH-linked assays. Both the V389L and T103S HH mutations have an increased RAI when compared to wild-type (6.0 and 8.4 respectively vs. 1.0). This was largely driven by an increased affinity for glucose demonstrated by reduced $S_{0.5}$ values (3.5±0.1 and 3.3±0.1 respectively vs. 7.5±0.1mM). The ATP_{Km}, n_H and K_{cat} values for these HH mutations were similar to that obtained with wild-type enzyme. Conversely, the MODY mutations V389D and T103I had greatly reduced RAI values (both <0.1 vs. 1.0 for wild-type). Again, this effect was largely driven by a decreased affinity for glucose ($S_{0.5}$ 78.6±4.5 for V389D, 24.9±0.6mM for T103I). However, with the V389D mutant, there was also a reduced affinity for the second substrate ATP when compared to wild-type (ATP_{Km} 1.5±0.1 vs. 0.4±<0.1mM) and turnover number (K_{cat} 10.3±1.1 vs. 62.9±1.2s^{-1}). It was

also of note that the yield of V389D recombinant protein was greatly reduced compared to the other enzymes (only 0.9 ± 0.3mg).

GCK	$S_{0.5}$ (mM)	n_H	ATP_{Km} (mM)	K_{cat} (s^{-1})	RAI	GSIS threshold (mM)	Yield (mg)	Activity/mg
wild-type	7.5 ± 0.1	1.6 ± <0.1	0.4 ± <0.1	62.9 ± 1.2	1.0	5.0	17.7 ± 0.7	6.6 ± 0.5
V389L	3.5 ± 0.1	1.6 ± <0.1	0.5 ± <0.1	67.8 ± 1.6	6.0	2.9	20.3 ± 1.8	11.3 ± 0.8
V389D	78.6 ± 4.5	1.5 ± <0.1	1.5 ± 0.1	10.3 ± 1.1	<0.1	7.1	0.9 ± 0.3	0.16 ± 0.1
T103S	3.3 ± 0.1	1.6 ± <0.1	0.4 ± <0.1	59.2 ± 1.7	8.4	2.9	9.5 ± <0.1	11.4 ± 2.2
T103I	24.9 ± 0.6	1.7 ± <0.1	0.6 ± <0.1	68.1 ± 0.1	<0.1	6.7	12.5 ± 0.5	11.2 ± 0.5

Table 3.4: Kinetic data for wild-type and four naturally occurring GCK mutants. Data presented are mean ± SEM (n=18) and are representative of two preparations of wild-type and each mutant protein.

Table 3.5 shows the results of kinetic assays characterising a range of amino acid substitutions designed to further probe the physiochemical requirements of positions 389 and 103. There is a clear preference for hydrophobic residues at position 389, with hydrophilic substitutions having a deleterious effect on protein function. Substitutions at residue 103 result in a spectrum of effects on enzyme function with activating, near-normal and inactivating kinetics.

GCK	Hydro-	$S_{0.5}$ (mM)	n_H	ATP_{Km} (mM)	K_{cat} (s^{-1})	RAI	GSIS threshold (mM)
wild-type	phobe	7.5 ± 0.1	1.6 ± <0.1	0.4 ± <0.1	62.9 ± 1.2	1.0	5.0
T103S	phobe	3.3 ± 0.1	1.6 ± <0.1	0.4 ± <0.1	59.2 ± 1.7	8.4	2.9
T103P	phobe	6.0 ± 0.1	1.9 ± <0.1	0.6 ± <0.1	51.9 ± 0.7	1.2	4.7
T103Y	phobe	9.1 ± 1.0	1.3 ± 0.1	0.4 ± <0.1	39.0 ± 0.9	0.9	5.5
T103C	phobe	11.2 ± 0.2	1.5 ± <0.1	0.5 ± <0.1	52.3 ± 1.4	0.3	5.7
T103I	phobe	24.9 ± 0.6	1.7 ± <0.1	0.6 ± <0.1	68.1 ± 0.1	<0.1	6.7
T103N	phile	5.8 ± 0.1	1.6 ± <0.1	0.5 ± <0.1	51.5 ± 1.1	1.2	4.5
T103K	phile	7.5 ± 0.3	1.3 ± <0.1	0.4 ± <0.1	25.6 ± 0.7	0.9	5.6
V389L	phobe	3.5 ± 0.1	1.6 ± <0.1	0.5 ± <0.1	67.8 ± 1.6	6.0	2.9
V389C	phobe	8.3 ± 0.3	1.5 ± <0.1	0.5 ± <0.1	58.5 ± 2.4	0.8	5.0
V389S	phobe	23.9 ± 2.5	1.3 ± <0.1	0.7 ± <0.1	39.9 ± 2.1	<0.1	6.5
V389P	phobe	39.4 ± 0.6	1.8 ± <0.1	0.6 ± <0.1	31.3 ± 0.3	<0.1	6.8
V389D	phile	78.6 ± 4.5	1.5 ± <0.1	1.5 ± 0.1	10.3 ± 1.1	<0.1	7.1
V389R	phile	68.8 ± 0.8	1.6 ± <0.1	0.5 ± <0.1	42.3 ± 0.4	<0.1	6.8

Table 3.5: Kinetic data for wild-type and a range of amino acid substitutions at positions 103 and 389. Mean data ± SEM are shown (n=9) and are representative of two preparations of wild-type and one preparation of each mutant protein.

3.3.3 In silico calculation of glucose-stimulated insulin secretion thresholds

Table 3.4 shows the calculated GSIS thresholds for patients with the four individual mutations. Both HH mutations give a predicted GSIS threshold of 2.9mM, which accurately mirrors the FPG levels of 2.9 and 2.8mM observed in the V389L and T103S probands respectively. Calculated GSIS thresholds of 7.1 and 6.7mM for the V389D and T103I mutations are in line with the expected FPG levels in patients with MODY mutations (~5.5-8.0mmol/L) (Fajans, et al., 2001; Osbak, et al., 2009). The severely deleterious effect of the V389D mutation on enzyme function is not reflected in the predicted FPG level due to compensation by the wild-type allele (Liang, et al., 1992).

3.3.4 Response to the physiological hepatic inhibitor glucokinase regulatory protein

Assays including GKRP were carried out in order to assess how the mutant proteins responded to the endogenous hepatic GCK inhibitor. Figure 3.3 shows the results of inhibition assays where 10m-units GCK (wild-type, V389L, T103S and T103I) were incubated with 0.5, 1.0 and 1.5units GKRP. V389D was not included in these experiments as its activity/mg was so low that to see measurable inhibition by regulatory protein, inappropriately large quantities of the enzyme would be needed. As expected, the IC_{50} value for wild-type was 1.1±0.1units GKRP, the HH mutant V389L yielding identical results. GKRP was less able to inhibit mutant T103S and T103I activity, with IC_{50} values of 1.5±<0.1 and 4.7±0.5 GKRP units respectively.

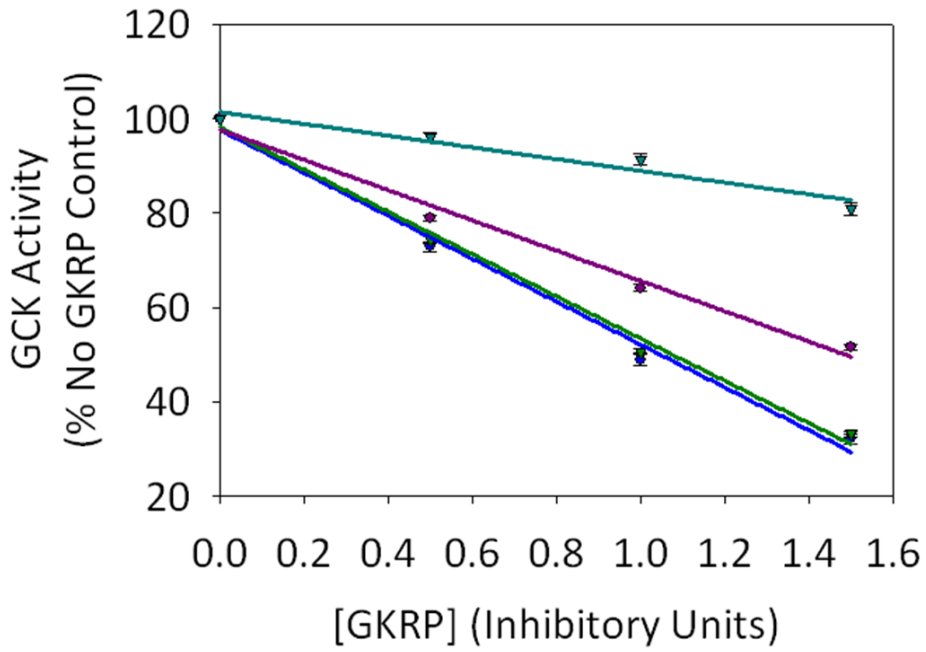


Figure 3.3: Wild-type (blue), V389L (green), T103S (purple), and T103I (cyan) GCK were incubated with 0.5, 1.0 and 1.5units GKRP. GCK activity was normalised to a control in which the reaction conditions were identical but GKRP was absent. IC_{50} values were calculated using this data, and defined as the half maximal inhibitory concentration of GKRP in each experiment. Data are shown as mean ± standard error of the mean (SEM) for n=6 experiments, and are representative of two preparations of wild-type and each mutant protein.

3.3.5 Activation by the small molecular glucokinase activator RO0281675

To observe how the four mutants responded to pharmacological manipulation, these enzymes were assayed in the presence of the Hoffman-La Roche GKA RO0281675. Table 3.6 shows fold activation of each enzyme in terms of $K_{cat}/S_{0.5}$ in the presence of 60µM GKA. The MODY mutation T103I showed the greatest fold increase in activity, roughly six times that of the wild-type enzyme (90.9±4.0 vs. 14.4±0.3 respectively). The V389D MODY mutation also demonstrated a high fold increase in activity of 33.9±2.1. Correspondingly, both MODY mutations required a greater concentration of the GKA to stimulate activity, with EC_{50} values of 34.6 ± <0.1 for V389D, 12.8 ± 4.2 for T103I (versus 5.8 ± 1.9µM for wild-type). Unsurprisingly, both HH mutations were activated to a lesser degree, with fold increases in activity of 5.7±0.5 for T103S and 7.4±1.3 for V389L. This finding was replicated in the low EC_{50} values obtained for both HH mutants (2.6 ± 1.3 and 3.9 ± 0.1µM respectively).

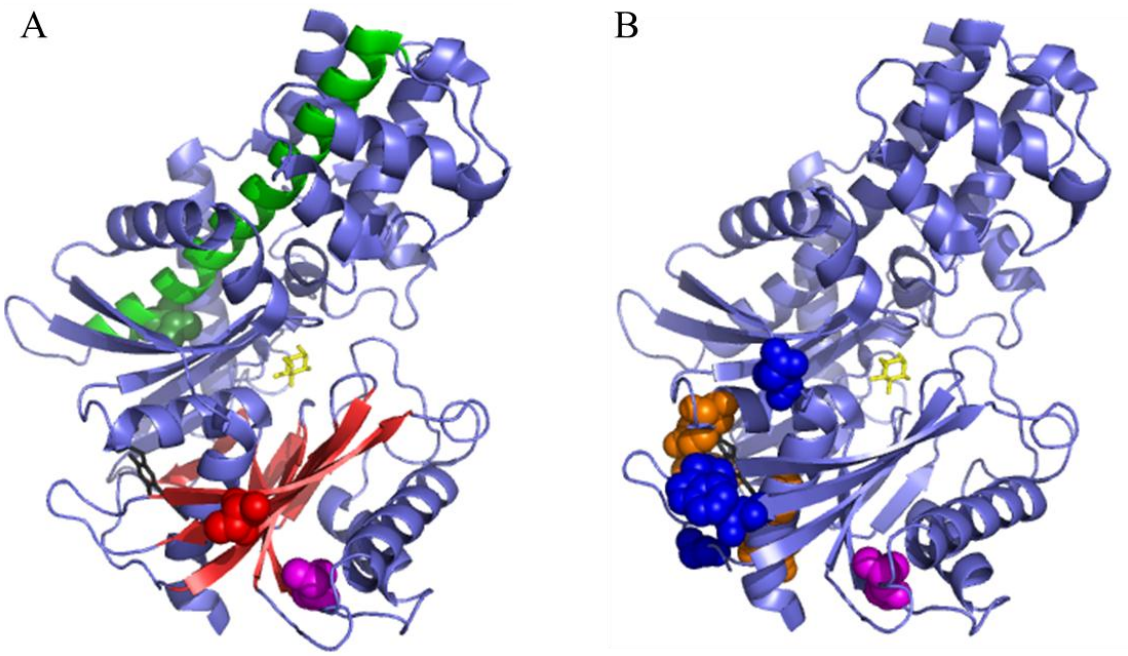
GCK	Fold activation ($K_{cat}/S_{0.5}$)	EC_{50} (µM)
WT	14.4 ± 0.3	5.8 ± 1.9
V389L	7.4 ± 1.3	3.9 ± 0.1
V389D	33.9 ± 2.1	34.6 ± <0.1
T103S	5.7 ± 0.5	2.6 ± 1.3
T103I	90.9 ± 4.0	12.8 ± 4.2

Table 3.6: Data obtained from assaying wild-type and mutant GCKs in the presence of the GKA RO0281675. Fold activation describes the ratio between the $K_{cat}/S_{0.5}$ determined in the presence and absence of saturating levels of GKA (60µM), and EC_{50} is the concentration of drug that gives half maximal GCK activity. Data are mean values ± SEM for n=6 experiments, and are representative of two preparations of wild-type and each mutant protein.

3.3.6 Structural modelling – T103 but not V389 maps to the allosteric activator site

To elucidate how the different mutations were able to elicit opposite effects on enzyme activity, analysis of the GCK crystal structure was carried out to identify the structural positions where the

amino acids substitutions occurred. Figure 3.4A and B shows the location of residues T103 and V389 within the closed conformation of human GCK (Kamata, et al., 2004). T103 lies within the allosteric activator site, specifically within beta-strand 4 of the small domain, a region where the majority of other activating mutations are reported (figure 3.4B). However, this residue does not form direct contacts with bound GKAs, and so T103S can be described as a group two activating mutation (Pal and Miller, 2009). Surprisingly, residue V389 is located far from the allosteric activator site in alpha-helix 11 of the large domain (figure 3.4A). Not only is this distal from the site of GKA binding, but it is also far from the only other group three activating mutation M197I (Kamata, et al., 2004; Sayed, et al., 2009), highlighting a novel regulatory region within the enzyme.



Figures 3.4A and B: Figure 3A shows the positions of residues Thr-103 (red) and Val-389 (green) within the crystal structure of closed human GCK (Kamata, et al., 2004). Thr-103 maps to the allosteric activator site close to where GKAs (black) bind, specifically within beta-strand four of the small domain beta-sheet (red). Val-389 (green) is both distal from this site and residue Met-197 (magenta) (Sayed, et al., 2009), and instead maps to alpha-helix 11 (green) within the large domain. Figure 3B shows the position of all other naturally occurring activating mutations reported. Group 1 mutations (those in allosteric activator site making direct contact with GKA) are shown in orange, group 2 mutations (those in allosteric activator site but not contacting GKA) are in blue, whilst group 3 (residues greater than 15Å from the allosteric activator site) are in magenta (Pal and Miller, 2009). The binding sites of the GKA 'compound A' (black) and glucose (yellow) are also shown in both figures (Kamata, et al., 2004).

3.4 Discussion

3.4.1 Characterisation of naturally occurring glucokinase mutations provides further insight into the regulation of this important drug target

The unmet medical need for more effective T2D treatments capable of sustainably lowering glycaemia means the quest for novel therapeutic targets both timely and important (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte; Zimmet, et al., 2001). The fundamental role of GCK in glucose homeostasis specifically within the beta-cell and liver (Matschinsky, 2002; Matschinsky, et al., 2006), as well as its mutation causing monogenic syndromes of aberrant glucose homeostasis (Osbak, et al., 2009), makes it an interesting and well-established drug target. The functional characterisation of two novel GCK-HH activating mutations and the corresponding MODY mutations at the same amino acid residues (Osbak, et al., 2009) allows us to study important regulatory regions within GCK, more specifically sites where the functional balance can be tipped in either direction.

3.4.2 Altered kinetics explain the pathogenic mechanisms behind the glucokinase mutants V389L, V389D, T103S, and T103I

Intrinsic kinetic parameters for wild-type and the four mutant GCKs measured via spectrophotometric *in vitro* assays revealed that the increased activity index of both HH mutations (V389L and T103S) was largely driven by an increased affinity for glucose (table 3.4), a result observed with previously reported naturally occurring GCK-HH mutations (Christesen, et al., 2002; Cuesta-Munoz, et al., 2004; Glaser, et al., 1998; Gloyn, et al., 2003a; Meissner, et al., 2009; Osbak, et al., 2009; Sayed, et al., 2009; Wabitsch, et al., 2007). The reduced activity index of the MODY-mutant T103I followed this theme, however the substitution of an aspartate for a valine at residue 389 (V389D) had disastrous consequences with regards to enzyme function, with affinity for the two substrates glucose and ATP decreased and a substantially reduced overall rate of catalysis. This

deleterious effect on GCK function was also mirrored by the protein yield in both recombinant V389D protein preparations. This suggests that not only does V389D directly alter GCK activity, but protein stability as a whole may be reduced. The result of this kinetic characterisation provides a potential explanation for the pathogenesis behind these mutations, as they correspond to both an elevated or diminished capacity to bind and turnover glucose (V389L/T103S and V389D/T103I respectively).

3.4.3 Both the activating mutations V389L and T103S co-segregate with hypoglycaemia

The four naturally occurring *GCK* mutants functionally characterised in this study clearly demonstrate altered kinetic properties which fit with the observed phenotypes in these patients; both HH mutations V389L and T103S are activating, whilst the two MODY mutants V389D and T103I are inactivating. In addition to this, there is clear co-segregation with of the activating mutations with hypoglycaemia in both the UK and Canadian families (figure 3.2). It is perhaps surprising that the paternal grandmother of family 2 who is heterozygous for the T103S mutation was diagnosed with T2D, although notably she is of Metis descent [a First Nation Canadian population at high risk of developing diabetes (Bruce, 2000)].

There is little evidence in the literature to suggest GCK-HH mutations lead to beta-cell exhaustion unlike HH caused through mutations in *ABCC8* in which there is unregulated excessive insulin secretion (Huopio, et al., 2003), or with *HNF4A* mutations where there is transient hyperinsulinism and corresponding hypoglycaemia prior to the development of HNF-4A MODY (Pearson, et al., 2007). Additionally, it has been shown that GCK-MODY patients only have a marginal decline in beta-cell function in line with the general population (Stride, et al., 2002), further suggesting GCK-HH does not fatigue beta-cells, but just re-sets the GSIS-threshold.

3.4.4 Mutations can affect enzymatic and protein properties secondary to glucokinase kinetic activity

As GCK activity is fundamental to glucose metabolism in both the beta-cell and hepatocyte (Matschinsky, 2002; Matschinsky, et al., 2006), it is subject to a number of different transcriptional (Lynedjian, 2004) and post-translational regulatory mechanisms (Alvarez, et al., 2002; Baltrusch, et al., 2001; Bjorkhaug, et al., 2007; Danial, et al., 2003; Danial, et al., 2008; Massa, et al., 2004; Shiota, et al., 1999; Van Schaftingen and Veiga da Cunha, 2004). Therefore, the pathogenic mechanism behind a GCK mutation may not just lie at the level of altered enzyme kinetics. This has been shown with mutations which co-segregate with hyperglycaemia in MODY families, but with kinetic parameters that are near-normal or even mildly activating. Two such examples are V62M (Arden, et al., 2007; Gloyn, et al., 2005) and G72R (Arden, et al., 2007; Sagen, et al., 2006). Kinetic analysis revealed both mutations to be mildly activating, and with only marginal thermolability versus wild-type and the previously reported instability mutant E300K (Burke, et al., 1999; Gloyn, et al., 2004b; Miller, et al., 1999). Further investigation revealed both mutants exhibited defective binding of the endogenous hepatic inhibitor GKRP and a small molecular GKA (Arden, et al., 2007; Gloyn, et al., 2005; Grimsby, et al., 2003).

As well as its direct inhibitory role, GKRP is thought to paradoxically bind and maintain a ‘reserve pool’ of GCK in the hepatocyte nucleus which may be released rapidly upon postprandial increases in glucose (Slosberg, et al., 2001). Additionally, small molecular GKAs are thought to bind at the same allosteric site as an as-yet unidentified endogenous GCK regulator within the beta-cell (Grimsby, et al., 2003). Therefore it was postulated that loss of these interactions upon GCK mutation could have physiologically pathogenic consequences, a result which was confirmed in Min6 cells where both the V62M and G72R mutants showed reduced catalytic and protein stability compared to wild-type enzyme (Arden, et al., 2007).

In vitro assays studying the interaction of GCK mutants with both GKRP and GKAs can therefore provide an insight into whether the pathogenic mechanism behind a particular mutation lies at levels additional to directly altered kinetics. Studies performed by Heredia and colleagues investigated the regulation of a number of naturally occurring GCK activating mutants by GKRP (Heredia, et al., 2006). Their data showed differences in GKRP-binding affinity between the mutants which was proposed to reflect the mutational mechanism. For example, mutations which enhanced the interaction between glucose and the apoenzyme had the lowest GKRP-affinity (as these substitutions stabilised a more compact structure in which the GKRP-binding site was altered), whilst those which enhanced glucose-bound GCK conformational change demonstrated only slightly reduced GKRP affinity versus wild-type enzyme (Heredia, et al., 2006).

3.4.5 Mutations at residue T103 but not V389 alter the affinity of glucokinase for the regulatory protein

The results of the GKRP-inhibition assays performed in this study (figure 3.3) show that GKRP was able to inhibit wild-type GCK and the V389L mutant equally. However, GKRP-mediated inhibition of both T103 mutants was less effective compared with the wild-type enzyme, with the binding to mutant T103I affected the most. This is consistent with data suggesting the interaction of GKRP with GCK occurs at leucine-asparagine motifs within the hinge region of the enzyme (Baltrusch, et al., 2005), which maps next to the allosteric activator site (within which T103 is found). Additionally, based on the findings of Heredia and colleagues, these data suggest that the T103S activating mutation directly increases glucose binding to the apoenzyme, whilst V389L may enhance the conformational change from the inactive to active structure once glucose is bound (Heredia, et al., 2006). As GKRP is specific to the liver and not expressed within the beta-cell [see **chapter 4** and (Grimsby, et al., 2000; Zawalich, et al., 1977)], it is difficult to postulate the precise consequence of a reduced GKRP binding-affinity on overall GCK activity, particularly as the ability to bind this inhibitor contributes to, but is not a determinant of clinical phenotype (Arden, et al., 2007; Gloyn, et al., 2005;

Heredia, et al., 2006; Sagen, et al., 2006; Sayed, et al., 2009). As GKRP binds T103S less effectively, it could be assumed that there is less inhibition of GCK activity in the liver, thus leading to an increase in hepatocyte glycolytic flux (O'Doherty, et al., 1999), which contributes to the hypoglycaemia of this HH patient. Equally, the greatly decreased GKRP-affinity observed with T103I may mean that there are markedly reduced hepatic nuclear reserves of this mutant enzyme, consequently hindering postprandial glucose clearance by the liver (Slosberg, et al., 2001), contributing to the hyperglycaemia in this MODY patient.

3.4.6 The mutants V389L, V389D, T103S, and T103I, responded to GKA-mediated stimulation

Table 3.6 shows the results of the GKA RO0281675 mediated-stimulation of wild-type and the four GCK mutants. Interestingly, all four mutants responded to the drug in a dose-dependent manner as had been seen previously (Grimsby, et al., 2003), suggesting that not even the particularly deleterious V389D substitution resulted in an enzyme that could not be converted into the active conformation. The MODY mutants V389D and T103I showed the greatest fold increase in activity. This is perhaps a reflection of the reduced activity of the MODY-mutants prior to stimulation, a hypothesis completed by both HH mutants in this study which showed the smallest fold increases in activity, and also by the previous findings of Grimsby and Grippo who demonstrated that the V455M GCK activating mutant was resistant to GKA-mediated stimulation (Glaser, et al., 1998; Grimsby and Grippo, 2004). The much greater response of the inactivating T103I mutant versus that of V389D may be explained by the physiochemical properties of these substituted residues. Both threonine (T) and isoleucine (I) are hydrophobic meaning it may be easier to 'force' GCK into the closed active structure using small molecular activators, whilst the V389D mutant sees a hydrophilic aspartate residue (D) being pushed into a hydrophobic pocket normally occupied by valine (V). This is also reflected in V389D having the highest EC_{50} value (concentration of GKA that gives half maximal stimulation) of the four mutants.

3.4.7 Structural modelling provides insight into the pathogenic mechanisms behind substitutions at residues V389 and T103

Molecular modelling of the mutations in this study within the GCK crystal structure (Kamata, et al., 2004) allows the mechanisms behind their pathogenesis to be further probed. The three-conformation model proposed by Kamata and colleagues suggest an explanation for both the sigmoidal binding curve and cooperativity with respect to glucose observed with GCK. Transition between these functional states requires global conformational change and the breaking and reforming of numerous intramolecular bonds (Kamata, et al., 2004), both of which are easily affected by the physiochemical properties of substituted residues at sites critical to function.

Valine 389 is situated near the C-terminal end of alpha-helix 11 (figure 3.4A). It is surrounded by hydrophobic residues; Leu386, Ile390, Met393, Met41, Tyr234, Trp257, and Ala232. The observation that certain amino acid substitutions at residue 389 may increase GCK activity identifies this position and alpha-helix 11 as a novel site regulating GCK activity, one which is both distal from the allosteric activator site and the only other reported naturally occurring group three activating mutation M197I (figures 3.4A and B) (Kamata, et al., 2004; Pal and Miller, 2009; Sayed, et al., 2009). Residue 389 lies within a solvent-excluded region of the molecule, a location suited by hydrophobic valine (V) and the even more hydrophobic leucine (L), but not hydrophilic aspartate (D). Table 3.5 shows the kinetic parameters measured for a series of mutations at position 389. These data showed all hydrophilic substitutions drastically reduced GCK activity, whilst all hydrophobic replacements except serine (S) gave activating or near-normal kinetics (bar the substitution of proline [P] which may break this alpha-helix). This finding is similar to that observed with the other group three activating mutant M197I. This residue is also located within a hydrophobic pocket where the introduction of hydrophilic residues is deleterious to GCK function (Kamata, et al., 2004; Pal and Miller, 2009; Sayed, et al., 2009).

The mutational mechanisms behind the substitutions at residue 103 are much harder to explain than those at residue 389. Thr-103 is a surface residue (figure 3.4A); however it is unclear how a conservative mutation such as the replacement of a threonine (T) with a serine (S) would affect GCK activity. Mutating the threonine to an aliphatic isoleucine (I) puts a non-polar hydrophobic residue in a solvent-exposed environment, which may account for the inactivating kinetics of T103I. A series of amino acid substitutions were carried out at this position to see how this altered GCK activity. Table 3.5 shows these mutants to demonstrate a spectrum of kinetic properties. T103S was the only activating mutation observed. The mutants T103N, K, P and Y all demonstrate near-normal kinetics, meaning that either i) these mutations would not be pathogenic, or ii) their mutational mechanism would not lie at the level of altered glucose catalysis (Arden, et al., 2007; Gloyn, et al., 2005; Sagen, et al., 2006). Importantly, only one of these mutations (T103N) has been identified in a GCK-MODY patient (Massa, et al., 2001). Finally, the mutants T103I and C had a reduced activity index, reflecting the potential intolerance of an aliphatic side-chain in this solvent-exposed region.

3.4.8 Identification of alpha-helix 11 as a novel site regulating glucokinase activity has implications for future drug development

In summary, the newly-identified importance of alpha-helix 11 suggests that further activating mutations are likely to exist and that they may not necessarily map to the allosteric activator site. Our results build upon earlier work by Pal and Miller who pre-emptively identified further activating mutations by genetic screening (Pal and Miller, 2009), as well as that by Sayed and colleagues who characterised the first activating mutation outside of the allosteric activator site (Sayed, et al., 2009). This study highlights the importance of the continued study of naturally occurring *GCK* activating mutations, as detailed kinetic analysis at these residues may enable the fine tuning of GKAs for the treatment of diabetes.

Chapter 4

The P446L variant in *GCKR* associated with fasting plasma glucose and triglyceride levels exerts its effect through increased glucokinase activity in the liver[2]

[2 2 2] Work in this chapter was published as a first author manuscript in Human Molecular Genetics [Beer NL, Tribble ND, McCulloch LJ, Roos C, Johnson PR, Orho-Melander M, Gloyn AL, 2009. Hum Mol Genet 18 (21) 4081-8]. All work was performed by Nicola Beer, as was the writing of the manuscript.

4.1 Introduction

4.1.1 The study of genetic variation in complex human disease can identify important pathogenic pathways and novel therapeutic targets

Complex human diseases like type 2 diabetes (T2D) and clinically important quantitative traits such as fasting plasma glucose (FPG) result from the effect of environmental, lifestyle and genetic factors (Hirschhorn and Daly, 2005; Zimmet, et al., 2001). Understanding the genetic aetiology behind these phenotypes is at the forefront of research into complex disease, with the hope that it will lead to novel approaches to the way they are treated and clinically managed (McCarthy and Hirschhorn, 2008a; McCarthy and Hirschhorn, 2008b). Over the last decade there have been major advances in genetic research which have made the genome wide association (GWA) study possible (Hardy and Singleton, 2009; Hirschhorn and Daly, 2005; McCarthy and Hirschhorn, 2008b). GWA studies been pivotal in increasing the number of susceptibility loci identified for complex diseases, for example the number associated with T2D has increased from 3 prior to the first GWA study in 2006 to roughly 40 in the last four years (Altshuler, et al., 2000; Bouatia-Naji, et al., 2009; Consortium, 2007; Dupuis, et al., 2010; Franks, et al., 2008; Frayling, et al., 2007; Gloyn, et al., 2003b; Grant, et al., 2006; Gudmundsson, et al., 2007; Kong, et al., 2009; Lyssenko, et al., 2009; Prokopenko, et al., 2009; Sandhu, et al., 2007; Saxena, et al., 2007; Sladek, et al., 2007; Steinthorsdottir, et al., 2007; Unoki, et al., 2008; Winckler, et al., 2007; Yasuda, et al., 2008; Zeggini, et al., 2008; Zeggini, et al., 2007).

The advent of GWA studies has opened the floodgates to a wealth of previously-unobtainable genetic information regarding the aetiology of complex diseases and quantitative traits. The large sample sizes used in these studies make them well-powered to identify common variants (minor allele frequency [MAF] >1%) which are believed to influence risk of many complex diseases including T2D (Collins, et al., 1997; Cornelis, et al., 2009; Lango, et al., 2008; Manolio, et al., 2009; Pearson and Manolio, 2008; Reich and Lander, 2001; Risch and Merikangas, 1996; Risch, 2000). They work by genotyping large numbers (~100,000) of single nucleotide polymorphisms (SNPs) in thousands of

unrelated individuals, these SNPs capturing all genetic variation across the genome (Pearson and Manolio, 2008). This method has drastically advanced our understanding of complex human diseases, however as the common variants identified in GWA studies only have small effect sizes, they do not provide enough genetic information to allow predictions about an individual's susceptibility to T2D (Lango, et al., 2008). Nevertheless, they still provide important information on the biology and pathways involved in the pathogenesis of this disease.

4.1.2 Genome-wide association study highlights glucokinase regulatory protein as a regulator of fasting plasma glucose and triglyceride levels

One such GWA study looking for loci associated with T2D and related quantitative traits identified glucokinase regulatory protein (*GCKR*; protein GKRP) as a potential modulator of FPG and triglyceride levels in the general population (Saxena, et al., 2007). Fine-mapping revealed the causal SNP to be non-synonymous (rs1260326, c.1403C>T, p.P446L, MAF 34%) (Orho-Melander, et al., 2008), with individuals homozygous for the risk 'L' allele having a mean decrease in FPG of 0.06mmol/ and increase in triglycerides of 0.15mmol/L compared to people with two copies of the wild-type 'P' allele (Orho-Melander, et al., 2008). This association was also replicated in subsequent studies (Sparso, et al., 2008; Vaxillaire, et al., 2008). Lowered FPG and raised triglycerides is an unusual phenotype because elevated triglycerides normally accompany insulin resistance and hyperglycaemia as occurs in T2D (Reaven, 1988).

Traditionally, the functional follow-up of GWA study signals has been challenging, as many of the association signals come from SNPs within non-coding regions of the genome (Hindorff, et al., 2009; McCarthy and Hirschhorn, 2008b; van de Bunt and Gloyn, 2010). However, unlike most signals arising from GWA studies, the *GCKR* SNP driving the genetic association with FPG and triglyceride levels is both non-synonymous and in a strong biological candidate gene (Orho-Melander, et al., 2008), thus facilitating further functional studies. However, even with such tractable variants, identifying the mutational mechanism for common risk alleles presents a challenge due to their

small physiological effects (Manolio, et al., 2009; McCarthy and Hirschhorn, 2008b; van de Bunt and Gloyn, 2010) (only 0.06mmol/L reduction and 0.15mmol/L elevation in FPG and triglyceride levels respectively per allele) (Orho-Melander, et al., 2008). It is therefore essential that the 'correct assays' are selected and performed in order for small differences in protein function to be observed.

4.1.3 Glucokinase regulatory protein negatively regulates hepatic glucokinase activity

Glucokinase (GCK) is a key regulator of glucose storage and disposal in the liver (Matschinsky, 2002; Matschinsky, et al., 2006). GKRP negatively regulates GCK activity competitively with respect to the substrate glucose, and its action is in turn controlled by the phosphate esters fructose 6- and fructose 1-phosphate (F6P and F1P), which compete with each other for binding, and enhance or inhibit regulatory protein-mediated GCK inhibition respectively (Mookhtiar, et al., 1996; Van Schaftingen, 1989; Van Schaftingen and Vandercammen, 1989; Vandercammen, et al., 1992; Vandercammen and Van Schaftingen, 1990). Controversy remains in the literature as to whether GKRP regulates GCK activity in both hepatocytes and pancreatic beta-cells (Alvarez, et al., 2002; Grimsby, et al., 2000; Zawalich, et al., 1977). The majority of studies state that *GCKR* is only expressed in the liver and not in rodent beta-cells (Grimsby, et al., 2000; Zawalich, et al., 1977), however no studies have been reported which investigate *GCKR* expression in human pancreatic islets.

4.1.4 Rodent models support the association of the glucokinase regulatory protein variant with low fasting plasma glucose and raised triglycerides

Biological evidence to support the association of the P446L-GKRP variant with the unusual low FPG and raised triglyceride phenotype comes from both cellular and rodent models. Slosberg and colleagues used an adenovirus to over-express *GCKR* in a human liver cell line, and counter-intuitively showed an increase in GCK activity and expression at the protein level (Slosberg, et al., 2001). Additionally, both hetero- and homozygous *Gckr* knockout mice show normal FPG levels but

marked hyperglycaemia postprandially or upon challenge, which is consistent with defective hepatic glucose utilisation (Farrelly, et al., 1999; Grimsby, et al., 2000) and is a similar phenotype to that observed in liver-specific *Gck* knockout mice (Postic, et al., 1999). Further to this, a similar reduction in hepatic Gck activity occurs in cats, an animal which does not express *Gckr* endogenously in the liver (Hiskett, et al., 2009). It has therefore been suggested that GKRP has a posttranscriptional GCK-stabilisation role which is paradoxical to its direct inhibitory action on GCK activity, maintaining adequate protein levels of this enzyme within the liver to cope with postprandial glucose challenges (Farrelly, et al., 1999; Grimsby, et al., 2000). Additionally, hepatic *Gck* over-expression in mice yields insulin-independent lowering of FPG levels (Jackerott, et al., 2002; Niswender, et al., 1997b), and in a study by O'Doherty and colleagues it was shown that *in vivo* over-expression of *Gck* in rat hepatocytes led to the expected drop in FPG levels, but was also accompanied by an increase in circulating triglycerides (O'Doherty, et al., 1999). Therefore, these rodent and cellular model findings suggest that 1) GKRP expression has a direct effect on GCK activity and protein levels, and 2) there is a link between hepatic GCK activity and plasma glucose/circulating triglyceride levels.

4.1.5 Functional studies using rat P446L glucokinase regulatory protein provided no insight into the mutational mechanism behind the genetic association

A number of studies investigating the interaction of GCK and its regulatory protein have been reported, but due to the technical difficulties in purifying human recombinant GKRP (Brocklehurst, et al., 2004a), all have used the rat isoform either directly isolated from hepatocytes or the recombinant protein (Detheux, et al., 1991; Mookhtiar, et al., 1996; Van Schaftingen, 1989; Vandercammen and Van Schaftingen, 1990; Vandercammen and Van Schaftingen, 1991; Vandercammen and Van Schaftingen, 1993; Veiga-da-Cunha, et al., 2003; Veiga-da-Cunha and Van Schaftingen, 2002; Veiga-da-Cunha, et al., 1996b). One of these studies investigated the effect of recombinant rodent P446L-GKRP on GCK activity using spectrophotometric assays, and reported no differences in the inhibitory ability of the variant protein or its response to phosphate esters versus

wild-type (Veiga-da-Cunha, et al., 2003). However, despite their high sequence homology (~88%) (Warner, et al., 1995), there are important differences in the regulation of rat and human GKRP (Brocklehurst, et al., 2004a). Firstly, human regulatory protein inhibits GCK even in the absence of F6P, an effect not observed with the same concentration of rat GKRP. Secondly, human regulatory protein was shown to have a higher affinity for this phosphate ester compared to the rodent protein (Brocklehurst, et al., 2004a).

Therefore, the aims of this study are to firstly investigate the effect of both human wild-type and P446L-GKRP on human GCK activity, and secondly to determine whether their response to and regulation by the phosphate esters F6P and F1P is altered. Finally, as previous studies have only been performed using rat tissues (Alvarez, et al., 2002; Grimsby, et al., 2000; Zawalich, et al., 1977), we aim to definitively establish whether *GCKR* is expressed in human pancreatic islets, and if so, to look at its expression relative to *GCK*. We hypothesise that due to the small change in FPG and triglyceride levels in individuals homozygous for the 446L GKRP variant, there will only be a subtle functional difference between wild-type and variant regulatory protein. Additionally, as this variant is also associated with increased triglycerides, we hypothesise that this genetic signal is largely driven by a functional effect in the liver.

4.2 Materials & Methods

4.2.1 Introduction of the GCKR-P446L mutation via site-directed mutagenesis

The human *GCKR* coding sequence NM_001486 was cloned into the pFLAG-CTC vector (Sigma Aldrich Company Ltd, Gillingham, UK) following its digestion with the restriction enzymes *Nde*I and *Sal*I (both New England Biolabs UK Ltd, Hitchin, UK). This construct was a gift from Katy Brocklehurst (AstraZeneca, Alderley Park, UK) (Brocklehurst, et al., 2004a). Polymerase chain reaction (PCR)-mediated site-directed mutagenesis (SDM) using the QuikChange SDM Kit (Stratagene Europe, Amsterdam, The Netherlands) and the primers in table 4.1 (Eurofins MWG Operon, Ebersberg, Germany) were used to introduce the P446L mutation into the *GCKR* coding sequence within this construct. Further protocol detail can be found in **section 2.4 chapter 2 materials & methods**.

Primer	Sequence (5'- 3')
SDM P446L	F - CAGACCTTGCTGATCCCTCTGAAGAAGCTCTTTCCCTCC
	R - GGAGGGAAAGAGCTTCTTCAGAGGGATCAGCAAGGTCTG

Table 4.2: SDM primers used to introduce P446L mutation into *GCKR* coding sequence. Mismatched bases are underlined, with 'F' and 'R' denoting forward and reverse primers respectively.

Following mutagenesis, samples were submitted to *DpnI*-mediated digestion (New England Biolabs UK Ltd) to remove un-mutagenised template. Mutagenised DNA was then amplified via transformation and overnight culture of SCS1 *Escherichia* (E) *coli* (Stratagene Europe), followed by DNA extraction using the QIAprep Spin Miniprep Kit (Qiagen Ltd, Crawley, UK). Extracted DNA quality and quantity was determined using the Nanodrop 2000 (ThermoScientific, Cramlington, UK).

The *GCKR* sequence within this extracted DNA was then amplified via PCR using the primers in table 4.2 and submitted to bi-directional sequencing to confirm that only the P446L mutation had been introduced.

Primer ID	Sequence (5'- 3')	Primer ID	Sequence (5'- 3')
GCKR frag1	F - GGAAGCTGTGGTATGGCTGT	*GCKR* frag6 F	F - TCTTCTCTTTCACCTGCTCCA
	R - GGTCAGAATGGATTCGCTGT		R - TGCTGATTTCCGAGATGTCC
GCKR frag2	F - GATGCTGAGATCTTCCAGGAG	*GCKR* frag7 F	F - TGCTAATCCGAAGGTCCAAC
	R - GTGCCAATCACGGAGAAGTC		R - TGGAGCAGGTGAAAGAGAAGA
GCKR frag3	F - TGTCGGAATGTTGAACTCCA	*GCKR* frag8 F	F - CCTGTTCCTTCTCATGTGCA
	R - AGGTGGTGACAGGTCTGTGG		R - ACCACATGTTGGACCTTCG
GCKR frag4	F - AATGTCCGCAAGATTTCCAG	*GCKR* frag9 F	F - TCCAGGTTGCACATGAGAAG
	R - ATTCCGACAAGTAGCAGAGC		R - CGCTTCTGCGTTCTGATTTA
GCKR frag5	F - ATGAGAAAGCCACGGACATC		
	R - ATTTGAGCGAGCTCATCAGG		

Table 4.3: PCR primers for amplifying the *GCKR* coding sequence within the pFLAG-CTC vector. All primers contained M13 tail attachments for ease of sequencing; forward $^{5'}$TGTAAAACGACGGCCAGT$^{3'}$, reverse $^{5'}$CAGGAAACAGCTATGACC$^{3'}$. 'F' denotes forward primer, 'R' the reverse primer. The primers spanned the entire *GCKR* coding sequence, apart from '*GCKR* frag1 F' and '*GCKR* frag9 R' which were designed against the vector sequence.

PCR products were viewed on a 2% agarose gel using ethidium bromide staining (Sigma Aldrich Company Ltd), and then submitted to bidirectional sequencing using M13 primers (Eurofins MWG Operon). Further detail on these protocols is provided in **chapter 2 materials & methods**.

4.2.2 Glucokinase regulatory protein extraction

Three preparations each of wild-type and P446L recombinant FLAG-tagged GKRP were prepared following heterologous expression in *E.coli* using previously described protocols, but without the use of a DEAE-sepharose ion-exchange resin (Brocklehurst, et al., 2004a). Further details can be found in **chapter 2 materials & methods section 2.13**. Extracted GKRP purities were determined using the Agilent 230 Protein Kit (Agilent Technologies UK Ltd, Stockport, UK), with a cut-off for purity of ≥95%. Protein concentration was calculated using Bradford reagent (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), which averaged at 0.19mg/ml for wild-type and 0.12mg/ml for P446L-GKRP (mean, n=3).

4.2.3 Glucokinase protein extraction

Two preparations of glutathione S-transferase-tagged human neuroendocrine GCK protein were prepared following heterologous expression in *E.coli* based on previously published protocols (Liang, et al., 1995), which are described further in **chapter 2 materials & methods section 2.12**. The neuroendocrine GCK isoform was used as it has the same affinity for GKRP as the liver enzyme (Veiga-da-Cunha, et al., 1996b). Protein purity and concentration were again checked using the Agilent 230 Protein Kit and Bradford reagent respectively (mean concentration of 1.2mg/ml [n=2]).

4.2.4 Kinetic assays

Competitive inhibition assays

GKRP-mediated inhibition of GCK activity was determined spectrophotometrically using glucose 6-phosphate dehydrogenase-linked assays based on published protocols (Brocklehurst, et al., 2004a; Liang, et al., 1995; Veiga-da-Cunha, et al., 1996b). These are described in greater detail in **chapter 2 materials & methods section 2.16**. One m-unit GCK was defined as that which converted one nmole substrate per minute. One GKRP unit inhibited 10m-units GCK by 50% under the standard assay conditions of 5mM glucose, no phosphate esters (Brocklehurst, et al., 2004a). On average, one GKRP unit resulted from 15.87µg/ml of wild-type and 14.05µg/ml of P446L-GKRP (n=3 independent protein preparations). One GKRP unit of both wild-type and variant P446L regulatory protein were subsequently used per equal activity assay in the presence of 10m-units wild-type GCK. Experiments using both comparable activity (0, 0.5, 1.0 or 1.5 GKRP-units) or equimolar concentrations (0, 50, 100 or 150mM) (Veiga-da-Cunha and Van Schaftingen, 2002) of both wild-type and variant regulatory protein were conducted. Both methods were used in order to validate the approach adopted by Veiga-da-Cunha and colleagues in their previous study of rat P446L-GKRP (Veiga-da-Cunha and Van Schaftingen, 2002), and to control for differences in protein activity induced as artefacts of the purification process. Data was presented as 'percentage GCK activity' which was normalised to data

obtained with 100mM glucose and no GKRP. Final result graphs were plotted using Sigmaplot v11.0 (Systat Software Inc., Hounslow, UK).

Phosphate ester assays

F1P and F6P assays were performed using the same assay conditions as for competitive inhibition experiments, but reaction mix A contained only 5mM glucose instead of a 0-100mM concentration range (Brocklehurst, et al., 2004a). Mix A also contained wild-type-GKRP, P446L-GKRP, or no GKRP (negative control). Mix B contained 10m-units wild-type GCK and 0-500mM F1P or F6P (both from Sigma Aldrich Company Ltd). Data was presented as 'percentage GCK activity' which was normalised to that obtained at the same phosphate ester concentration but where GKRP was absent. IC_{50} values (concentration of F6P causing 50% inhibition of GCK activity) were also calculated from these data.

Calculating dissociation constants

Ki (inhibitor dissociation constants) for GKRP binding were determined from Dixon plots of 1/percentage GCK activity versus concentration of GKRP (Dixon, 1953a), as has been described previously for competitive inhibition assays (Brocklehurst, et al., 2004a). Plots of 1/percentage GCK activity versus F1P or F6P concentration were also used to determine apparent inhibitor dissociation constants for these assays (Ki_{app}). As the phosphate esters are secondary regulators of GCK activity and act via the inhibitor GKRP, Dixon plots provided only an insight into the dissociation of the phosphate esters, GKRP and GCK, but could not be used to distinguish specifically between the individual components of these interactions.

4.2.5 Gene expression analysis

GCKR and *GCK* mRNA expression were observed via quantitative reverse transcription-PCR (qRT-PCR) in human liver, human adipocytes, human skeletal muscle, human kidney, human pancreas, and human islets. All samples were part of the commercially available Human Total RNA Master Panel II (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), except for the human islets which

were collected as part of a previous study at the University of Oxford (full ethical consent). These islets were isolated by the Oxford Consortium for Islet Transplantation team. All laboratory benches and equipment were cleaned with RNaseZap before RNA work, and RNA samples were kept within RNase-free 1.5ml microcentrifuge tubes (both Applied Biosystems, Warrington, UK). RNA was extracted from the islets using TRI Reagent according to the manufacturer's guidelines (Applied Biosystems). All RNA samples were treated with DNAse I (Applied Biosystems) to remove genomic DNA contamination.

DNase-I treatment of RNA samples

RNA samples containing DNAse-I and its corresponding buffer were incubated at 37^{o}C for 20 minutes so that all genomic DNA contamination could be digested. Following incubation, DNAse-I inactivation resin was added to the RNA sample, and this mixture left at room temperature for two minutes (vortexing halfway through). The sample was then centrifuged at 16,000xg in a force 1618 desktop microcentrifuge (Labnet International, Rutland, UK) for 90 seconds to pellet the inactivation resin. Supernatant containing DNaseI-treated RNA was then removed and transferred to a fresh RNase-free 1.5ml microcentrifuge tube and stored at -20^{o}C until use.

DNaseI treatment was followed by random-primed first strand cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and 1µg of each RNA sample. Reactions in which the reverse transcriptase enzyme was absent were also carried out to produce negative controls for the final qRT-PCR experiment.

Random-primed cDNA synthesis

The constituents of the cDNA synthesis mastermix are given in table 4.3, all of which were added to thin-walled RNAse-free 0.2ml PCR tubes (Applied Biosystems). The tubes were then heated on a PTC-225 tetrad thermal-cycler (MJ Research, Waltham, Massachusetts, USA) using the cDNA transcription reaction conditions given in table 4.4.

Reagent	Volume added to RT+ reaction (µl)	Volume added to RT- reaction (µl)
10x reverse transcription buffer	2.0	2.0
25x dNTP mix	0.8	0.8
10x reverse transcription random primers	2.0	2.0
Nuclease-free water	4.2	5.2
Multiscribe reverse transcriptase	1.0	n/a
RNA (1µg)	1.0	1.0

Table 4.3: cDNA synthesis reagents (n=1 reaction). The 10x reverse transcription buffer, 25x dinucleoside triphosphates (dNTPs), 10x reverse transcription random primers, and mulitscribe reverse transcriptase were all part of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Nuclease–free water was also from Applied Biosystems. 'RT+' reactions contained the reverse transcriptase enzyme, whilst the 'RT-' ones did not and were the negative control to test for genomic DNA contamination.

Programme section	Number of cycles	Temperature (^{o}C)	Time (mins)
Primer annealing	1	25	10
Reverse transcription	1	37	120
Primer/enzyme dissociation	1	85	0.8

Table 4.4: cDNA synthesis thermal-cycler reaction conditions.

Once cDNA had been transcribed, 1µl from each sample were pooled together and then serially-diluted to make a set of standards (1/50, 1/100, 1/200, 1/400, 1/800) which were used to calculate the 'efficiency' of the individual gene expression assays. Additionally, a 'no template control' (NTC) well in which the sample was nuclease-free water was included to check for genomic contamination in the final reaction mix.

All cDNA samples were diluted 100-fold and added in triplicate to the appropriate wells of a 384well thermofast PCR plate (ThermoScientific), as well as the appropriate standards and NTC. To this was added TaqMan gene expression mastermix, and of the appropriate TaqMan gene expression assay listed in table 4.5 (both Applied Biosystems).

Gene	Applied Biosystems assay code
GCK	Hs00277220_m1
GCKR	Hs00609799_m1
HPRT1	Hs99999909_m1
B2M	Hs99999907_m1
ACTB	Hs99999903_m1

Table 4.5: Applied Biosystems assay codes for each of the Taqman gene expression assays used in this study. The 'Hs' prefix denotes that the probe is specific to the human gene sequence. The '_m1' suffix illustrates that the primer is specific for the mRNA sequence of this gene.

The 384 well plates were then read using a TaqMan 7900HT machine (Applied Biosystems). Expression of the 'house-keeping' genes hypoxanthine phosphoribosyltransferase-1 (*HPRT1*), beta-2-microglobulin (*B2M*), beta-actin (*ACTB*) (all assays from Applied Biosystems) were also observed in all tested tissues. Data were analysed using the 2(-Delta Delta C(T)) method (Livak and Schmittgen, 2001) but with an efficiency correction to account for how well each gene expression assay worked in the experiment. *GCK* and *GCKR* expression were made relative to the geometric mean expression of the three housekeeping genes (Vandesompele, et al., 2002) and normalised to the 1in100 dilution of the standard curve for each gene-specific assay.

4.2.6 Statistical analysis

Paired two-tail t-tests were used for statistical analysis of all assay data, with a cut-off value for statistical significance of 0.05. Statistical tests were performed using SPSS v16.0 (IBM, Portsmouth, UK).

4.3 Results

4.3.1 Wild-type and P446L glucokinase regulatory protein both inhibit glucokinase activity

To assess the inhibitory function of the purified recombinant human wild-type and P446L-GKRP proteins following heterologous expression in *E.coli*, one unit of each regulatory protein was used in assays to study competitive inhibition of GCK activity over a glucose concentration range of 0-100mM (figure 4.1A). Statistical analysis revealed no difference between the intrinsic inhibitory capacity of wild-type and P446L-GKRP. Equivalent assays with equimolar concentrations of both GKRPs were also conducted (100 and 150mM; figures 4.1B and C respectively). With 100nM, there was statistically no difference between the responses of the two regulatory proteins, although it was noted that the variance of this data was larger than seen for other experiments and only n=3. With 150nM, the intrinsic inhibitory capacity of P446L-GKRP was significantly lower than that of the wild-type regulatory protein at glucose concentrations over 25mM (p=0.03).

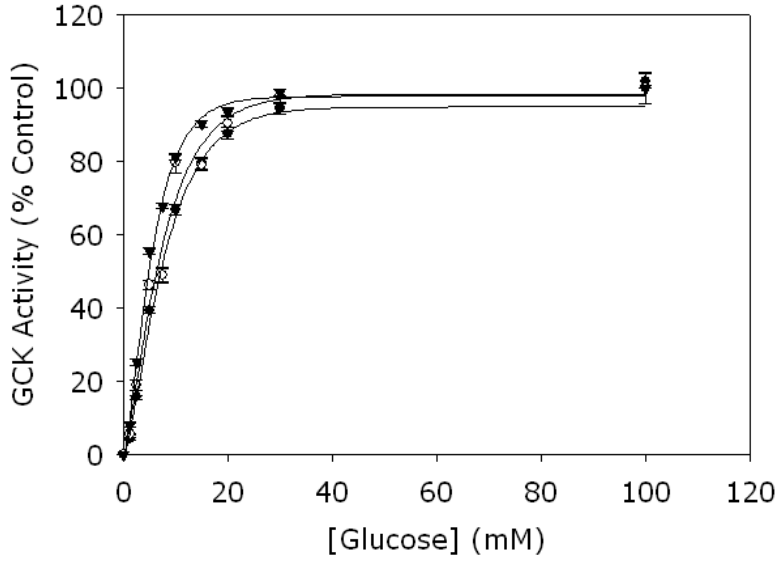
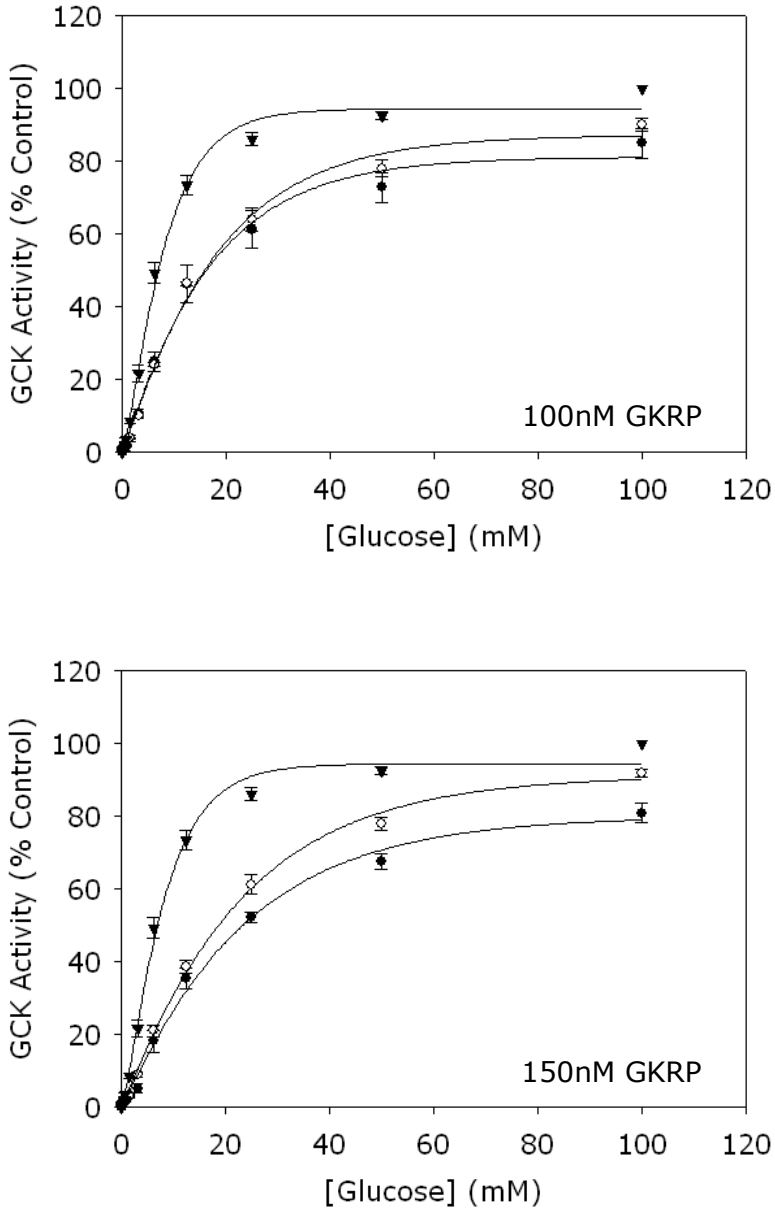


Figure 4.1A: Inhibition of GCK using equivalent activities of wild-type and P446L-GKRP (n=24). Inhibition of 10m-units GCK by one GKRP unit (wild-type = black circles, P446L = white circles, negative control = black triangles) was observed over a glucose concentration range of 0-100mM. Each data point was plotted as a percentage of the negative control in which GKRP was absent and glucose concentration equalled 100mM.



Figures 4.1B and C: Inhibition of GCK using equimolar concentrations of wild-type and P446L-GKRP (n=3). GCK activity was assayed in the presence of 0nM, 100nM and 150nM wild-type (black circles) and P446L-GKRP (white circles) over a glucose concentration range of 0-100mM. Each data point was plotted as a percentage of the negative control in which GKRP was absent (black triangles) and glucose concentration equalled 100mM.

Ki values calculated from the equimolar concentration experiments were not significantly different between the two regulatory proteins (24.0±40.7 for wild-type versus 23.1±40.8nM for P446L-GKRP; means ± standard error of the mean [SEM]; p=1).

Our data are consistent with GKRP acting as a competitive inhibitor of GCK activity (Vandercammen and Van Schaftingen, 1993). The maximal specific activity of GCK ($Kcat_{GCK}$) remained unchanged in the presence of 0-150nM regulatory protein (51.8±1.6 to 51.9±6.9 for wild-type versus 46.8±0.7 to 53.4±3.6S^{-1} for P446L-GKRP; means±SEM; n=3), whilst the $S_{0.5}$ (glucose concentration needed for half maximal rate) was raised with increasing concentrations of both GKRPs (8.7±0.2 to 16.8±1.3 for wild-type versus 8.0±0.3 to 18.2±1.1mM for P446L-GKRP).

4.3.2 Fructose 1-phosphate increases glucokinase activity with both wild-type and P446L-GKRP

Data obtained using comparable activities of regulatory protein demonstrated the expected reduction in GKRP-mediated inhibition of GCK activity across a F1P concentration range of 0-500µM (Figure 4.2A). This GKRP-mediated inhibition was decreased by the same extent for both wild-type and P446L-GKRP (53.7±1.1 to 92.8±1.2 GCK activity for wild-type versus 55.7±1.5 to 93.6±1.4% GCK activity with P446L; means±SEM; n=18; p>0.1). Experiments using fixed concentrations of both wild-type and variant regulatory protein confirmed that there was no difference in F1P-mediated inhibition of the two GKRPs at concentrations up to and including 500µM of phosphate ester (92.5±2.6 for wild-type versus 90.1±1.8% for P446L; n=9; p=0.2), despite the initial difference in intrinsic inhibitory capacity at 0µM F1P (Figure 4.2B).

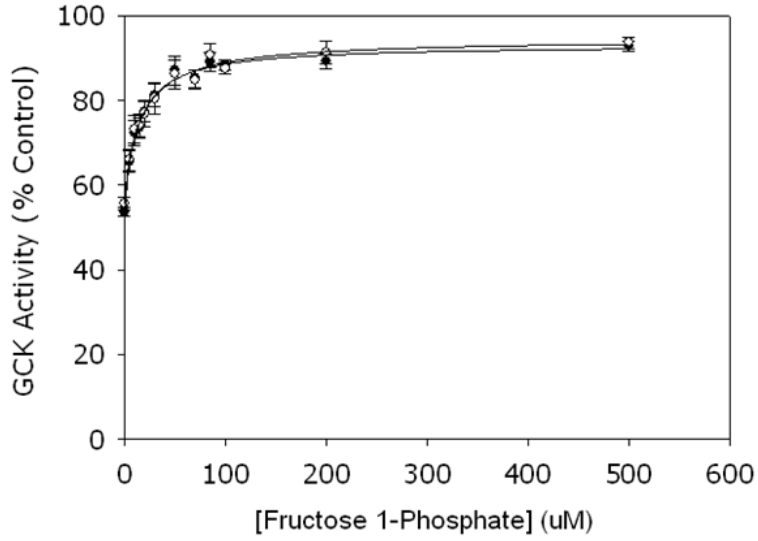


Figure 4.2A: F1P-mediated inhibition of wild-type and P446L-GKRP increases GCK activity. Inhibition of 10 m-units GCK by one GKRP unit (black circles = wild-type, white circles = P446L) was observed over 0-500µM F1P, 5mM glucose (n=18). GCK activity was plotted as a percentage of that obtained in the absence of either regulatory protein.

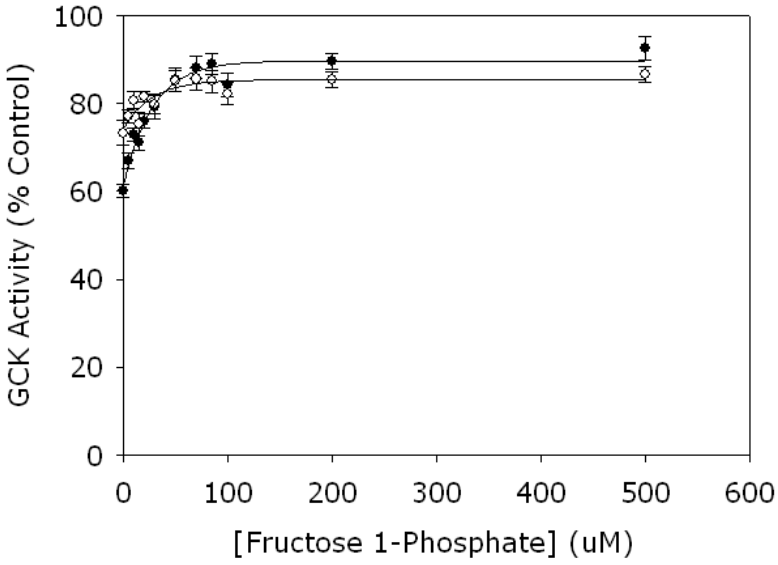


Figure 4.2B: One hundred nanomolar GKRP-mediated inhibition (black circles = wild-type, white circles = P446L) of 10m-units GCK was observed over 0-500µM F1P, 10mM glucose (n=9). GCK activity was plotted as a percentage of that obtained in the absence of either regulatory protein.

It has been suggested previously that F1P causes an increase in GCK activity by promoting dissociation of the GKRP:GCK complex (Brocklehurst, et al., 2004a). Therefore the 'apparent' inhibitor dissociation constant (Ki_{app}) would be expected to increase upon addition of F1P, as regulatory protein dissociation from the enzyme is promoted. In our studies, Ki_{app} from Dixon plots

of 1/percentage GCK activity versus F1P concentration reflected this. Also, these calculated Ki_{app} values did not significantly differ between the two regulatory proteins when using either comparable activity (24.5±2.0 for wild-type versus 31.2±4.3µM for P446L-GKRP; mean±SEM; p=0.1), or equimolar concentrations (54.0±10.8 for wild-type versus 46.3±4.2µM for P446L-GKRP; p=0.5), suggesting a similar dissociation of both regulatory proteins from GCK in response to F1P. This was also corroborated by observing the F1P concentration that caused reversal of GCK inhibition by half (i.e. 75% enzyme activity), which was approximately 15µM F1P for both GKRPs in the matched activity experiments, and approximately 15µM and 20µM F1P for P446L and wild-type GKRP respectively in the comparable concentration experiments.

4.3.3 Differences in fructose 6-phosphate-mediated regulation of P446L versus wild-type glucokinase regulatory protein

GCK activity was observed over 0-500µM F6P in the presence of either equal activities or equivalent concentrations of both GKRPs (Figure 4.3A and B respectively).

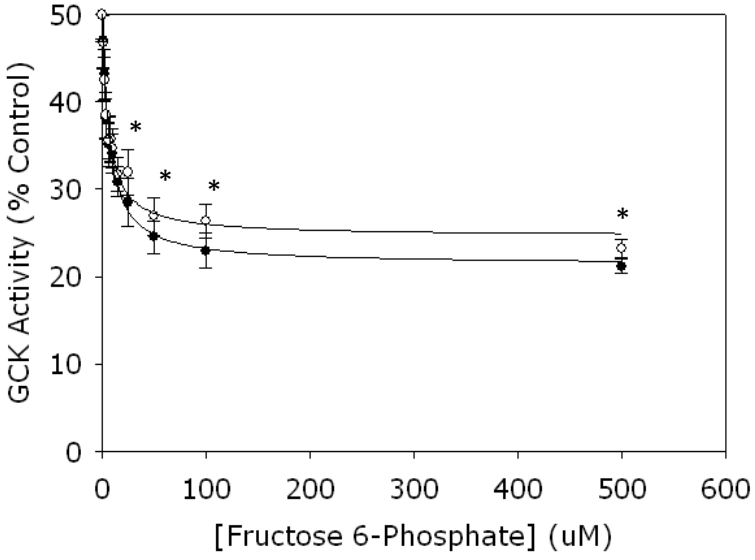


Figure 4.3A: F6P-mediated regulation of P446L-GKRP is significantly diminished compared to wild-type regulatory protein. Inhibition of 10 m-units GCK by one unit GKRP (black circles = wild-type, white circles = P446L) was observed over 0-500µM F6P, 5mM glucose. GCK activity was plotted as a percentage of that obtained in the absence of regulatory protein.

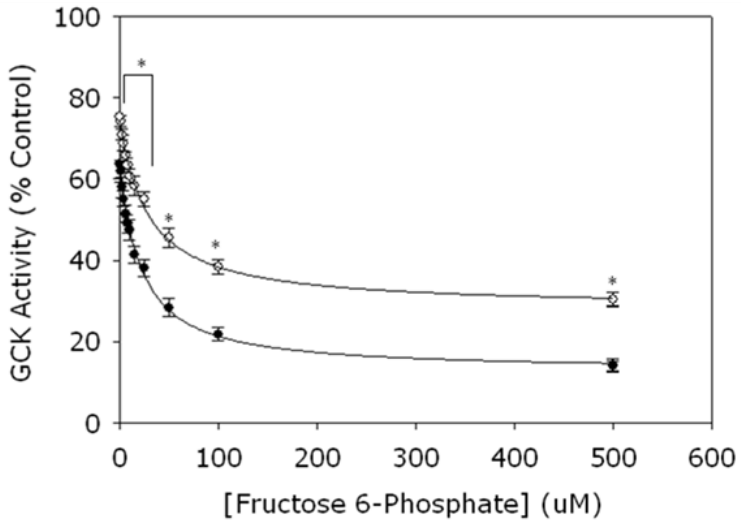


Figure 4.3B: Response of P446L-GKRP to the phosphate ester F6P is significantly reduced compared to wild-type. One hundred nanomolar GKRP-mediated inhibition (black circles = wild-type, white circles = P446L) of 10 m-units GCK was observed over 0-500µM F6P, 10mM glucose (n = 9). GCK activity was plotted as a percentage of that obtained in the absence of either regulatory protein.

It is well established that F6P-mediated regulation of GKRP leads to enhanced GCK inhibition, and hence a corresponding reduction in observed enzyme activity (Brocklehurst, et al., 2004a; Vandercammen and Van Schaftingen, 1993; Veiga-da-Cunha and Van Schaftingen, 2002). This was seen with both wild-type and P446L-GKRP when using the same activity of regulatory protein, although the reduction in GCK activity was significantly less over the physiologically relevant 25-500µM F6P concentration range with the variant protein (31.9±2.6 to 23.3±1.0 GCK activity versus 28.5±2.8 to 21.2±0.8% GCK activity for wild-type; means±SEM; n=18; p=0.04, 0.0001, 0.006 and 0.03 for 25, 50, 100 and 500µM F6P respectively). These results were confirmed in experiments using the same concentration of both regulatory proteins, as there was a significant difference between the response of wild-type and P446L-GKRP over the entire F6P concentration range (63.7±2.6 to 14.1±1.2 versus 71.9±1.4 to 33.8±3.1% respectively; n=9; p<0.001).

Increasing F6P concentration has been shown in this study to enhance GKRP-mediated inhibition of GCK activity, and thus it would be expected that Ki_{app} would be decreased as a result of reduced dissociation of the inhibitor GKRP from GCK (via tighter enzyme:regulatory protein binding). This

was reflected in our data, as Dixon plots of F6P concentration versus 1/percentage GCK activity showed a decrease in Ki_{app} upon addition of F6P. Ki_{app} was also significantly different between wild-type and P446L-GKRP for both the equal activity (31.0±4.4 versus 21.1±2.7μM respectively; mean ±SEM; p=0.5) and equimolar concentration experiments (16.6±1.7 versus 9.2±1.0μM respectively; p<0.001). The IC_{50} (concentration of F6P needed to inhibit enzyme activity by 50%) was also significantly different between wild-type and P446L-GKRP; 23.8 versus 35.0μM respectively for matched activity experiments (p=0.01), and 25.0 versus 56.3μM respectively for equal concentration experiments (p<0.001), reflecting a decreased affinity of the variant regulatory protein for F6P in both experiments.

4.3.4 Glucokinase regulatory protein is highly expressed relative to glucokinase in human liver, with very low levels in human islets

Due to the association of P446L-GKRP with FPG levels, we investigated whether the observed modulation in the variant regulatory protein response to physiologically-relevant F6P concentrations could have consequent metabolic affects in human pancreatic islets as well as human liver. To address this question, we quantified *GCKR* and *GCK* mRNA levels in appropriate human tissues (figure 4.4).

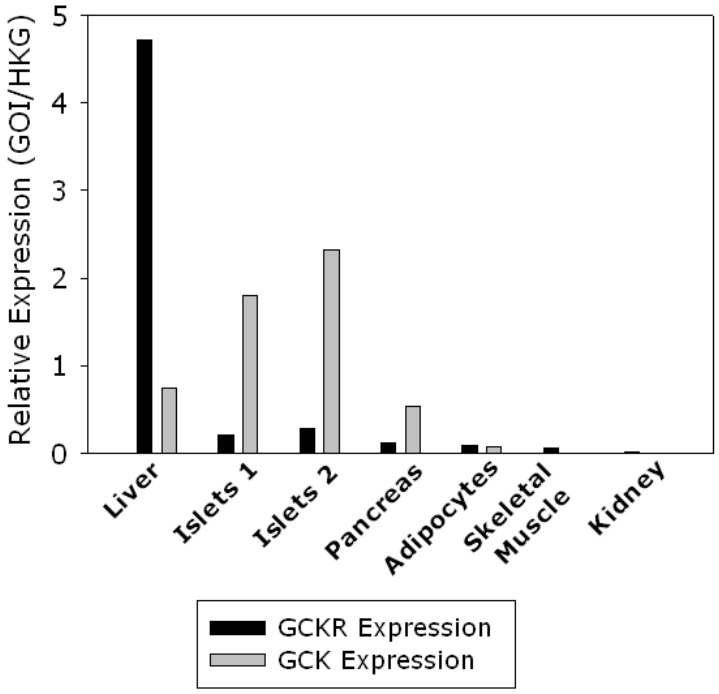


Figure 4.4: *GCKR* and GCK expression in human liver (n=1), adipocytes (n=1), kidney (n=1), skeletal muscle (n=1), whole pancreas (n=1), and isolated islets (n=2). One microgram of RNA was converted to cDNA via RT-PCR and expression of the human *GCKR* and *GCK* genes observed via Taqman gene expression analysis.

Quantitative RT-PCR analysis of human liver (n = 1), adipocytes (n = 1), kidney (n = 1), skeletal muscle (n=1), pancreas (n = 1), and islets (n = 2) showed that *GCKR* mRNA was expressed in molar excess in human liver compared to *GCK*, thus supporting a functional role for the regulatory protein in the hepatocyte (de la Iglesia, et al., 2000; Van Schaftingen and Veiga da Cunha, 2004; Vandercammen and Van Schaftingen, 1993). Conversely, although *GCKR* is expressed at low levels in human islets, whole pancreas, and adipocytes (only a tenth of that seen in liver), the much higher relative expression of *GCK* (~8-fold that of *GCKR* in islets) suggests regulation of this enzyme in these tissues is not GKRP-mediated. In adipocytes, skeletal muscle, and kidney both genes were only present at negligible levels.

4.4 Discussion

4.4.1 Human P446L-glucokinase regulatory protein exhibits diminished fructose 6-phosphate-mediated regulation versus the wild-type protein

In this study we performed the first functional characterisation of the human P446L-*GCKR* variant which had been reproducibly associated with an average 0.06mmol/L decrease in FPG levels and 0.15mmol/L increase in triglycerides in the general population (Orho-Melander, et al., 2008; Saxena, et al., 2007; Sparso, et al., 2008; Vaxillaire, et al., 2008). Our data demonstrated both human wild-type and variant regulatory protein to be functional and able to inhibit GCK activity in a dose-dependent manner. Both GKRPs were also equally sensitive to F1P-mediated regulation, which is consistent with that shown previously using rodent proteins (Veiga-da-Cunha, et al., 2003). However, our analyses revealed a previously unappreciated change in F6P-mediated modulation of the P446L-regulatory protein at physiologically relevant concentrations of this phosphate ester. These results differ from those reported in earlier rodent studies (Veiga-da-Cunha, et al., 2003) which showed no differences between the two proteins and confirms the important differences in the properties of human GKRP compared to rodent homologues (Brocklehurst, et al., 2004a).

4.4.2 Residue 446 lies close to phosphate ester binding motifs

A possible explanation as to why P 446L-GKRP exhibits altered F6P- but not F1P-mediated regulation comes from looking at the binding site of these molecules. The crystal structure of GKRP has not been solved, however it does have homology with other proteins of known crystal structure, such as the isomerase domain of bacterial glucosamine 6-phosphate synthase, which catalyses the conversion of F6P into either glucose 6-phosphate or glucosamine 6-phosphate (Teplyakov, et al., 1999; Teplyakov, et al., 1998; van Schaftingen, et al., 1997; Veiga-da-Cunha and Van Schaftingen, 2002). This isomerase domain consists of two sugar isomerase sub-domains (Bateman, 1999), and analysis of the GlmS crystal structure (Teplyakov, et al., 1998) and functional studies on rat GKRP mutants (Veiga-da-Cunha and Van Schaftingen, 2002) suggests a single substrate binding site lies

between the interface of these two sub-domains. It has also been suggested that GKRP adopts different structural conformations in order to accommodate either F1P or F6P at this single binding site, and only one of these (that favouring F6P-binding) can complex with GCK (Detheux, et al., 1991; Veiga-da-Cunha and Van Schaftingen, 2002). Residue 446 lies between two sequence motifs thought to be involved directly in the binding of phosphate esters. As previously reported, mutating certain residues outside of these motifs can still affect binding (and hence regulation of GKRP activity) (Veiga-da-Cunha and Van Schaftingen, 2002). Also, residue 446 is a conserved proline across human, rat and *Xenopus laevis* regulatory protein, and all bar the latter are responsive to both F1P and F6P (Brocklehurst, et al., 2004a; Veiga-da-Cunha and Van Schaftingen, 2002). Therefore, we proposed that residue 446 may not play a direct role in the binding of phosphate esters, but may be important structurally within GKRP. This amino acid substitution could then either reduce the ability of the regulatory protein to adopt the F6P-binding conformation, or hinder the F6P-GKRP complex from binding to and inhibiting GCK.

4.4.3 Diminished fructose 6-phosphate-mediated regulation of glucokinase regulatory protein could lead to increased hepatic glycolytic flux, thus accounting for the 446L phenotype

Our data showed F6P-mediated regulation of P446L-GKRP to be significantly attenuated compared to wild-type over a physiological range of F6P concentrations, which ultimately leads to a reduction in GCK-inhibition by the variant regulatory protein. This would be predicted to increase glycolytic flux and hence glucose uptake by the liver. This enhanced rate of glycolysis, as is seen with *Gck* over-expression in rats, may raise levels of other liver metabolites such as malonyl-CoA which would increase triglyceride levels via two mechanisms (O'Doherty, et al., 1999); firstly by acting as a substrate for *de novo* lipogenesis (Bloch and Vance, 1977; Girard, et al., 1994), and secondly by inhibiting carnitine-palmitoyl transferase-1 thus blocking fatty acid oxidation (McGarry and Foster, 1980). This perturbation of hepatic metabolism could account for the reduced glucose and raised triglycerides seen in L446-GKRP individuals . However, the very low *GCKR* mRNA levels detected in

human islets compared to human liver suggest that GKRP has only a very minor (if any) functional role in the regulation of GCK in the pancreas (de la Iglesia, et al., 2000). Consequently, pancreatic GCK is unlikely to be affected in individuals with this variant regulatory protein confirming that the phenotype observed is driven by a hepatic effect.

4.4.4 Altered regulation of hepatic glucokinase by P446L variant regulatory protein provides important information regarding novel approaches to diabetes treatment

Broader implications of these findings regard the modulation of GCK activity to treat diabetes. Aberrant GCK function has been implicated in monogenic forms of diabetes (Froguel, et al., 1992; Glaser, et al., 1998; Hattersley, et al., 1992; Njolstad, et al., 2001; Osbak, et al., 2009), and common variation within the gene has also been associated with both FPG levels (Prokopenko, et al., 2009; Weedon, et al., 2006; Weedon, et al., 2005) and T2D risk (Dupuis, et al.). As such, GCK is a well-established therapeutic target (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010).

The initial genetic association data concerning the P446L-*GCKR* variant (Orho-Melander, et al., 2008; Saxena, et al., 2007; Sparso, et al., 2008; Vaxillaire, et al., 2008) supported by our functional studies confirms that changing the regulation of GCK activity solely in the liver could alter other hepatic metabolic pathways, thus resulting in an unfavourable lipid profile. This has already been shown in one study in rodents, where over-expressing hepatic *Gck* lowers plasma glucose as well as raising triglyceride levels (O'Doherty, et al., 1999). This hypothesis is strengthened by the recent observation that rare *GCKR* non-synonymous variants are enriched in patients with hypertriglyceridaemia (Johansen, et al., 2010). Additionally, a recent survey of participants within the ClinSeq programme [a study into the use of large-scale genome sequencing for clinical research (Biesecker, et al., 2009)] identified 17 rare non-synonymous *GCKR* variants in individuals with increased triglycerides and decreased triglyceride/glucose ratios, although this did not reach significance (Rees, et al., 2010). However, a number of other studies have shown that hepatic over-

expression of *Gck* does not always result in an increase in triglycerides, suggesting that the degree of increased GCK activity is critical (Desai, et al., 2001; Ferre, et al., 1996a). As such, any potential new therapies targeting GKRP in the liver should be approached with caution (Agius, 2009).

4.4.5 The mutational mechanisms behind genetic variants associated with complex human disease and related quantitative traits can be uncovered if appropriate functional studies are performed

In conclusion, our functional studies demonstrate that altered F6P-mediated GKRP regulation in carriers of the L446 allele explain the association between the common P446L-*GCKR* variant and higher triglyceride and lower FPG levels in several human populations (Orho-Melander, et al., 2008; Saxena, et al., 2007; Sparso, et al., 2008; Vaxillaire, et al., 2008). This work demonstrates that with suitable functional studies it is possible to establish mutational mechanisms for common variants of modest effect size identified by GWA studies (van de Bunt and Gloyn, 2010), a route which we must now take in order to translate these genetic signals into deeper biological understanding and improved therapeutic strategies (McCarthy and Hirschhorn, 2008b).

Chapter 5

Characterisation of P446L-GKRP within a cellular system aims to provide further insight into the mutational mechanism behind this variant

5.1 Introduction

5.1.1 Kinetic characterisation of the common P446L glucokinase regulatory protein variant associated with fasting plasma glucose and triglyceride levels may not fully explain the mutational mechanism behind this genetic association

A recent genome-wide association study for type 2 diabetes (T2D) and associated traits identified *GCKR* as a novel regulator of fasting plasma glucose (FPG) and triglyceride levels (Saxena, et al., 2007). Further fine-mapping revealed that the signal came from a common non-synonymous single nucleotide polymorphism (SNP) within *GCKR*, this SNP encoding a proline to leucine amino acid change at residue 446 (rs1260326, c.1403C>T, p.P446L, minor allele frequency 34%) (Orho-Melander, et al., 2008). Subsequent studies were also able to replicate the association of this SNP with the unusual phenotype of lowered FPG and raised triglycerides (Sparso, et al., 2008; Vaxillaire, et al., 2008).

GCKR encodes the glucokinase regulatory protein (GKRP), which is an endogenous hepatic inhibitor of the glycolytic enzyme glucokinase (GCK). GCK fulfils a pivotal regulatory role over glucose metabolism in both the hepatocyte and beta-cell (Matschinsky, 2002; Matschinsky, 2009; Matschinsky, et al., 2006). Physiologically, the activity of the regulatory protein is modulated by fructose 6- and fructose 1-phosphate (F6P and F1P respectively), these phosphate esters competing with each other for binding and enhancing or diminishing GKRP-mediated inhibition of GCK activity respectively (Mookhtiar, et al., 1996; Van Schaftingen, 1989; Van Schaftingen and Vandercammen, 1989; Vandercammen, et al., 1992; Vandercammen and Van Schaftingen, 1990). In addition to its direct inhibitory role on GCK activity, experiments using *Gckr* knock-out mice demonstrated that GKRP also has a paradoxical role in which it binds and stabilises GCK, sequestering the enzyme in the cell nucleus away from proteolytic degradation (Farrelly, et al., 1999; Grimsby, et al., 2000). This serves to maintain nuclear 'reserve pools' of GCK which can be released rapidly upon postprandial

rises in blood glucose levels (Bosco, et al., 2000; de la Iglesia, et al., 1999; Fernandez-Novell, et al., 1999; Mukhtar, et al., 1999; Shiota, et al., 1999; Toyoda, et al., 1995)

Initial *in vitro* functional studies of rat P446L-GKRP suggested that this variant regulatory protein behaved no differently to wild-type, both with regards to inhibitory capacity and response to phosphate ester-mediated regulation (Veiga-da-Cunha, et al., 2003). The mutational mechanism behind this genetic association therefore remained elusive. However, subsequent studies directly comparing the activities and intrinsic properties of rat and human GKRP showed important differences between the regulatory proteins of both species (Brocklehurst, et al., 2004a) despite their high homology [88%; (Warner, et al., 1995)]. Firstly, human regulatory protein could inhibit GCK even in the absence of F6P, and secondly, human GKRP bound this phosphate ester with much higher affinity versus the rodent protein (Brocklehurst, et al., 2004a). This prompted re-characterisation of P446L-GKRP using human proteins, this study showing the variant regulatory protein to exhibit a significantly diminished response to F6P-mediated modulation over physiologically-relevant concentrations of this phosphate ester (**chapter 4 section 4.3.3**). This taken together with the results of expression studies which showed that *GCKR* is only expressed in molar excess versus *GCK* in the liver (**chapter 4 section 4.3.4**), suggested that reduced F6P-mediated inhibition of GCK activity in individuals homozygous for the risk 'L' *GCKR* allele would lead to enhanced hepatic glycolytic flux and lowered FPG levels. This was proposed to subsequently increase levels of other metabolites such as malonyl-CoA, which would serve to increase triglyceride levels by both acting as a substrate for *de novo* lipogenesis (Bloch and Vance, 1977; Girard, et al., 1994) and by blocking fatty acid oxidation (McGarry and Foster, 1980; O'Doherty, et al., 1999).

5.1.2 Cellular characterisation of P446L-glucokinase regulatory protein is crucial in order to fully understand the mutational mechanism behind this variant

Despite a putative mutational mechanism proposed for P446L-GKRP, characterisation of this variant remains incomplete, as its role in GCK stabilisation and nuclear sequestration has yet to be observed.

Therefore, the aim of this study is to further characterise P446L-GKRP within a cellular system, to note specifically whether the variant exhibits differences in its ability to bind and sequester GCK in the nucleus versus wild-type regulatory protein. Early experiments observing GCK and GKRP localisation have largely used immunostaining (Bosco, et al., 2000; Chu, et al., 2004; Toyoda, et al., 1994; Toyoda, et al., 1995), however more recent technological advances have allowed subsequent studies to view GCK:GKRP intracellular localisation and translocation using fusion proteins and fluorescent microscopy (Baltrusch, et al., 2005; Baltrusch and Lenzen, 2007; de la Iglesia, et al., 1999; Shiota, et al., 1999). Consequently, I aim to study P446L-GKRP using fluorescent fusion proteins in a similar manner. As previous fluorescent microscopy study protocols have been optimised using rat GCK and GKRP (Baltrusch, et al., 2005), I initially aim to replicate the glucose-responsive intracellular trafficking observed with rat fluorescent fusion proteins.

I hypothesise that there could be differences between the ability of wild-type and P446L-GKRP to bind GCK and sequester in the cell nucleus, thus affecting the amount of free 'metabolically-active' GCK in the cell cytoplasm, which would subsequently impact upon hepatic glycolytic flux in individuals carrying the variant *GCKR* allele.

5.2 Materials & Methods

5.2.1 Fluorescent fusion protein constructs

The experimental work used in this study was carried out in collaboration with Prof Francis Collins and Matthew Rees at the National Institutes for Health (Maryland, USA). The rat fluorescent fusion constructs were kindly supplied by Dr Simone Baltrusch (Department of Medical Biochemistry and Molecular Biology, University of Rostock, Germany) (Baltrusch, et al., 2005). These constructs contained wild-type rat *Gck* (NM_012565) and *Gckr* (NM_013120) coding sequences within the pEGFP-c3 and pmCherry-n1 vectors respectively (both Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France).

P446L site-directed mutagenesis on rat Gckr-pmCherry-n1

The P446L mutation was introduced into the rat *Gckr* coding sequence by polymerase chain reaction (PCR)-based site-directed mutagenesis (SDM) using the QuikChange SDM kit (Stratagene Europe, Amsterdam, The Netherlands) according to the manufacturer's guidelines (**chapter 2 section 2.4**). Details of the mutagenesis primers used are given in table 5.1 (Eurofins MWG Operon, Ebersberg, Germany).

Primer	**Sequence (5'- 3')**
Rat SDM P446L	F - CAGTCCTTGCTGGCCCCTCTAAAGAAACTCTTTCCCTCA
	R - TGAGGGAAAGAGTTTCTTTAGAGGGGCCAGCAAGGACTG

Table 5.1: SDM primers used to introduce P446L mutation into rat *Gckr* coding sequence. Mismatched bases are underlined, with 'F' and 'R' denoting forward and reverse primers respectively.

Template DNA was removed from the mutagenised samples via *Dpn*I-mediated digestion (New England Biolabs UK Ltd, Hitchin, UK). The mutated plasmid was then used to transform SCS1 *Escherichia coli*, and DNA extracted using the QIAprep Spin Miniprep Kit (Qiagen Ltd, Crawley, UK). For more information on these protocols refer to **chapter 2 materials & methods**.

Confirmation of correct coding sequences/mutations within final fluorescent constructs

PCR-mediated amplification of the fluorescent constructs used primers designed against the rat *Gck* and *Gckr* coding sequences (table 5.3), as well as those specific for the pEGFP-c3 and pmCherry-n1 vector sequences (table 5.4). All PCR primers were obtained from Eurofins MWG Operon.

Primer	Sequence (5'- 3')	Primer	Sequence (5'- 3')
R.*Gckr* 1	R – TCCCAGGCCTTTCATCAGCTGGTT	R.*Gck* 1	R – GCCAGGATCTGCTCGACCAGAGTC
R.*Gckr* 2	F – ATTGTGCCCACCTACCAGCGAC	R.*Gck* 2	F – GCCGGATGCAGAAGGAGATGGAC
	R – ACCTTCTTCAGCTCCTCGATCCCGT		R – GTTCACCATTGCCACCACATGCATC
R.*Gckr* 3	F – GCTGAGTGGAGGGGGAACCTCTG	R.*Gck* 3	F – CCATCCCCGAGGACGCCATGAC
	R – CGGCTCATCAGGTGACCTACAGTC		R – CCAGCTCGCCCATATACTTCCCACC
R.*Gckr* 4	F – CCGGCAAGTGGCAGAGCGGATG	R.*Gck* 4	F – CATGTGCGTCAACACGGAGTGGGG
	R – CAAGGACTGCCCCACAGTGTCTGT		R – TTCAGCCTGCGCACACTGGCGTGA
R.*Gckr* 5	F – GAACTCACCAACCAGGGTCCCCAG	R.*Gck 5*	F – CTGTGGGCGTGGATGGCTCC
	R –TCCCGCCTGTGGGACCCTGAATTG		
R.*Gckr* 6	F – GGCCATGTTGCAGAGGTTCTCTGG		

Table 5.3: PCR primers designed against the rat *Gckr* and rat *Gck* coding sequences. All primers contained M13 tail attachments for ease of sequencing; forward $^{5'}$TGTAAAACGACGGCCAGT$^{3'}$, reverse $^{5'}$CAGGAAACAGCTATGACC$^{3'}$. 'F' denotes forward primer, 'R' the reverse primer. All primers were supplied by Eurofins MWG Operon.

Primer ID	Sequence (5'- 3')
pEGFP-c3	F – CGACAACCACTACCTGAGCA
	R – TGAGTTTGGACAAACCACAAC
pmCherry-n1	F – CACCAAAATCAACGGGACTT
	R – CCTGTCCCCTCAGTTCATGT

Table 5.4: PCR primers designed against the pEGF-c3 and pmCherry-n1 vector sequences. Use of these primers ensured all coding sequence was included in the final sequencing reactions. All primers contained M13 tail attachments for ease of sequencing; forward $^{5'}$TGTAAAACGACGGCCAGT$^{3'}$, reverse $^{5'}$CAGGAAACAGCTATGACC$^{3'}$. 'F' denotes forward primer, 'R' the reverse primer. Forward vector primers were used with the desired coding sequence fragment 1 reverse primer, whilst the reverse vector primers were used with the forward primer covering the 3' end of appropriate coding sequence.

Following amplification, PCR products were observed via ethidium bromide staining on a 2% agarose gel (both Sigma Aldrich Company Ltd, Gillingham, UK), and then submitted to bidirectional sequencing using M13 primers (Eurofins MWG Operon). Further detail on these protocols is provided in **chapter 2 materials & methods**. This sequencing aimed to confirm that 1) the P446L

mutation had been accurately introduced into the *Gckr* coding sequence, and 2) that both the *Gck* and *Gckr* coding sequences were absent of all other mutations.

5.2.2 Cell culture

HepG2 cells are an immortal cell line derived from the hepatocellular carcinoma of an adolescent Caucasian male (Knowles, et al., 1980). Appropriate culture of these cells can induce their polarisation (Van, et al., 2000; van, et al., 1997), thus accurately mimicking polarised hepatocyte cells as occurs *in vivo*. This makes them a good model system for studying intracellular events specific to the liver. However, difficulties in obtaining appropriate levels of transfection with these cells and time constraints imposed on this study meant work with this cell line was discontinued. Henrietta Lacks (HeLa) cells (Scherer, et al., 1953) were used instead for several reasons. Firstly, these cells do not express *GCK* or *GCKR*, thus meaning intracellular binding and localisation of the fluorescent fusion proteins would not be affected by endogenous proteins. Secondly, both GCK and GKRP had been shown to interact in the nucleus of these cells (Shiota, et al., 1999), and finally, HeLa cells have been shown to take-up glucose at measurable levels (Rodriguez-Enriquez, et al., 2009).

Culture and maintenance of HeLa cells

HeLa cells were maintained in culture at 37^{o}C, 5% CO_2, in 75cm² rectangular canted neck cell culture flasks with vented caps (Corning, Lowell, Massachusetts, USA) using Dulbecco Modified Eagle's Medium (DMEM) containing; 25mM glucose, 10% foetal bovine serum, and 1% penicillin/streptomycin (all Invitrogen, Carlsbad, California, USA). Note: DMEM medium containing 5.5mM glucose was also used to culture cells where GCK:GKRP localisation was to be observed under conditions of low sugar, and there were no pre-transfection differences noted between cells cultured at these differing glucose concentrations. Passage of cells involved removal of culture medium via aspiration, followed by washing with sterile phosphate-buffered saline solution (Invitrogen). Subsequent incubation with trypsin (Invitrogen) for 3 minutes at 37^{o}C caused cell

detachment. The detached cells were then re-suspended in culture medium to dilute and neutralise the trypsin, before centrifugation at ~200xg for 5 minutes to pellet the cells. The pelleted cells were then finally re-suspended in the desired amount of fresh culture medium and passaged as appropriate.

Cells were counted using a Neubauer haemocytometer (Invitrogen) to determine the number of cells/ml culture medium. As before, trypsin was used to detach cells from the culture flask wall, and this mixture neutralised by the addition of fresh medium as discussed previously. Trypan blue (Invitrogen) was used to stain the cells so that they would be visible under the light microscope.

5.2.3 Transient transfection of HeLa cells

The following transfection protocols were established following the optimisation of numerous parameters including; the amount of DNA transfected, the volume- and type of transfection reagent used, time allowed for recombinant protein expression, etc.

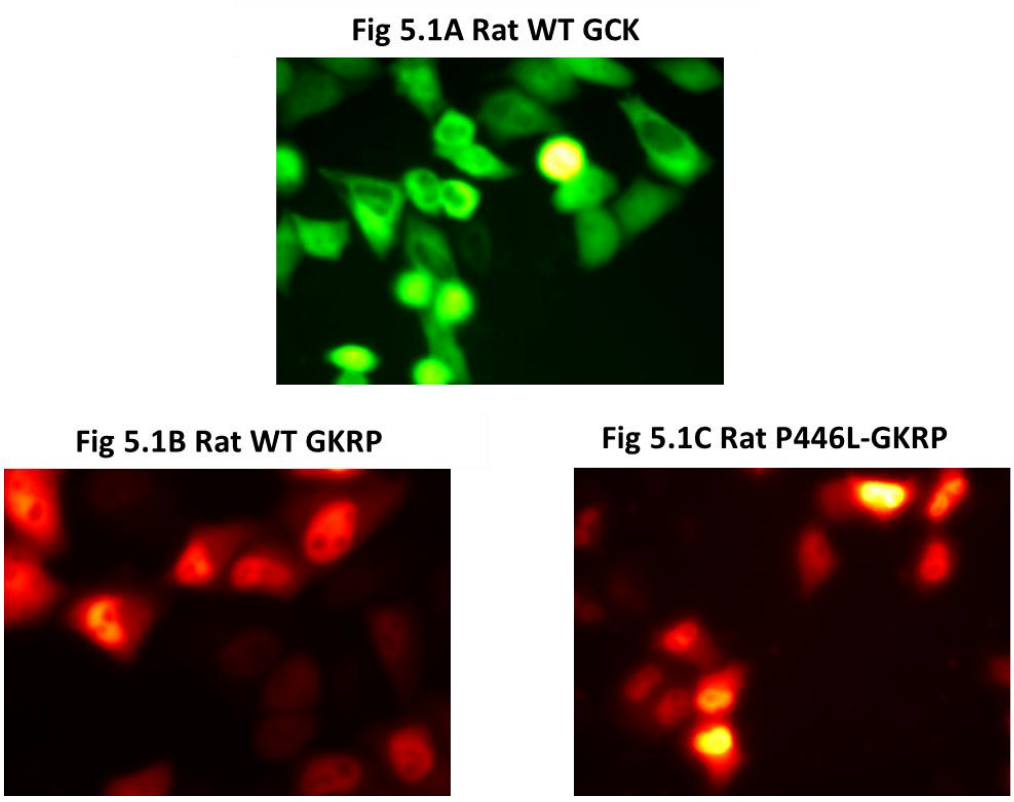
For transfection, a single glass coverslip (BD Biosciences, Maryland, USA) was placed in the bottom of each well of a six well clear cell culture plate (Corning). For cell seeding, $4x10^6$ cells suspended in 2ml medium (either high [25mM] or low [5.5mM] glucose) were added to each well. Incubation at 37^oC, 5% CO_2, for 24 hours allowed these cells to divide and attach to the coverslips. Transfection was carried out the next day as follows. Firstly, 40ng *Gck* and 160ng *Gckr* plasmid DNA were added to 3µl Lipofectamine-2000 (Invitrogen) in a sterile micro-centrifuge tube and incubated for 30 minutes at room temperature. Two hundred nanograms total plasmid DNA was found to be optimal for fluorescent protein expression and cell viability in these experiments, and the 1:4 *Gck*:*Gckr* expression ratio was proposed to reflect physiological mRNA levels for both genes (**chapter 4 section 4.3.4**). Following incubation, the DNA-Lipofectamine mixture was added to 2ml Opti-MEM reduced serum medium (Invitrogen). This was then used to replace the medium in each well of the cell-seeded plate, and the cells were then incubated with this transfection mixture for 5 hours at 37^oC,

5% CO_2. Following incubation, the transfection mixture was removed from the cells and replaced with 2ml of normal DMEM culture medium (high [25mM] or low [5.5mM] glucose). Cells were finally incubated for 24 hours at 37^{o}C, 5% CO_2 to allow for fluorescent fusion protein expression, before fixing to the coverslips with 4% formaldehyde (Sigma Aldrich Corp, St Louis, Missouri, USA) and mounting with VectaShield mounting medium (Vector Laboratories, Burlingame, California, USA). All cell images were viewed and recorded using a Zeiss 510 NLO confocal microscope with the same laser settings (Carl Zeiss Microimaging, Thornwood, New York, USA). Each transfection experiment was carried out at least two times.

5.3 Results

5.3.1 Independent transfections of the rat constructs confirm that glucokinase cannot enter the nucleus without glucokinase regulatory protein

Initially, the constructs encoding both rat wild-type GCK and wild-type or P446L-GKRP were used in independent transfection experiments to assess how these proteins behaved when expressed by themselves. Figure 5.1A shows that transiently-transfected wild-type rat GCK-GFP is purely cytoplasmic (5.1A), as had been seen previously (Baltrusch, et al., 2005). This was true for experiments carried out at both high and low glucose concentrations (Farrelly, et al., 1999). Images obtained following the transient transfection of HeLa cells with plasmids encoding rat wild-type or P446L-GKRP-cherry (figures 5.1B and C respectively) show that both regulatory proteins are predominantly nuclear-bound when expressed by themselves (5.1B and C).



Figures 5.1A-C: Transient transfection of HeLa cells with rat wild-type pEGFP-c3-*Gck* (A), wild-type (B), or P446L-pmCherry-n1-*Gckr* (C). Recombinant GCK is shown by green fluorescence, recombinant GKRP by red. The above images are representative of that obtained at both high and low glucose.

5.3.2 Co-expression of rat glucokinase and glucokinase regulatory protein results in glucose-responsive intracellular trafficking of the complexed proteins

Figures 5.2A-D show images obtained following the co-transfection of rat wild-type pEGFP-c3-*Gck* with either rat wild-type or P446L-pmCherry-n1-*Gckr.* The results of these experiments show that at low glucose (5.5mM, figures 5.2A and B), rat GCK and both rat GKRPs co-localise to the nucleus (yellow fluorescence). At high extracellular glucose concentrations of 25mM (figures 5.2C and D), rat GCK translocated into the cytoplasm, whilst both regulatory proteins remained retained in the nucleus. Time-course experiments were also conducted where the cell culture medium was changed from low to high glucose following the collection of the initial microscopic observations and image recording, and vice-versa. Following 24 hour incubation under the 'new' extracellular glucose conditions, it was noted that rat GCK localisation was dynamic, the enzyme moving from the nucleus to the cytoplasm with increasing glucose concentrations, and vice-versa if the medium glucose levels were reduced. This dynamic glucose-responsive intracellular trafficking occurred regardless of whether the rat enzyme was co-expressed with rat wild-type or P446L-GKRP.

Fig 5.2A Rat WT GCK & WT GKRP – 5mM Glucose

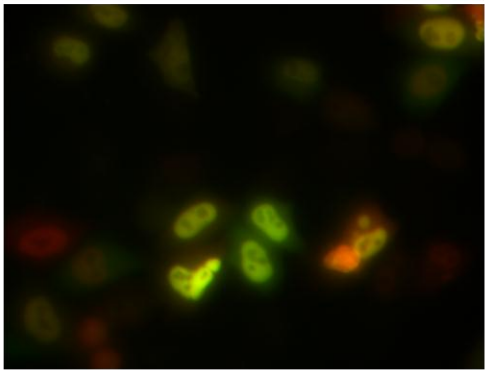


Fig 5.2B Rat WT GCK & P446L-GKRP – 5mM Glucose

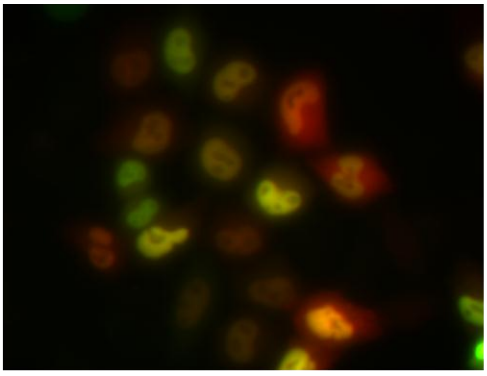


Fig 5.2C Rat WT GCK & WT GKRP – 25mM Glucose

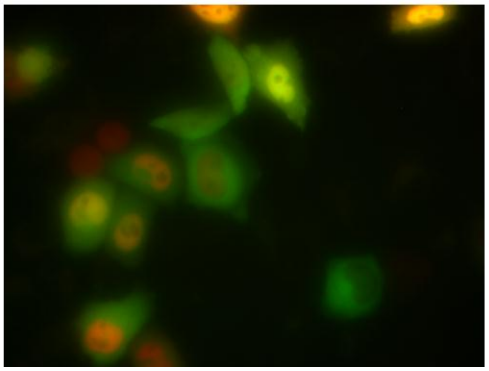
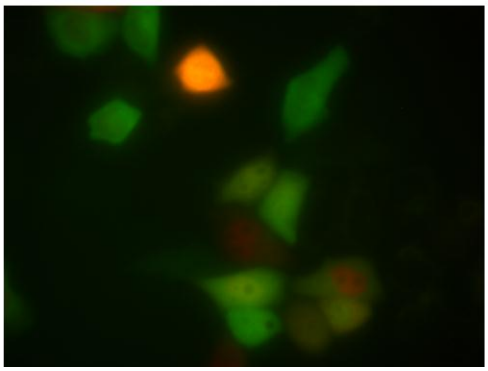


Fig 5.2D Rat WT GCK & P446L-GKRP – 25mM Glucose



Figures 5.2A-D: Microscopy images obtained following transient co-transfection of HeLa cells with rat wild-type pEGFP-c3-*Gck* construct and either rat wild-type or P446L-pmCherry-n1-*Gckr*, in conditions of high (25mM) or low (5.5mM) glucose. Recombinant Gck is shown as green fluorescence, and Gckr as cherry. Yellow fluorescence signifies co-localisation of the two proteins. All images were taken with the same laser settings, and are representative of the two repeats carried out for each experiment.

5.3.3 No differences between the ability of rat wild-type and P446L-regulatory protein to bind and sequester rat wild-type glucokinase in the cell nucleus

Under conditions of both high and low glucose, there appeared to be no difference between the ability of rat wild-type and P446L-GKRP to enter the cell nucleus when expressed in the absence of GCK. Additionally, co-expression of both regulatory proteins with rat GCK saw approximately equivalent amounts of GKRP-mediated enzyme sequestration in the cell nucleus. However, due to the time constraints of this project, these observations were not accurately quantified.

5.4 Discussion

5.4.1 Cellular studies may help further explain the mutational mechanism behind the P446L glucokinase regulatory protein variant associated with fasting plasma glucose and triglycerides

The initial identification of *GCKR* as a novel locus regulating FPG and triglyceride levels shed new light on the potential role of hepatic GCK activity in both glucose and lipid metabolism within the liver (Saxena, et al., 2007). Preliminary kinetic characterisation of the variant behind this genetic association showed that human P446L-GKRP exhibited diminished F6P-mediated GCK binding (**chapter 4**), thus providing a plausible mutational mechanism behind the associated low FPG and raised triglyceride phenotype (O'Doherty, et al., 1999). However, evidence from *Gckr* knock-out mice show that loss of the GKRP-GCK interaction means that the enzyme is not sequestered in the cell nucleus away from proteolytic degradation, paradoxically reducing hepatic GCK levels (Farrelly, et al., 1999; Grimsby, et al., 2000). This study therefore aimed to complete the cellular characterisation of P446L-GKRP in order to take into account its additional nuclear chaperone role.

5.4.2 Glucokinase regulatory protein is essential for nuclear localisation of glucokinase

Initially, the independent transfection of the rat and human *GCK*- and *GCKR*-encoding constructs was carried out in a bid to replicate the results of previous studies (Baltrusch, et al., 2005), and to validate that the fluorescent tags did not affect the cellular behaviour and localisation of the fusion proteins. Data obtained with the rat proteins showed that in the absence of regulatory protein, GCK remained cytoplasmic (figure 5.1). This agrees with the findings of previous studies using insulinoma-derived cell lines endogenously expressing *Gck* (Bosco, et al., 2000), those in cells where both the enzyme and regulatory protein are naturally absent (Bosco, et al., 2000; Shiota, et al., 1999), *Gckr* knockout mice (Farrelly, et al., 1999; Grimsby, et al., 2000), and cells expressing *X.laevis Gck* mutants with reduced affinity for GKRP (de la Iglesia, et al., 1999). Additionally, the lack of a GCK nuclear

localisation signal further suggests that the regulatory protein is needed to act as a nuclear chaperone for the enzyme (Shiota, et al., 1999).

Experiments in which the *GCKR*-constructs were transfected independently of those encoding the enzyme saw predominantly nuclear localisation of the rat regulatory protein. In the limited time-frame of this study it was however difficult to detect whether the nuclear GKRP pool increased upon co-expression of GCK as has been observed in other studies (Bosco, et al., 2000). Co-expression of rat GKRP and GCK saw the enzyme relocate from the cytoplasm to the nucleus under conditions of low glucose as has been seen previously (Baltrusch, et al., 2005).

5.4.3 No differences in the ability of rat wild-type and P446L regulatory protein to bind and sequester glucokinase in the nucleus

Data obtained with the rat proteins suggested that there were no difference between the amount of GCK retained in the nucleus by either wild-type or P446L-GKRP. This finding complements that obtained following the previous kinetic characterisation of the recombinant rat variant regulatory protein, which concluded it behaved no differently to wild-type GKRP (Veiga-da-Cunha, et al., 2003). However, it is important to remember that in the limited time-frame of this study we were unable to quantify this result, and this is particularly important considering that the cellular localisation of GKRP has been shown to be heterogeneous (Mukhtar, et al., 1999; Watanabe and Furuya, 2010). Additionally, any differences that may have been anticipated based on the previous kinetic characterisation of human P446L-GKRP may have been too small to see 'by eye', as the P446L *GCKR* variant has only a small effect size on FPG and triglyceride levels, and the human protein shows only a marginally attenuated response to F6P (**chapter 4**).Therefore in order to confirm this result, these experiments would need to be repeated and the fluorescence intensity of wild-type and P446L-GKRP within the two cellular compartments measured.

5.4.4 Intrinsic biochemical differences between the rat and human regulatory proteins mean that human P446L must be characterised at the cellular level in order to fully explain the mutational mechanism behind this variant

Figure 5.2 shows the data obtained upon co-expression of rat wild-type GCK and rat wild-type or P446L-GKRP in HeLa cells cultured at both high and low glucose conditions. When glucose levels were low, both GCK and wild-type/variant GKRP co-localised to the nucleus (figures 5.2A and B). However, when these transfected cells were then cultured in high glucose medium, GCK appeared to redistribute to the cytoplasm whilst GKRP remained predominantly nuclear-bound (figures 5.2C and D). These data corroborate findings from previous studies also investigating the cellular location of the rat proteins (Baltrusch, et al., 2005; Bosco, et al., 2000; de la Iglesia, et al., 1999; Shiota, et al., 1999), as well as those studying this intracellular GCK trafficking *in vivo* (Fernandez-Novell, et al., 1999). Therefore, we propose that data obtained using the rat proteins accurately mimics GCK and GKRP intracellular trafficking as occurs physiologically.

However, the crucial differences between rat and human GCK (Brocklehurst, et al., 2004a) make the use of rodent proteins for the functional characterisation of genetic variants associated with disease in humans less than ideal. This has been demonstrated previously with the kinetic characterisation of P446L-GKRP using recombinant human (**chapter 4**) and rat (Veiga-da-Cunha, et al., 2003) proteins, with the results of these experiments yielding different outcomes. Therefore, although we were able to replicate the intracellular trafficking of previous rat protein-based experiments (Baltrusch, et al., 2005; Bosco, et al., 2000; de la Iglesia, et al., 1999; Shiota, et al., 1999), no conclusions can be drawn about the mutational mechanism of this variant without also studying the cellular behaviour of human P446L-GKRP.

During the time-frame available for this project which was carried out during a secondment to NIH, we were unable to conduct these experiments with human proteins. This has however been studied by others, although this has yet to be optimised so as to observe glucose-responsive intracellular

trafficking. For example, experiments in a similar HeLa cell system have only observed 'glucose-unresponsive' localisation of both human GKRP and GCK (Rees, et al., 2010; Shiota, et al., 1999). This could however be due to a number of reasons, including the requirement of liver-specific factors by the human proteins in order for targeted intracellular trafficking to occur (Shiota, et al., 1999) [these may be lacking in the cervical carcinoma-derived HeLa cell line (Scherer, et al., 1953)]. Secondly, the intracellular redox state of HeLa cells is different to that in hepatocytes, which may affect human GCK and/or GKRP intracellular activity and trafficking (Tiedge, et al., 1997; Tiedge, et al., 2000; Tippett and Neet, 1983). These obstacles could be overcome by prioritising the optimisation of HepG2 culture and transfection, however studies in which this has been conducted report no difference in GCK/GKRP localisation with the use of these cells (Rees, et al., 2010).

Nevertheless, the human fluorescent proteins have since been used in a cellular system to characterise rare *GCKR* variants identified in individuals from the ClinSeq programme (Biesecker, et al., 2009) who have elevated triglycerides and other glucose or lipid phenotypes (Rees, et al., 2010). The authors of the study proposed that despite the lack of glucose-responsive intracellular trafficking, this cellular system involving human proteins was still valid as any localisation defects observed in HeLa cells were potentially an underestimate of that occurring in hepatocytes (Rees, et al., 2010; Shiota, et al., 1999), and that kinetic characterisation of all human *GCKR* variants to date had shown none to affect GCK glucose-dependence (Rees, et al., 2010). The subsequent results of this study showed a variety of intracellular localisation/trafficking defects with the naturally occurring *GCKR* variants which could be contributing to the triglyceride-phenotype of these individuals (Rees, et al., 2010). This hypothesis is also supported by another study in which rare *GCKR* variants were found to be enriched in individuals with hypertriglyceridaemia (Johansen, et al., 2010). This theory also supports data from rodent models where defective GCK translocation is proposed to contribute to the T2D in these animals (Abdel-Halim, et al., 1993; Fujimoto, et al., 2004; Kawano, et al., 1992; Shin, et al., 2007; Toyoda, et al., 2000).

5.4.5 The cellular characterisation of human P446L-glucokinase regulatory protein remains to be definitively established

The rat data obtained from the cellular experiments in this study must be interpreted with caution considering 1) the intrinsic differences between rat and human GCK (Brocklehurst, et al., 2004a), 2) the small anticipated effect size of the P446L-variant (Orho-Melander, et al., 2008; Saxena, et al., 2007), and 3) the inability to quantitate these anticipated marginal differences in protein localisation. Subsequent studies have aimed to remedy these problems by optimising their protocols so that the localisation of human proteins can be observed, as well as employing intricate computational quantitation techniques so as to accurately detect any minor differences in protein function (Rees, et al., 2010). Such studies have suggested that human P446L-GKRP is less capable of sequestering GCK in the nucleus versus the wild-type regulatory protein, thus increasing the cytoplasmic pool of 'catalytically active' GCK, leading to increased hepatic glycolytic flux in individuals homozygous for the 446L *GCKR* allele (Rees, et al., 2010).

Additionally, particularly important is the fact that *in vitro* P446L-GKRP kinetic studies showed the only difference between human wild-type and variant regulatory protein lay at the level of altered F6P-mediated regulation (**chapter 4**), and the experimental cellular system used in this study did not take into account/allow for observations to be made in different concentrations of phosphate ester. The limitations of this study therefore provide further evidence as to the difficulties in following up genetic variants associated with human disease and quantitative traits, and shows there is still much to learn about the intricate biology of GKRP-mediated GCK regulation and nuclear sequestration.

Chapter 6

Mutations within *GCKR* do not cause monogenic forms of beta-cell dysfunction

6.1 Introduction

6.1.1 Maturity-onset diabetes of the young is caused by mutations in genes critical to glucose homeostasis

Maturity-onset diabetes of the young (MODY) is a heterogeneous group of disorders characterised by autosomal dominant inheritance, non-insulin-dependent diabetes, and early onset (<25 years of age) (Tattersall, 1974; Tattersall and Fajans, 1975). It accounts for 1-2% of diabetes, and the majority of cases are caused by mutations in eight genes that are expressed in the beta-cell and other tissues of insulin action (Ellard, et al., 2008; Fajans, et al., 2001; Frayling, et al., 2001; Frayling, et al., 2003; Ledermann, 1995). One of these genes encodes the glycolytic enzyme glucokinase (*GCK*), for which one allele is mutated in patients with MODY (GCK-MODY) (Froguel, et al., 1992; Hattersley, et al., 1992). Five of the other MODY genes encode transcription factors; hepatocyte nuclear factor 4-alpha [*HNF4,* HNF4A-MODY (Yamagata, et al., 1996a)], hepatocyte nuclear factor 1-alpha [*HNF1A*, HNF1A-MODY (Yamagata, et al., 1996b)], insulin-promoter factor-1 [*PDX1*, IPF1-MODY (Stoffers, et al., 1997)], hepatocyte nuclear factor 1-beta [*HNF1B*, HNF1B-MODY (Horikawa, et al., 1997)], and neurogenic differentiation factor-1 [*NEUROD1*, NEUROD1-MODY (Malecki, et al., 1999)]. These transcription factors regulate the expression of genes crucial to beta-cell development and glucose metabolism (Fajans, et al., 2001; Naya, et al., 1997; St-Onge, et al., 1999; Stoffel and Duncan, 1997). Mutations within the insulin gene (*INS*) (Molven, et al., 2008) and that encoding carboxyl ester lipase (*CEL*) (Raeder, et al., 2006) are also proposed to cause different subtypes of MODY. Finally, it has been suggested that mutations within the *ABCC8* gene which encodes the sulphonylurea receptor-1 (SUR1) subunits of the beta-cell K^{+}_{ATP} channel can cause dominantly-inherited diabetes that present with a MODY-like phenotype (Flanagan, et al., 2009; Gonsorcikova, et al., 2011). Mutations within *GCK* and *HNF1A* are the most common cause of MODY (Ellard, et al., 2008).

It is important to correctly diagnose these monogenic forms of diabetes, as the different sub-types vary in their clinical prognoses and treatment regimens. For example, patients with GCK-MODY have

only a mild elevation in their fasting plasma glucose (FPG) and experience beta-cell decline at a rate in line with the general population, thus meaning they can often be treated by diet alone (Ellard, et al., 2008; Frayling, et al., 2001; Gill-Carey, et al., 2007; Stride, et al., 2002). Conversely, patients with *HNF1A* mutations experience a progressive decline in beta-cell function which results in diabetes, and require treatment with oral anti-diabetic agents and eventually insulin (Frayling, et al., 2001; Lehto, et al., 1997; Pearson, et al., 2001; Velho, et al., 1996b). It has been shown that HNF1A-MODY patients are much more sensitive to sulphonylureas than those with type 2 diabetes (T2D), as the defect in beta-cell function in these patients is upstream of the K^{+}_{ATP} channel. Therefore HNF1A-MODY patients should be preferentially treated with sulphonylureas as opposed to other oral anti-diabetic agents such as the insulin-sensitiser metformin (Pearson, et al., 2003).

6.1.2 Not all cases of maturity-onset diabetes of the young can be explained by mutations in these eight genes

Despite most MODY cases being of known genetic aetiology, 15-20% of cases do not contain mutations in the eight genes listed above, and these are so-called 'MODY-X' patients (Chevre, et al., 1998; Frayling, et al., 2001; Frayling, et al., 2003; Lehto, et al., 1999). This suggests that there are additional MODY genes to be identified, particularly because of the autosomal dominant inheritance and early age of diagnosis of the condition. Early linkage studies in MODY families aimed at identifying these MODY-X genes were unsuccessful (Vaxillaire, et al., 1994), as was screening of beta-cell candidate genes including *SLC2A2* (Moller, et al., 2001), *HNF6* (Moller, et al., 1999), and *HNF3B* (Abderrahmani, et al., 2000), as these studies did not identify any variants that conclusively co-segregated with the MODY phenotype. However, in 2003 Frayling and colleagues carried out a meta-analysis on the results of three genome-wide linkage scans which included 23 MODY families from the UK, France and Sweden, and showed putative linkage with regions on chromosomes 3, 6, and 10. These data suggested that there was heterogeneity within MODY-X cases, but nonetheless

provided the ground-work for further investigation into the different genetic aetiologies within this patient group (Frayling, et al., 2003).

6.1.3 Genome-wide linkage scan identifies chromosome 2p as a novel locus for maturity-onset diabetes of the young

Lindgren and colleagues hypothesised that despite the heterogeneity within MODY-X patients, families could be separated into homogeneous groups by refining the phenotypic/clinical inclusion criteria used when recruiting them for a genome-wide linkage study (Lindgren, et al., 2007). For example, GCK-MODY has a distinctive phenotype versus the other MODY-subtypes (Ellard, et al., 2008; Frayling, et al., 2001; Gill-Carey, et al., 2007; Stride, et al., 2002), thus it was proposed that patients who were negative for mutations within *GCK*, but who were diagnosed at a young age (<15 years) with at least one affected family member and who were treated by diet modification alone represented a 'GCK-like MODY' group (Lindgren, et al., 2007). After excluding mutations within the next two most common MODY genes (*HNF4A* and *HNF1A*) and *GCK* whole or partial deletions, a genome-wide linkage study was performed using four families (32 individuals, 18 with diabetes, 6 diagnosed <15 years). The results of this study showed the highest non-parametric linkage (NPL) scores (all > 3) came from chromosomes 3q, 4q, and 10q, as well as a locus on chromosome 2p which had not been reported previously (Lindgren, et al., 2007).

6.1.4 The gene encoding glucokinase regulatory protein maps to chromosome 2p

Amongst the other biologically-plausible candidate genes found within these newly-identified MODY loci, the gene encoding glucokinase regulatory protein (*GCKR*, protein GKRP) was particularly interesting. Firstly, GKRP negatively modulates hepatic GCK activity, which is the key regulator of liver glucose uptake and utilisation (Matschinsky, 2009; Matschinsky, et al., 2006; Van Schaftingen, 1989; Van Schaftingen and Vandercammen, 1989). Additionally, G72R and V62M are *GCK* mutations which co-segregate with hyperglycaemia in MODY families, but which have paradoxically activating

kinetics upon *in vitro* assay (Arden, et al., 2007; Gloyn, et al., 2005). Importantly, both G72R and V62M exhibit defective GKRP-binding, and this has been shown to have pathogenic consequences on overall catalytic and protein stability in a cellular system (Arden, et al., 2007). However, alterations to GKRP-GCK binding have been shown to contribute to- but not be an overall determinant of clinical phenotype, thus suggesting that additional mutational mechanisms may also be involved in the pathogenesis of the G72R and V62M mutations (Arden, et al., 2007; Gloyn, et al., 2005; Heredia, et al., 2006; Sagen, et al., 2006; Sayed, et al., 2009).

Further evidence supporting the biological candidacy of *GCKR* as a MODY gene comes from rodent models. *Gckr* knockout mice have decreased levels of hepatic GCK, and consequently suffer from defective glucose utilisation and clearance by the liver (Farrelly, et al., 1999; Grimsby, et al., 2000). This phenotype was surprising as it was hypothesised that lack of GKRP-mediated inhibition would lead to enhanced hepatic GCK activity. However, it was subsequently shown that GKRP serves a paradoxical role in which GCK is sequestered in the hepatocyte nucleus at low glucose levels to save it from proteolytic degradation (Farrelly, et al., 1999; Grimsby, et al., 2000; Slosberg, et al., 2001). Consequently, rodent models in which this GKRP:GCK nuclear sequestration is indirectly affected have a diabetic phenotype (Fujimoto, et al., 2004; Fujimoto, et al., 2006; Shin, et al., 2007; Toyoda, et al., 2000). This theory is also supported by experiments in which over-expression of *Gckr* in the liver leads to both hypoglycaemia in normal rats, and corrects the hyperglycaemic phenotype of high-fat diet-induced diabetic mice (O'Doherty, et al., 1999; Slosberg, et al., 2001).

Additionally, there was some evidence that *GCKR* could be expressed in islets (Becker, et al., 1996) and beta-cells (Alvarez, et al., 2002), with one study proposing that an alternatively spliced regulatory protein variant was present in beta-cells of rodent origin (Alvarez, et al., 2002). This remained controversial as numerous studies had suggested that *Gckr* was not expressed in rodent beta-cells (Grimsby, et al., 2000; Vandercammen and Van Schaftingen, 1993; Zawalich, et al., 1977). However, further evidence in support of pancreatic GKRP came from the observation that fructose

1-phosphate (a known inactivator of GKRP) stimulated GCK activity in beta-cell extracts, (Malaisse, et al., 1990), and the identification of a protein within rodent beta-cells which fulfilled a similar role to GKRP (although this was shown to have different properties to the hepatic GKRP) (Tiedge, et al., 1999).

Finally, a study aimed at determining the control coefficient of GKRP on glucose metabolism in hepatocytes showed that GCK and GKRP exhibited reciprocal control, and subsequently suggested that *GCKR* mutation would therefore impact upon glycaemic phenotype (de la Iglesia, et al., 2000).

6.1.5 Mutations within glucokinase regulatory protein could cause a spectrum of glycaemic phenotypes

Over 600 mutations within *GCK* have been reported to date, the majority exhibiting inactivating enzyme kinetics upon *in vitro* characterisation. As such, the glucose-induced insulin secretion (GSIS)-threshold is elevated resulting in the hyperglycaemia characteristic of MODY and permanent neonatal diabetes mellitus patients, the latter disorder present in individuals homozygous/compound heterozygous for *GCK* mutations and characterised by a much more severe diabetic phenotype (Froguel, et al., 1992; Hattersley, et al., 1992; Njolstad, et al., 2001; Osbak, et al., 2009). Only thirteen activating mutations have been reported in *GCK*, these lowering the GSIS-threshold causing hyperinsulinaemic hypoglycaemia (HH) (Glaser, et al., 1998; Osbak, et al., 2009). Therefore, if mutations within *GCKR* could cause a phenotype similar to GCK-MODY, it is also possible that they may cause a spectrum of glycaemic disorders as occurs with *GCK* mutations.

I subsequently aim to screen all 19 exons and exon-intron boundaries of *GCKR* to see if rare mutations in this gene could cause monogenic beta-cell dysfunction. More specifically, I aim to interrogate the following groups; GCK-like MODY patients (n=44), HH patients (n=33), and normoglycaemic controls (n=200). My hypothesis is that mutations within *GCKR* could be the cause of the aberrant glycaemia in these patients via defective regulation of GCK activity.

6.2 Materials & Methods

6.2.1 Subjects studied

The GCK-like MODY patients were part of existing cohorts set-up by Prof Sian Ellard (Peninsula Medical School, University of Exeter, UK) and Dr Anna Gloyn (Oxford Centre for Diabetes Endocrinology and Metabolism [OCDEM], University of Oxford, UK), and included DNA from probands for the four families used in the initial genome-wide linkage study which showed linkage between MODY-X and chromosome 2p (Lindgren, et al., 2007). The HH patients were collected by Prof Khalid Hussain (Great Ormond Street Childrens' Hospital, UK). The cohort inclusion criteria for each patient sub-group are given below.

- **GCK-like MODY patients (n=44)** – selected patients had a phenotype similar to that of GCK-MODY, but mutations within their *GCK* coding sequence had previously been excluded as a cause of this phenotype, as had those in the next two most common MODY genes *HNF1A* and *HNF4A* (Ellard, et al., 2008). All patients had mild fasting hyperglycaemia (5.5-8.0mmol/L [mean 6.6mmol/L, range 5.5-7.8mmol.L]), and were diagnosed <30years of age (mean 18years). Mean BMI was 23.6kg/m^2. All patients had detectable C-Peptide levels and where tested were negative for autoantibodies (33% of patients had antibody data available). This suggested that they did not have type 1 diabetes and could secrete insulin.
- **HH patients (n=33)** – selected patients secreted excessive insulin despite hypoglycaemia, with median age of diagnosis at 0.02years (range 0.02-35years). Mutations within *GCK* and the other genes known to cause HH [*ABCC8, KCNJ11* and *HNF4A* (Hussain, 2010)] had been excluded as a cause of this disease. This work was carried out by Laura McCulloch (OCDEM, University of Oxford, UK).
- **Normoglycaemic controls (n=200)** – all subjects in this group had FPG <6mmol/L, and were matched for ethnicity with the patient cohorts (all Caucasian). These subjects formed part of the population-based database Oxford BioBank (Tan, et al., 2006), and were included in this study

through collaboration with Prof Frederik Karpe (Oxford Centre for Diabetes Endocrinology and Metabolism, University of Oxford, UK).

6.2.2 Extraction of patient DNA

DNA from the GCK-like MODY and HH patients was extracted from peripheral leukocytes by Dr Kevin Colclough (Royal Devon and Exeter NHS Foundation, UK), whilst that from the normoglycaemic controls was obtained by Dr Matt Neville (OCDEM, University of Oxford). All extractions used standard protocols. Purity/concentration of DNA was determined using the Nanodrop 2000 (ThermoScientific, Cramlington, UK).

6.2.3 Screening the glucokinase regulatory protein gene in patient DNA

Primers for all 19 *GCKR* exons and intronic boundaries were designed using Primer3 and OligoCalc (http://frodo.wi.mit.edu/ and http://www.basic.northwestern.edu/biotools/oligocalc.html respectively) using the human genomic *GCKR* sequence NG_028024 obtained from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/nuccore/). Primers (table 5.1) were obtained from Eurofins MWG Operon (Ebersberg, Germany), and universal M13 sequences added onto the 5'-end of each for ease of subsequent sequencing (forward $^{5'}$TGTAAAACGACGGCCAGT$^{3'}$, reverse $^{5'}$CAGGAAACAGCTATGACC$^{3'}$).

Polymerase chain reaction (PCR) was carried out using AmpliTaq Gold enzyme (Applied Biosystems, Warrington, UK) and the standard protocol described in **chapter 2 materials & methods section 2.1**. Following amplification, products were observed on a 2% agarose gel using ethidium bromide staining (Sigma Aldrich Company Ltd, Gillingham, UK) as described in **section 2.2**. Due to the large number of normoglycaemic subjects to be tested, the only exons screened in these controls were those in which variants had been identified within in either of the patient groups.

Sequencing was carried out as described in **section 2.3**. Briefly, PCR products were treated with exonuclease-I (New England Biolabs, Hichin, UK) and shrimp alkaline phosphatase (Promega,

Southampton, UK) to remove background template and deoxynucleotide triphosphates. Amplification was then carried out using BigDye v1.1 (Applied Biosystems), and reaction products cleaned using Performa V3 plates (EdgeBiosystems, Gateshead, UK). Samples were run by Lynne Richardson at the University's core sequencing facility with the Department of Zoology on an ABI3730xl DNA Analyser (standard run module, 15 seconds injection, v1.1 dye set and analysis module). All results were analysed using Mutation Surveyor v3.4 (Soft Genetics, Cambridge, UK) using NG_028024 as a reference for the human genomic *GCKR* sequence.

Primer	**Sequence (5'- 3')**	**Primer**	**Sequence (5'- 3')**
Exon1	F - TCAGATAAGGCCCTGCCAACTGGA	Exon11&12	F - AGCTTTCTGAGGGGGAATTG
	R - TCAGCACAGAAAGCCAACATGAAG		R - TCAGGAGGAAGCAGGAAGAA
Exon2&3	F - GAATGAGGCTTTGGCTTCCT	Exon13	F - CCTTTCCTATATAGCCAATCACTC
	R - GAAAAGTTTAGGCAGGGAACA		R - AGCTTCCCCAAGTCCTGTTCTTCT
Exon4&5	F - ATTCTTCTCCCAACCTGAGC	Exon 14-16	F - GGCCCCCTTTAGTCCTCA
	R - AAGACAAGCCAGACACAATCTC		R - CACCCTCTGAGCCCCTTACT
Exon6&7	F - CACTGCCACTGACCTTGAGT	Exon17	F - AGGGCCCTGTTCACTCTGCTGCTT
	R - CCATTCCCACTCACTTCCTC		R - CTCCTCTGGCAGCCTAAGCATTG
Exon 8	F - TGGGCACCACTCAGAGTAATGACC	Exon18	F - GGCCTTTAGCCTGACACTGA
	R - AGGGCTGCCCAGTCATTCCAGAA		R - TGCCGAACAAACTTCCTGAC
Exon 9	F - GGCCCTTCTTTGAGATCCTCATAG	Exon19	F - TCCCTCAGGCTTCAGTGC
	R - GTGTTGGAGGGGTGGAGAACATAG		R - CCCACCTCTGATCTACTGTTTG
Exon 10	F - AATTCCTCTTGGGTGTCTTCACCT		
	R - CTGAGCTCTCTCTTATCCAGATCC		

Table 6.4: *GCKR* sequencing primers covering all 19 exons and exon-intron boundaries. 'F' denotes forward primer whilst 'R' denotes reverse.

6.2.4 Variant analysis

The effect of intronic variants on transcript splicing was carried out by Dr Sarah Flanagan (Peninsula Medical School, University of Exeter, UK) using Alamut Interactive Biosoftware v1.5 (Rouen, France)

(Fairbrother, et al., 2002; Pertea, et al., 2001; Reese, et al., 1997; Zhang, 1998). Silent mutations were analysed using Exonic Splicer Enhancer (ESE) finder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) (Cartegni, et al., 2003; Smith, et al., 2006). Predictions about the pathogenicity of non-synonymous variants were made using Sorting Intolerant From Tolerant (SIFT) and Polymorphism Phenotyping (PolyPhen) web-based tools (http://sift.jcvi.org/ and http://genetics.bwh.harvard.edu/pph/ respectively) (Flanagan, et al., 2010; Ng and Henikoff, 2001; Ng and Henikoff, 2003; Ramensky, et al., 2002; Sunyaev, et al., 2001).

6.3 Results

6.3.1 Fourteen single nucleotide polymorphisms identified within the glucokinase regulatory protein gene

Fourteen SNPs were identified in *GCKR* (table 6.2) which were found throughout the entire gene. Seven of these SNPs were novel. The rs numbers for the other seven previously-identified variants were obtained from the publically-available database dbSNP (http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp). The majority (ten) of the variants were deemed 'rare' as their MAFs were ≤0.05.

Non-coding variants

Eight intronic SNPs were found, three of which were novel (IVS750+10C>T, IVS428+102A>G, IVS1241-44T>C). IVS1241-44T>C was only found in normoglycaemic controls, and thus was not deemed pathogenic. IVS750+10C>T and IVS428+102A>G were however rare and only found within either the GCK-like MODY or HH patients respectively (absent in 400 chromosomes from normoglycaemic controls). The effect of these SNPs was investigated using Alamut v1.5, a programme which uses several splice site prediction tools to analyse intronic variants (Fairbrother, et al., 2002; Pertea, et al., 2001; Reese, et al., 1997; Zhang, 1998). Alamut analysis predicted that IVS750+10C>T would not affect any splice sites and hence suggested that this variant was unpathogenic. Additionally, although it was shown that IVS428+102A>G abolished a putative splice acceptor site, this variant was also proposed to be unpathogenic as Alamut analysis of the sequence around this variant predicted that this site was not used in normal splicing.

Another intronic variant also encoded a nucleotide change close to the exon-intron splice site IVS1423-22C>G, and was only found with rare frequency (MAF 0.05) in the GCK-like MODY cohort (absent in 400 chromosomes from normoglycaemic controls). However, its previous identification

and presence in dbSNP (rs8179236) suggested that it was unlikely to be pathogenic. Alamut testing also predicted that this substitution would not affect transcript splicing.

Coding variants

Six variants were identified within the coding sequence, three of which had not been found previously. Of these, two were synonymous (c.1215T>C, p.T405T; c.1722C>T, p.A574A). T405T was only identified in the normoglycaemic controls, suggesting this variant was not pathogenic. The A574A variant was however novel, and found with rare frequency in the GCK-like MODY cohort only (MAF 0.01). ESE finder can predict whether silent mutations would affect binding of serine/arginine proteins to the transcript. These proteins regulate both constitutive and alternative transcript splicing by binding to enhancer sequences and recruiting the splicing machinery (Cartegni, et al., 2003; Graveley, 2000; Smith, et al., 2006). ESE finder analysis of A574A predicted that this silent mutation would not affect SRP protein binding, and hence transcript splicing.

A novel non-synonymous (c.583A>C, p.M195L,) and previously-reported non-synonymous (c.1685G>A, p.R540Q) variant were found with rare frequency in the GCK-like MODY patients only (MAF 0.01 and 0.03 respectively) (Veiga-da-Cunha, et al., 2003). Additionally, a previously-reported nonsense variant (c.745C>T, p.R227X) was found in the GCK-like MODY patients with much higher frequency versus the normoglycaemic controls (Veiga-da-Cunha, et al., 2003). These variants thus warranted further investigation.

SNP	Location	MAF in Cohort			MAF in HapMap CEU
		GCK-like	PHHI	Controls	
IVS217-44C>CT rs8179205	Intron 4	0.01	0.05	n/a	0.05
IVS428+102A>G Novel	Intron 6	0.04	0.30	n/a	Undefined
c.649A>C, p.M195L Novel	Exon 8	0.01	0	0	Undefined
c.745C>T, p.R227X Novel	Exon 9	0.01	0	n/a	Undefined
IVS750+10C>T Novel	Intron 11	0.01	0	n/a	Undefined
IVS968+34G>A rs2293571	Intron 13	0.27	0.52	n/a	0.44
IVS1241-44T>C Novel	Intron 14	0	0	0.003	Undefined
IVS1241-27G>A rs8179219	Intron 16	0.01	0.06	0.04	0.09
c.1281T>C, p.T405T Novel	Exon 14	0	0	0.003	Undefined
c.1403C>T, p.P446L rs1260326	Exon 15	0.29	0.35	0.35	0.40
IVS1423-22C>G rs8179236	Intron 18	0.05	0	n/a	0.02
c.1685G>A p.R540Q rs34316866	Exon 18	0.03	0	0.003	0.004
c.1788C>T, p.A574A Novel	Exon 19	0.01	0	n/a	Undefined
IVS1878+88G>T rs41291159	3' Untranslated Region	0.01	0.02	n/a	Undefined

Table 6.2: Results of *GCKR* screen. Listed are the intronic and coding SNPs identified in either the GCK-like MODY patients, HH patients, or normoglycaemic controls. Population MAF and rs numbers obtained from dbSNP.

6.3.2 Follow-up of the M195L variant

To determine whether the M195L substitution was pathogenic, DNA from the family of this proband was screened to see if the variant co-segregated with the GCK-like MODY phenotype. This family was one of the four used in the earlier genome-wide linkage study performed by Lindgren and colleagues (Lindgren, et al., 2007).

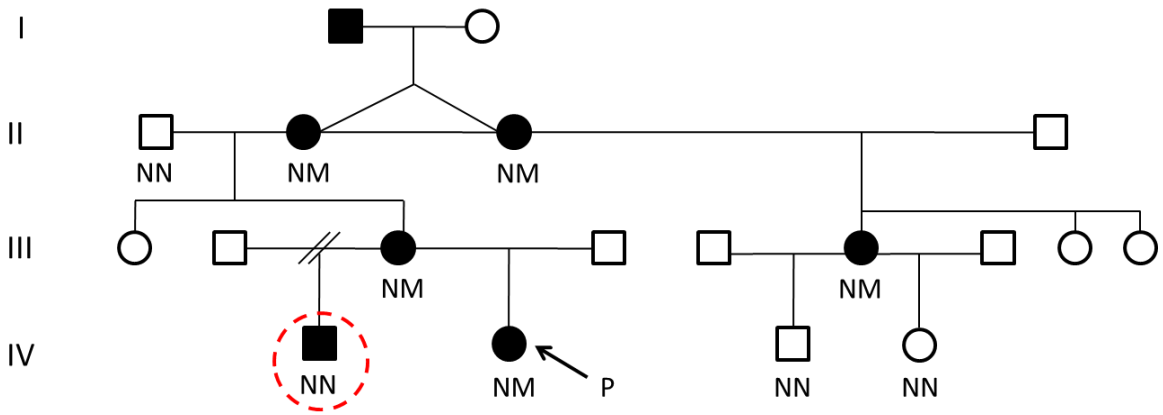


Figure 6.1: Extended pedigree for *GCKR* M195L. Family of proband (identified by P) was screened following discovery of M195L variant. Filled symbols indicate GCK-like MODY phenotype, empty symbols show normoglycaemic family-members. The *GCKR* mutation status is shown under each symbol; NN denotes homozygous for wild-type allele, NM shows individuals heterozygous for the M195L variant. The proband's half-brother who is hyperglycaemic but who does not carry the M195L is circled in red.

The pedigree in figure 6.1 shows that although most affected family members carry the M195L variant, the proband's affected half-brother is homozygous for the wild-type allele. This suggests that either the phenotype does not completely co-segregate with the variant, that M195L could be a rare incompletely penetrant variant, or that the disease phenotype is being driven by something other than this variant. Functional characterisation of this variant would provide further insight as to whether the methionine to leucine substitution at position 195 within the regulatory protein is pathogenic and contributing to the hyperglycaemic phenotype in this family.

SIFT and PolyPhen are web-based tools for predicting whether a non-synonymous variant is likely to affect protein function. The first programme bases its predictions purely on sequence conservation, whilst the latter also uses annotated UniProt entries to predict whether specific structural/functional

sites may be affected (Flanagan, et al., 2010; Ng and Henikoff, 2001; Ng and Henikoff, 2003; Ramensky, et al., 2002; Sunyaev, et al., 2001). Both SIFT and PolyPhen predicted that the leucine substitution would be benign and tolerated at this position. However, sequence alignment of the top ten mammalian GKRP homologues as determined from a protein blast of human GKRP sequence Q14397 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) did show that residue methionine-195 lies within a highly conserved sequence, suggesting it may have functional/structural importance.



Figure 6.2: Alignment of the top ten mammalian human GKRP homologues. Residue M195 is highlighted with a red circle, and is conserved amongst all species observed.

6.3.3 Follow-up of the R227X variant

Co-segregation of the R227X variant with the GCK-like MODY phenotype was checked using DNA from the proband's family [again originally obtained as part of the earlier genome-wide linkage study (Lindgren, et al., 2007)].

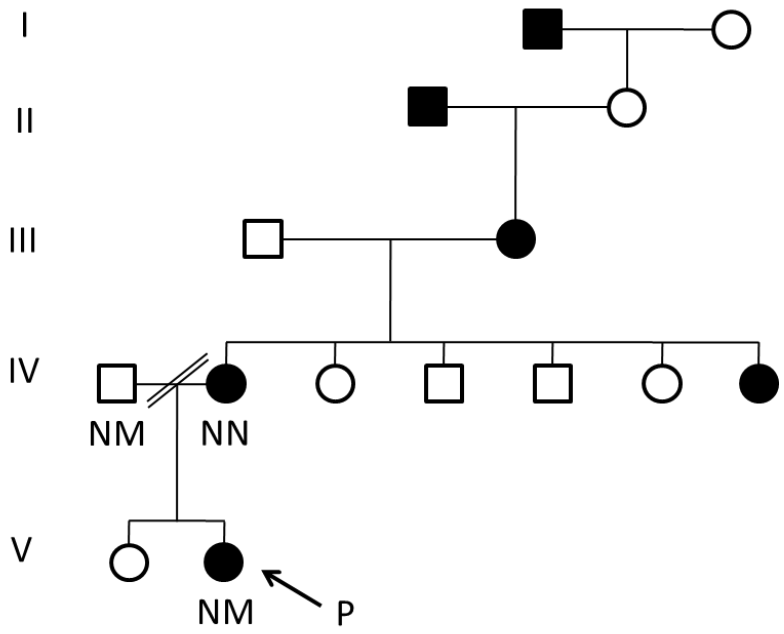


Figure 6.3: Extended pedigree for *GCKR* R227X. Family of proband (identified by P) was screened following discovery of R227X variant. Filled symbols indicate GCK-like MODY phenotype, empty symbols show normoglycaemic family-members. The *GCKR* mutation status is shown under each symbol; NN denotes homozygous for wild-type allele, NM shows individuals heterozygous for the R227X variant.

Figure 6.3 shows that R227X is inherited from the proband's unaffected father, therefore it is unlikely that this nonsense mutation is causing the GCK-like MODY phenotype.

6.3.4 Follow-up of the R540Q variant

Follow-up of R540Q again involved looking for co-segregation of this variant with the GCK-like MODY phenotype in the proband's family (Lindgren, et al., 2007).

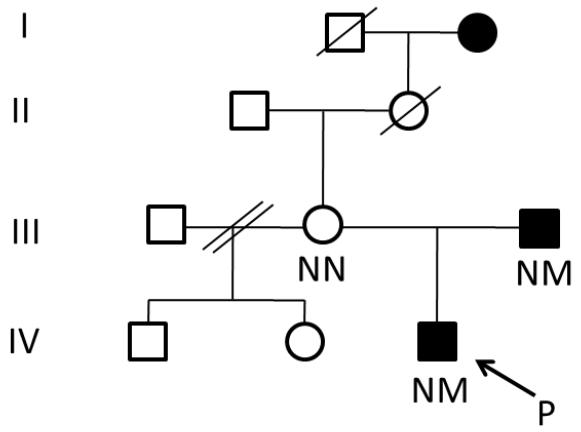


Figure 6.4: Extended pedigree for *GCKR* R540Q. Family of proband (identified by P) was screened following discovery of R540Q variant. Filled symbols indicate GCK-like MODY phenotype, empty symbols show normoglycaemic family-members. The *GCKR* mutation status is shown under each symbol; NN denotes homozygous for wild-type allele, NM shows individuals heterozygous for the R540Q variant.

Figure 6.4 shows *GCKR* R540Q is inherited from the proband's affected father. This variant has a MAF of 0.004 in the general population and the normoglycaemic controls screened in this study. In the 46 GCK-like MODY subjects we observed, this MAF was raised to 0.03, thus suggesting this variant could confer some level of susceptibility. However, further study using larger numbers of patients would be needed to confirm this, as would characterisation of this variant protein. Preliminary analysis using SIFT and PolyPhen both predicted that this mutation would be benign and tolerated. Sequence alignment interestingly showed that R540 was semi-conserved, and that in mammals where this residue was not an arginine (R), it was glutamine (Q). This bioinformatic analysis supports the conclusion that the R540Q variant is non-pathogenic.

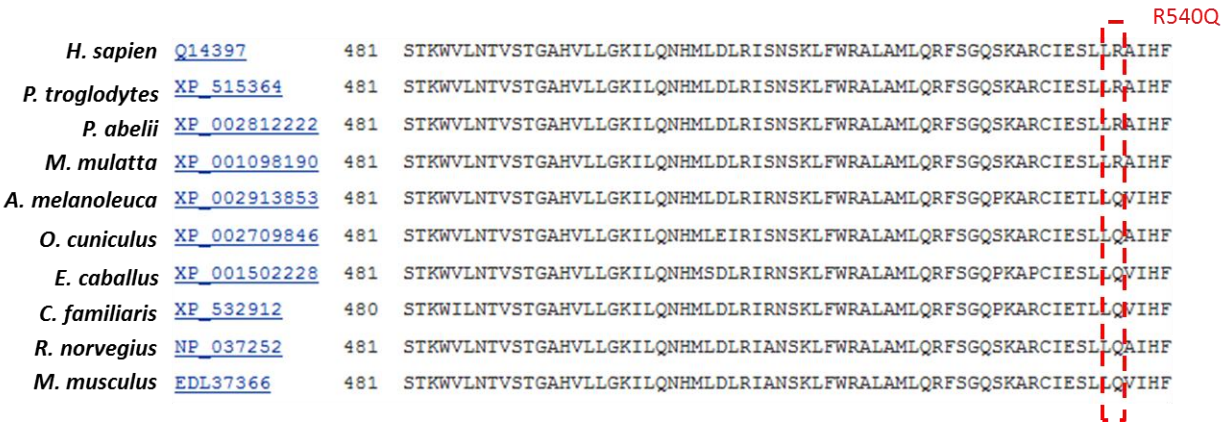


Figure 6.5: Alignment of the top ten mammalian human GKRP homologues. Residue R540 is highlighted with a red circle, and is conserved amongst four of the observed species. The other six species have a glutamine (Q) at this position instead.

6.4 Discussion

6.4.1 Novel coding sequence and intronic variants identified within the glucokinase regulatory protein gene are unlikely to be a cause of monogenic beta-cell dysfunction

Through screening of *GCKR* in patients with GCK-like MODY, HH, and 200 ethnically-matched normoglycaemic controls, we were able to identify six non-synonymous SNPs, three of which were instantly deemed unpathogenic for the following reasons. P446L is common to the general population (MAF 0.40), whilst the two novel silent variants were either present in the normoglycaemic controls or predicted as unable to affect transcript splicing as deemed by ESE finder (Cartegni, et al., 2003; Smith, et al., 2006). It was important to check these silent variants, as pathogenic mutations affecting transcript splicing have been identified in *GCK* previously (although these pathogenic variants were not silent) (Ellard, et al., 2008; Osbak, et al., 2009; Sun, et al., 1993).

Conversely, three non-synonymous variants M195L, R227X, and R540Q were found that were only present in the GCK-like MODY cohort, or at a ten-fold higher frequency than in the normoglycaemic controls in the case of R540Q. Further family testing was subsequently carried out to see if these variants co-segregated with hyperglycaemia. Despite inheritance from the affected father, M195L was absent from the proband's affected half-brother (figure 6.1), thus suggesting that this variant was not driving the hyperglycaemic phenotype. Bioinformatic analysis also suggested that this mutation would be benign, although sequence analysis showed that this residue was highly conserved amongst mammals. The ability of M195L GKRP to bind and sequester GCK in the nucleus has since been investigated in a cellular setting by Matthew Rees (Rees, et al., 2010). This data shows that the variant regulatory protein behaves no differently to wild-type, and although it has yet to be observed at the kinetic level, only one GKRP variant studied to date (R612C in the GKRP C-terminus) has defects in just one of these parameters alone (Rees, et al., 2010). Therefore, it is unlikely that the M195L *GCKR* variant is causing the MODY-phenotype in this proband.

Genetic screening of the R540Q proband's family showed that this variant co-segregated with hyperglycaemia. However, it was also identified in the normoglycaemic controls, although this was at a much lower frequency (MAF 0.03 vs. 0.0043 respectively). R540Q had also been identified in previous studies (Veiga-da-Cunha, et al., 2003) and was present in the dbSNP database. It was concluded that this variant could confer some level of susceptibility, although larger numbers of individuals would need to be tested to confirm this. As this variant has yet to be captured by genome-wide association studies it seems unlikely that it is contributing to the hyperglycaemic phenotype. Bioinformatic analysis suggested that this amino acid substitution would be tolerated, and sequence alignment showed that in mammals where arginine(R)-540 was not conserved, there was instead a glutamine (Q) at this position. These data provide further evidence to support this variant as non-pathogenic. Additionally, characterisation of R540Q at the cellular level has shown this protein to behave no differently to wild-type GKRP (Rees, et al., 2010). This variant has also been found in non-obese controls in a study aimed at screening *GCKR* for mutations following the association of chromosome 2p21-23 with leptin levels and fat mass (Comuzzie, et al., 1997; Hager, et al., 1998; Rotimi, et al., 1999; Veiga-da-Cunha, et al., 2003).

The R227X nonsense mutation yields an insoluble protein (Rees, et al., 2011; Veiga-da-Cunha, et al., 2003), thus heterozygous individuals are haploinsufficient for *GCKR*. Despite this, R227X was inherited from the proband's unaffected father in this study, thus meaning that this variant was not the cause of hyperglycaemia in this family. This corroborates findings from hetero- and homozygous *Gckr* knockout mice which shows that these rodents only have elevated blood glucose levels following a glucose challenge, as reduced GKRP:GCK sequestration means they have a diminished nuclear reserve of the enzyme which cannot be recruited as required postprandially. This hyperglycaemia is not present without this challenge as the effect of the reduced liver GCK protein and activity levels are cancelled out by the lack of GKRP-mediated inhibition on glycolytic flux (Farrelly, et al., 1999; Grimsby, et al., 2000).

We were also able to identify three novel intronic SNPs. These were not thought to be pathogenic, and were excluded as such either on their presence in normoglycaemic controls, or by their predicted inability to affect transcript splicing (Fairbrother, et al., 2002; Pertea, et al., 2001; Reese, et al., 1997; Zhang, 1998). The previously-identified IVS1423-22C>G variant (rs8179236) was only found in the GCK-like MODY patients and not in the normoglycaemic controls, however analysis using Alamut software again predicted that this variant would not affect transcript splicing (Fairbrother, et al., 2002; Pertea, et al., 2001; Reese, et al., 1997; Zhang, 1998).

Our results therefore suggest that *GCKR* mutations are not a cause of MODY. This is supported by the earlier findings of Vaxillaire and colleagues who carried out linkage studies on both insulin-secretion and –action genes in an attempt to identify novel MODY genes (Vaxillaire, et al., 1994). This study used 15 MODY families in which all known genetic aetiologies had been excluded, however the authors were unable to show linkage between the *GCKR* locus and disease (Vaxillaire, et al., 1994).

Since our study, the association of a common *GCKR* variant (rs1260326, c.1403C>T, p.P446L) with FPG and triglyceride levels has been shown (Orho-Melander, et al., 2008; Saxena, et al., 2007; Sparso, et al., 2008; Vaxillaire, et al., 2008). A similar effect on FPG has also been observed with the beta-cell promoter-specific -30G>A *GCK* variant (Weedon, et al., 2006; Weedon, et al., 2005). However unlike *GCK*, evidence from *Gckr* knockout mice suggests that the regulatory protein effect could be due to defective hepatic glucose utilisation, with hyperglycaemia only present upon glucose challenge (Farrelly, et al., 1999; Grimsby, et al., 2000). Additionally, it has since been confirmed that *GCKR* is only expressed at negligible levels versus *GCK* within human pancreas (**chapter 4**), and in order for GKRP to have a functional effect it must be expressed in molar excess compared to the enzyme (de la Iglesia, et al., 2000). This further suggests that the regulatory protein only plays a functional role within the liver.

Interestingly, despite the association of *GCKR* with triglycerides, all probands and family members heterozygous for the M195L, R540Q, and R227X variants had triglyceride and cholesterol levels within the normal 'healthy' range (data not shown). This further suggests that these *GCKR* mutations do not sufficiently perturb hepatic glucose uptake and metabolic flux enough to see marked changes to *de novo* lipogenesis and circulating triglyceride levels [as has conversely been observed following *Gck* over-expression in rat hepatocytes (O'Doherty, et al., 1999)].

6.4.2 Further fine-mapping of the chromosome 2p linkage signal and/or sequencing of biologically-relevant candidate genes in this locus may highlight novel maturity-onset diabetes of the young genes

Our data strongly suggest that *GCKR* mutations are not a common cause of MODY, particularly as even *GCKR* haploinsufficiency did not lead to this phenotype. Therefore, further fine-mapping of the chromosome 2p locus (as well as the chromosome 3q, 4q, and 10q loci) identified in the original genome-wide linkage study of early-onset MODY families (Lindgren, et al., 2007) is needed to tease out the variants/genes implicated in this disorder. Additionally, further screening of biologically-plausible candidate genes within the chromosome 2p locus in patients with MODY of unknown genetic aetiology may shed light on the pathogenesis behind their disorder. However, it must also be considered that the chromosome 2p signal from the original linkage scan could in fact be a false positive due to the low power of this study (Lindgren, et al., 2007).

Chapter 7

Discussion

7. Discussion

In the quest for novel type 2 diabetes (T2D) therapeutic strategies, studying the physiology behind the glycolytic enzyme glucokinase (GCK) and its interaction with the endogenous hepatic inhibitor glucokinase regulatory protein (GKRP, gene *GCKR*) will provide crucial information on one of the most important control points of glucose homeostasis. GCK possesses near-total control of glycolytic flux within the pancreatic beta-cell and liver hepatocyte (Matschinsky, 2002; Matschinsky, et al., 2006), with *Gck* knock-out mice exhibiting greatly perturbed glucose homeostasis. For example, homozygous global- or beta-cell-specific-*Gck* knock-outs have markedly elevated fasting plasma glucose (FPG) levels, and promptly die within the perinatal period (Grupe, et al., 1995; Postic, et al., 1999; Terauchi, et al., 1995). Alteration of GCK activity within the hepatocyte also impacts heavily upon hepatic glucose utilisation, as liver-specific *Gck* knock-out mice exhibit mild fasting hyperglycaemia and defective glycogen synthesis (Postic, et al., 1999). A similar phenotype is observed when *Gckr* is ablated, as these mice exhibit defective hepatic glucose uptake during an intraperitoneal glucose tolerance test versus their wild-type littermates (Farrelly, et al., 1999; Grimsby, et al., 2000).

Mutations often have a much more subtle effect on GCK activity versus the gene knock-out models, however they can still have pathogenic consequences on glucose homeostasis. For example this is clearly demonstrated in the human monogenic disorders of beta-cell dysfunction caused by *GCK* mutations; hypoglycaemia hyperinsulinaemia (HH), maturity-onset diabetes of the young (MODY), and permanent neonatal diabetes mellitus (PNDM). Consequently, the ability of GCK to control beta-cell insulin secretion and hepatic glucose clearance, and the clear pathogenic consequences that occur when these processes are deregulated, make GCK activity (and the regulation of) greatly interesting therapeutically (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010; Matschinsky, et al., 2011).

The most recent efforts in developing GCK-targeted therapeutics have become more focussed on 'activating' the enzyme as opposed to relieving GKRP-mediated inhibition. This is because a number of pathogenic side-effects have been recorded following modulation of GCK activity solely in the liver, and data included in **chapter 4** shows that *GCKR* is not expressed at functional levels within human islets. For example, *Gckr* knock-outs exhibit an unexpected decrease in hepatic GCK activity, as despite acting as an inhibitor, the regulatory protein also paradoxically stabilises the enzyme and protects it from degradation (Farrelly, et al., 1999; Grimsby, et al., 2000). Additionally, increased GCK activity in the hepatocyte alone as the result of either naturally-occurring genetic variation (Saxena, et al., 2007) or artificial gene manipulation (O'Doherty, et al., 1999) have shown that this may augment liver metabolism enough to cause an unfavourable lipid profile. Nevertheless, study of GKRP is still crucial in the quest for novel T2D therapeutic strategies, as any pharmacological manipulators of GCK which effect the interaction of this enzyme with its physiological regulators could indirectly cause pathogenic consequences as described above.

Preliminary studies of pharmacological small molecular GCK activators (GKAs) in cell lines and rodents (Brocklehurst, et al., 2004b; Castelhano, et al., 2005; Coope, et al., 2006; Efanov, et al., 2005; Fyfe, et al., 2007; Grimsby, et al., 2003), as well as more recent clinical trials in humans (Bonadonna, et al., 2010), have provided promising data showing a dose-dependent lowering of blood glucose levels upon administration of these drugs. However, the proposed risk of hypoglycaemia as was apparent during GKA administration to patients with T2D (Bonadonna, et al., 2010), suggests that further trials are needed to ensure any GKAs brought to market will be of optimal clinical benefit.

The GKAs designed to date bind to the same site within GCK where the majority of naturally-occurring activating mutations have been reported (Coghlan and Leighton, 2008; Kamata, et al., 2004; Matschinsky, 2009; Matschinsky and Porte, 2010; Matschinsky, et al., 2011). Only one activating mutation has been found greater than 15Å away from this site (Kamata, et al., 2004; Sayed, et al., 2009). Through collaborations with the molecular genetics laboratory of Prof Sian Ellard

(Peninsula Medical School, University of Exeter) we were able to identify two novel *GCK* mutations within HH families; V389L (c.1165C>G; p.Val389Leu) and T103S (c.308C>G; p.Thr103Ser). This was in itself an exciting find considering that even though >600 mutations have been identified in *GCK* to date, only 13 of these are activating (Barbetti, et al., 2009; Kassem, et al., 2010; Osbak, et al., 2009). More interesting still was the observation that these activating mutations occurred at residues where GCK-MODY mutations had also been reported; V389D (c.1166T>A; p.Val389Asp) and T103I (c.308C>T; p.Thr103Ile) respectively (Osbak, et al., 2009). This suggested that these sites were of great functional importance and thus warranted further investigation as to the mutational mechanisms behind these phenotypes.

My characterisation of these GCK mutants showed that their pathogenicity lay at the level of altered kinetics, with activating and inactivating mutations increasing or decreasing the enzyme's affinity for the substrate glucose respectively. Additionally, these mutants showed differing responses to both physiological (GKRP) and pharmacological (GKA) regulation. For example, GKRP was able to inhibit wild-type and V389L equally, whilst both T103 mutants showed defective regulatory protein binding. These dissimilarities were thought to reflect differences in the mutational mechanisms behind the activating mutations at the specific positions; V389L proposed to increase glucose-bound GCK conformational change to the active structure, and T103S enhancing glucose-binding to the apoenzyme (Heredia, et al., 2006). Interestingly, all four mutants responded to GKA-mediated stimulation in assays involving the Hoffman-La Roche GKA compound RO0281675 (Grimsby, et al., 2003), including the particularly deleterious V389D MODY mutation. This provided evidence to suggest that GKAs can be used to treat patients with even the most severe functional defects in GCK. Analysis of the GCK crystal structure showed that although residue T103 mapped to the allosteric activator region, V389 lay within alpha-helix 11 of the large sub-domain, which is a previously-unappreciated regulatory region within GCK, and also one where no other activating mutations have been reported to date.

These findings are of great interest to those seeking to modulate GCK pharmacologically, as they provide new insight into structural regions critical to enzyme activity. This is particularly important as even though GKAs have been the subject of much pharmaceutical research and development, they have yet to reach the market for use in patient treatment (Coghlan and Leighton, 2008; Matschinsky, 2009; Matschinsky and Porte, 2010; Matschinsky, et al., 2011). This suggests that perhaps there is still room for improvement in developing effective GKAs, particularly as clinical trials of previous/current compounds have suggested that these drugs may induce hypoglycaemia (Bonadonna, et al., 2010). The observation that manipulating alpha-helix 11 can enhance enzyme activity therefore provides new food for thought with regards to designing the next wave of these drugs.

An obvious continuation of these findings would be to further investigate the precise role of alpha-helix 11 in the regulation of GCK activity. By carrying out sequential site-directed mutagenesis on each amino acid within alpha-helix 11, it could be elucidated which part, if not all, of this secondary structure element is crucial to enzyme function. Substitution of amino acids with a range of physiochemical properties as has already been carried out for residue 389 would yield further information regarding the required chemistry within alpha-helix 11 and the surrounding molecular environment. Submitting these mutants to basic kinetic characterisation (glucose- and adenosine triphosphate-dependent assays) would also provide information on how substitutions within alpha-helix 11 affect the affinity of the enzyme for its substrates and the structural conformation adopted by these GCK mutants. Additionally, the functional stability of these alpha-helix 11 mutants could be tested using assays designed to measure the activity of the enzyme over a temperature range. This type of characterisation has already been used to show that the GCK-MODY mutant E300K which has near-normal kinetics was markedly unstable at the protein level versus the wild-type enzyme (Burke, et al., 1999), this subsequently being the mutational mechanism behinds its pathogenesis. These

assays would thus be a useful tool to see if the effects of any mutations on enzyme kinetics correlated with altered protein stability.

From a therapeutic perspective, it would also be interesting to determine whether there are any putative GKA-binding sites in and around the large sub-domain/alpha-helix 11. Analysis of the GCK crystal structure using programmes such as CavSearch would map the location of any 'cavities' in this region (Stahl, et al., 1999), and identify those potentially-accessible to GKAs on the assumption that most orally-active drugs satisfy distinct physiochemical criteria (Ghose, et al., 1999; Lipinski, et al., 2001). Further thought would also be needed on what the benefit of pharmacologically targeting alpha-helix 11 would be as opposed to the conventional allosteric activator site, an obvious benefit would be if this allowed GCK to retain interactions with its physiological regulators including GKRP.

The maintenance of GKRP:GCK interactions in patients treated with GKAs is important because even though the set-points for insulin secretion and FPG levels are largely determined by beta-cell GCK (Matschinsky, 2002; Matschinsky, et al., 2006), the association of the common P446L *GCKR* variant with FPG (Orho-Melander, et al., 2008; Saxena, et al., 2007) suggested that even small perturbations to hepatic GCK activity could affect liver glucose utilisation enough to cause a noticeable impact upon blood glucose levels. As the P446L *GCKR* variant was additionally associated with triglyceride levels (Orho-Melander, et al., 2008; Saxena, et al., 2007), this suggested that altered hepatic GCK activity and glycolytic flux also affected downstream metabolic pathways within the liver (O'Doherty, et al., 1999). This theory is supported by more recent observations which suggest that there is a high preponderance of rare *GCKR* variants in patients with hypertriglyceridaemia (Johansen, et al., 2010). Therefore, elucidating the mutational mechanism behind the P446L-GKRP variant aimed to provide further insight into how altered regulation of hepatic GCK activity, as could be achieved with GKAs aiming to alleviate hyperglycaemia by stimulating glucose uptake into the liver, may have knock-on effects on hepatic metabolism. This provided a significant challenge, as although the causal variant behind this genetic association was non-synonymous and located within a gene of known function,

the small effect size in individuals homozygous for the L allele (FPG ± 0.06mmol and triglycerides ± 0.15mmol/L) (Orho-Melander, et al., 2008; Saxena, et al., 2007) meant that any experiments conducted would need to be sensitive enough to observe the predicted marginal differences in wild-type and variant GKRP function.

Previous attempts at kinetically characterising P446L-GKRP had used recombinant rat proteins (Veiga-da-Cunha, et al., 2003) due to technical difficulties in purifying the human form (Brocklehurst, et al., 2004a; Mookhtiar, et al., 1996). However, despite high sequence homology between the regulatory proteins from both species (Warner, et al., 1995), there are crucial differences in their phosphate ester-mediated regulation (Brocklehurst, et al., 2004a). Additionally, no investigations as to the effect of this variant on GKRP-mediated nuclear sequestration of GCK had been undertaken, even though the ability to observe GKRP:GCK intracellular trafficking and localisation was possible (Baltrusch, et al., 2005).

My characterisation of P446L-GKRP revealed significant differences between the wild-type and variant regulatory protein at the kinetic level. Firstly, I was able to show that P446L-GKRP had a marginal but significantly diminished response to modulation by the phosphate ester fructose 6-phosphate (F6P). F6P enhances the inhibitory effect of GKRP on GCK activity (Van Schaftingen, 1989), therefore individuals homozygous for 446L-GKRP would have attenuated GCK inhibition, and thus an increase in hepatic glucose uptake and glycolytic flux. This could account for the unusual lowered FPG and raised triglyceride phenotype, with enhanced glucose turnover in the liver increasing the supply of intermediate metabolites such as malonyl-CoA; a substrate for *de novo* lipogenesis (O'Doherty, et al., 1999; Saxena, et al., 2007). Analysis of the crystal structure of a GKRP homologue (Teplyakov, et al., 1998) as well as data from previous mutant regulatory protein assays (Veiga-da-Cunha and Van Schaftingen, 2002) led to the hypothesis that the substitution of a leucine at amino acid position 446 altered the regulatory protein structure significantly enough to inhibit

F6P binding and/or the ability of GKRP to adopt the structural conformation which favours binding of this phosphate ester.

Unfortunately the cellular characterisation of P446L-GKRP remains incomplete. Initial experiments using rat fluorescent GCK- and GKRP-fusion proteins were able to reproduce the dynamic glucose-responsive intracellular trafficking that had been observed previously in similar studies (Baltrusch, et al., 2005). Data obtained in this system showed no difference between rat wild-type and P446L-GKRP mediated nuclear sequestration of rat GCK. However, in the limited time available for this study I was unable to observe human P446L-GKRP in this cellular system, and this is critically important when considering the difference in the intrinsic properties of the regulatory proteins from both species.

The results of the kinetic characterisation of P446L-GKRP (**chapter 4**) confirm that modulation of GCK activity purely in the liver could produce the beneficial effect of lowering blood glucose levels (Saxena, et al., 2007), as does the correction of the hyperglycaemic phenotype of high fat diet-induced diabetic mice upon *Gckr* over-expression (Slosberg, et al., 2001). Although this may come at the cost of elevating triglyceride levels (O'Doherty, et al., 1999; Saxena, et al., 2007), Slosberg and colleagues have suggested that this may not always be the case, and that the degree of *Gckr* over-expression/increase in hepatic GCK is important in determining the triglyceride effect (Slosberg, et al., 2001). It also remains to be clarified as to whether any increase in triglycerides could actually lead to increased cardiovascular risk (Kozian, et al., 2010; Teslovich, et al., 2010). For example, it has been shown that although the P446L-*GCKR* variant is associated with increased triglycerides in 3500 patients from the Ludwigshafen Risk and Cardiovascular Health Study, it is not associated with an elevated risk of cardiovascular disease (Kozian, et al., 2010). This finding was corroborated by another large-scale study which carried out a meta-analysis of 46 lipid GWAs (totalling >100,000 individuals) showed that although the P446L-*GCKR* variant was associated with both triglycerides and total cholesterol, it was not associated with increased coronary artery disease (Teslovich, et al.,

2010). Regardless of this, these data suggest that any putative GKAs designed to target hepatic GCK specifically should be approached with caution. Finally, my characterisation of P446L-GKRP has shown that even common variants of small effect size can still be characterised if the correct tests are done, and these functional studies are crucial in elucidating the molecular mechanism behind a genetic association signal, which will ultimately contribute to the development of novel therapeutic outcomes.

My cellular characterisation studies involved the use of fluorescently-tagged rat GCK and GKRP, as the time constraints imposed on this study meant I was unable to carry out any experiments with the human proteins. In order to fully explain the mutational mechanism behind the P446L-*GCKR* variant, these cellular studies should be repeated to study human regulatory protein. Additionally, these experiments were conducted in HeLa cells (Scherer, et al., 1953) as they do not endogenously express *GCK* or *GCKR*, and both the enzyme and regulatory protein had been shown to interact in the nucleus of these cells (Shiota, et al., 1999). However the use of these cells means that any liver-specific factors that may contribute to- and/or influence GKRP:GCK trafficking were absent in this experimental system. The HepG2 human hepatocellular carcinoma cell-line could therefore be used instead as a more 'physiologically representative' cellular environment (Knowles, et al., 1980), however initial attempts to transfect these cells with the fluorescent-*GCK* and *GCKR* constructs were unsuccessful. Finally, the difference between the ability of wild-type and P446L-GKRP to enter the cell nucleus should be quantitated so that accurate comparisons can be made. These issues have all subsequently been addressed by Matthew Rees and colleagues (Rees, et al., 2010) and confirm that human P446L-GKRP is less capable of entering the nucleus versus human wild-type regulatory protein, further supporting the mutational mechanism proposed upon P446L-GKRP kinetic characterisation which suggests the variant regulatory protein is less capable of inhibiting/sequestering hepatic GCK activity.

Another example of where perturbation of GCK activity has a drastic effect on FPG levels are the monogenic disorders of aberrant glucose homeostasis resulting from mutations within the gene encoding this enzyme (Osbak, et al., 2009). The effect of the *GCK* mutation on enzyme activity dictates the resultant clinical phenotype observed, and ranges from; the hypoglycaemia of HH (GCK activity increased) (Glaser, et al., 1998), the mild hyperglycaemia of MODY (patients are heterozygous for *GCK* mutation and so even though GCK is inactivated there is compensation by the wild-type allele) (Froguel, et al., 1992; Hattersley, et al., 1992), and the extreme hyperglycaemia of PNDM (no fully-functional GCK) (Njolstad, et al., 2003; Njolstad, et al., 2001). Common genetic variation within *GCK* is also associated with FPG (Weedon, et al., 2006; Weedon, et al., 2005) and T2D (Dupuis, et al., 2010). Therefore, considering 1) the direct role that GKRP plays in modulating GCK activity within the liver as is shown in *Gckr* knock-out models (Farrelly, et al., 1999; Grimsby, et al., 2000; O'Doherty, et al., 1999), 2) that common variation within this gene is also associated with FPG (Saxena, et al., 2007) and T2D (Dupuis, et al., 2010), and 3) that a genome-wide linkage study involving MODY patients of unknown genetic aetiology identified linkage between the disease and a locus on chromosome 2p (to which *GCKR* maps) (Lindgren, et al., 2007), I hypothesised that *GCKR* mutations may cause monogenic disorders of aberrant glucose homeostasis with similar phenotypes to GCK-MODY and GCK-HH.

It was important to determine whether mutations within the regulatory protein could account for some of the ~20% of MODY cases where the genetic aetiology is undetermined (Chevre, et al., 1998; Frayling, et al., 2001; Frayling, et al., 2003; Lehto, et al., 1999), as the different sub-types of this disorder vary in their clinical prognoses and treatment regimens. For example, if assumed that patients with '*GCKR*-MODY' have a similar phenotype to those with GCK-MODY, it would be anticipated that these patients would experience only mild stable fasting hyperglycaemia, and would be treatable by diet-modification alone (Ellard, et al., 2008; Frayling, et al., 2001; Gill-Carey, et al., 2007; Stride, et al., 2002). The presence of a *GCKR* mutation could also have more detrimental

clinical ramifications, such as an unfavourable lipid profile. It has been shown with the P446L-GCKR variant that reduced hepatic GCK inhibition can elevate triglycerides (Orho-Melander, et al., 2008; Saxena, et al., 2007), therefore patients with MODY and HH resulting from *GCKR* mutations could subsequently have increased/decreased triglycerides respectively. Finally, establishing a role for GKRP in monogenic diabetes and hypoglycaemia would provide further insight into how modulation of GCK activity in the liver could have drastic effects on glucose homeostasis and FPG levels.

Consequently, I screened *GCKR* (all exons and exon-intron boundaries) in 44 patients with 'GCK-like' MODY, 33 with HH, and 200 normoglycaemic controls. Although we identified novel intronic and non-synonymous coding variants in these individuals, we were unable to obtain clear evidence of co-segregation with either hyper- or hypoglycaemia for any of them. We therefore concluded that rare mutations within *GCKR* were not a common cause of monogenic disorders of aberrant glucose homeostasis. Therefore, as the original genome-wide linkage scan by Lindgren and colleagues showed linkage between chromosome 2p and MODY (Lindgren, et al., 2007), it is possible that the disease in these original 'MODY-X' patients was caused by a different gene/s within the same locus within which *GCKR* is found. For example, GeneSniffer (http://www.genesniffer.org/) is a gene candidacy and bioinformatics database mining tool which could be used to interrogate the chromosome 2p locus for other genes that could be implicated in MODY and related phenotypes (Thornblad, et al., 2007). Any additional genes identified as good 'biological candidates' could then be sequenced in these patients to see if they harboured mutations which co-segregated with hyperglycaemia and showed functional defects upon characterisation. Additionally and from a different perspective, the 44 patients with 'GCK-like' MODY screened in my study could have mutations within the genes encoding other physiological regulators of GCK activity, such as the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase which is expressed in both the hepatocyte and beta-cell (Baltrusch, et al., 2001; Massa, et al., 2004; Payne, et al., 2005), or the pro-apoptotic protein B-cell lymphoma 2-associated death promoter (BAD) which activates GCK

in beta-cells (Danial, et al., 2003; Danial, et al., 2008). Again, screening of these genes in these patients would highlight whether they harboured any pathogenic mutations.

In summary the data included in this thesis suggests that investigating and functionally characterising genetic variation within *GCK* and *GCKR* helps further our understanding of one of the most important regulatory points in glucose homeostasis, and gives new insights into our approach towards pharmacologically manipulating corresponding metabolic pathways. This is both timely and crucial if we are to combat the ever-growing global diabetes epidemic (Zimmet, et al., 2001) with more effective therapeutic strategies.

Appendices

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Discovery of a Novel Site Regulating Glucokinase Activity following Characterization of a New Mutation Causing Hyperinsulinemic Hypoglycemia in Humans*

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Type 2 diabetes is a global problem, and current ineffective therapeutic strategies pave the way for novel treatments like small molecular activators targeting glucokinase (GCK). GCK activity is fundamental to beta cell and hepatocyte glucose metabolism, and heterozygous activating and inactivating *GCK* mutations cause hyperinsulinemic hypoglycemia (HH) and maturity onset diabetes of the young (MODY) respectively. Over 600 naturally occurring inactivating mutations have been reported, whereas only 13 activating mutations are documented to date. We report two novel *GCK* HH mutations (V389L and T103S) at residues where MODY mutations also occur (V389D and T103I). Using recombinant proteins with *in vitro* assays, we demonstrated that both HH mutants had a greater relative activity index than wild type (6.0 for V389L, 8.4 for T103S, and 1.0 for wild type). This was driven by an increased affinity for glucose ($S_{0.5}$, 3.3 ± 0.1 and 3.5 ± 0.1 mM, respectively) *versus* wild type (7.5 ± 0.1 mM). Correspondingly, the V389D and T103I MODY mutants had markedly reduced relative activity indexes (<0.1). T103I had an altered affinity for glucose ($S_{0.5}$, 24.9 ± 0.6 mM), whereas V389D also exhibited a reduced affinity for ATP and decreased catalysis rate ($S_{0.5}$, 78.6 ± 4.5 mM; ATP_{K_m}, 1.5 ± 0.1 mM; K_{cat}, 10.3 ± 1.1s^{-1}) compared with wild type (ATP_{K_m}, 0.4 ± <0.1; K_{cat}, 62.9 ± 1.2). Both Thr-103 mutants showed reduced inhibition by the endogenous hepatic inhibitor glucokinase regulatory protein. Molecular modeling demonstrated that Thr-103 maps to the allosteric activator site, whereas Val-389 is located remotely to this position and all other previously reported activating mutations, highlighting α-helix 11 as a novel region regulating GCK activity. Our data suggest that pharmacological manipulation of GCK activity at locations distal from the allosteric activator site is possible.

The global rise in the prevalence of type 2 diabetes (T2D)[2] not only reflects worldwide changes in lifestyle, behavior, and diet but perhaps also suggests that our current therapeutic strategies against this disease are ineffective (1). This leaves an unmet medical need for treatments that are more effective at lowering glycemia with sustained efficacy (2–4). One drug target that has generated much interest over the last decade is glucokinase (GCK). Mutations within *GCK* have long been established as a cause of monogenic disorders of aberrant glucose homeostasis (5). This includes a spectrum of clinical phenotypes ranging from hyperinsulinemic hypoglycemia (HH) with heterozygous activating mutations (5–15) to maturity onset diabetes of the young (MODY) (5, 16, 17) and permanent neonatal diabetes mellitus (5, 18–22) with heterozygous and homozygous/compound heterozygous inactivating mutations, respectively. The association of common variation within this gene with both fasting plasma glucose (fpg) levels (23, 24) and T2D risk (25) also highlights the significance of this enzyme in maintaining normoglycemia. Additionally, the expression of *GCK* in both the pancreatic beta cell and liver hepatocyte (26, 27) provides the promise of a two-pronged attack on hyperglycemia with the opportunity of not only lowering the threshold for glucose-stimulated insulin secretion (GSIS) but also increasing hepatic glucose uptake, firmly establishing GCK as an important therapeutic target (2, 4). Several small molecular GCK activators (GKAs) have been patented and are in clinical trials (2–4, 28–39) with the results of one study showing a dose-dependent reduction in glycemia following administration to patients with T2D (30).

The elucidation of the GCK crystal structure has allowed us to study naturally occurring mutations in much greater molecular detail (40). Over 600 inactivating *GCK* mutations have

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[2] The abbreviations used are: T2D, type 2 diabetes; GCK, glucokinase; HH, hyperinsulinemic hypoglycemia; MODY, maturity onset diabetes of the young; fpg, fasting plasma glucose; GSIS, glucose-stimulated insulin secretion; GKA, small molecular glucokinase activator; $S_{0.5}$, glucose concentration needed for half-maximal GCK activity; n_H, Hill number (measure of cooperativity); ATP_{K_m}, concentration of ATP needed for half-maximal enzyme activity; K_{cat}, turnover number; EC_{50}, GKA half-maximal effect concentration; RAI, relative activity index; GKRP, glucokinase regulatory protein.

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been identified to date that are located throughout the enzyme, whereas only 13 naturally occurring activating substitutions are reported, the majority of which cluster at a discrete structural location deemed the allosteric activator site (5, 6, 12). Interestingly, this is also the site at which pharmacological GKAs bind (40). However, on greater scrutiny, the location of these activating mutations can be categorized in further detail. Group 1 includes residues that make direct contact with GKAs, group 2 encompasses residues that lie within the allosteric activator site but that do not contact the GKA, and group 3 includes residues greater than 15 Å from the allosteric activator site (40, 41). Importantly to date, only one GCK-HH mutation has been identified that falls within group 3: M197I (14, 40, 41).

GCK possesses certain biochemical properties that allow it to act as a glucose sensor in the pancreatic beta cell where its activity is coupled to GSIS. First, it has a low affinity for its substrate glucose (half-maximal activity at 7.5 mM (deemed the "$S_{0.5}$ value")) compared with other hexokinases, which become saturated well below physiological levels of this sugar. Additionally, lack of product inhibition by glucose 6-phosphate and cooperativity with respect to glucose (Hill number (n_H), 1.7) despite functioning as a monomer mean that GCK is uniquely placed to fulfill its critical role in coupling fpg levels to insulin secretion (26, 27).

In this study, we report two novel GCK-HH mutations: V389L (c.1165C→G, p.Val-389 → Leu) and T103S (c.308C→G, p.Thr-103 → Ser). Importantly, these mutations occur at residues where GCK-MODY (formerly known as MODY-2) mutations V389D (c.1166T→A, p.Val-389 → Asp) and T103I (c.308C→T, p.Thr-103 → Ile), respectively, have also been reported (5), suggesting that these positions are of great regulatory importance (as has been shown with previous studies investigating contiguous mutations causing opposite phenotypes (6)). Therefore, we aimed to characterize all four naturally occurring mutations to further understand the mutational mechanisms behind these substitutions and learn more about these critical sites where the balance of enzyme activity can be "tipped" in opposite directions. We hypothesized that the structural location and physiochemical properties of the substituted residue would influence function and the corresponding clinical phenotype observed.

EXPERIMENTAL PROCEDURES

Patients—The male proband of family 1 is Caucasian and from the United Kingdom and was born by caesarean section at 38 weeks gestation weighing 5.4 kg. There are postnatal records of low capillary blood glucose levels, but no action was taken. He presented with seizures at 2 years of age and was diagnosed at 3.6 years with hyperinsulinemia (39.8 pM) and hypoglycemia (fpg, 2.9 mM). Following diagnosis, the proband was treated with diazoxide (5 mg/kg/day divided into three doses) and chlorothiazide (7 mg/kg/day divided into two doses). Within 3 days, diazoxide had to be increased to 7.5 and then 10 mg/kg/day to adequately control his blood glucose levels. The patient was then discharged from the hospital a week after his initial diagnosis with stable sugars. However, over the following month, the dose of diazoxide was increased further to 15 mg/kg/day. At the age of 5.2 years, the diazoxide dose per kg was reduced to 10 mg/kg/day but within a month had to be increased back up to 14 and then 15 mg/kg/day in response to periods of increased frequency of (typically morning) hypoglycemic episodes with symptoms (not seizures). Chlorothiazide was reduced to 3.5 mg/kg/day a fortnight after initial diagnosis, although it was quickly raised back up to 7 mg/kg/day a week later. At the age of 4.3 years, the chlorothiazide dose was gradually reduced and has now been discontinued. The proband now only suffers a handful of fasting hypoglycemic episodes per month (consumption of carbohydrate supplement restores normoglycemia promptly). Unfortunately, the proband has marked hypertrichosis as a side effect of the diazoxide treatment. The proband's father also carries the mutation. Following long periods without food, he feels "lightheaded" and "weak" and has capillary glucose levels of ~2.9 mM.

The female proband of family 2 is part Caucasian, part Metis and from Canada. She was born at 38 weeks gestation weighing 3.2 kg. There was no history of neonatal hypoglycemia, but in retrospect, she had an episode of symptomatic hypoglycemia at 15 years after a long hike. She presented at 22 years with frequent episodes of fatigue, weakness, and unexplained sweating, which could be prevented by frequent snacks in addition to her regular meals (body mass index of 22 kg/m^2), and was subsequently diagnosed with hyperinsulinemia (36.1 pM) and hypoglycemia (2.8 mM). Triglyceride levels were in the normal range (1.38 mM). Investigations including endoscopic ultrasound, computed tomography, and MRI of abdomen and an exploratory laparotomy were negative for an insulioma. While the proband was in the hospital, her 27-year-old sister was found to have hypoglycemia, and subsequent investigations confirmed hyperinsulinemia (143.1 pM) with hypoglycemia (2.5 mM). She was born at 42 weeks gestation weighing 4.1 kg. With both sisters having symptomatic hypoglycemia when glucose readings are lower than 3 mM, they are both currently managed with frequent small meals, 50–75 mg of diazoxide twice daily, and 12.5–25 mg of hydrochlorothiazide once a day. Screening of the family revealed that the proband inherited the mutation from her 56-year-old father who also has two affected male siblings, and her 78-year-old paternal grandmother who was diagnosed with T2D at age 75. The father's fasting glucose values are between 3.1 and 4.0 mM with corresponding insulin levels of 51.4 and 56.3 pM. Although he does not have symptomatic fasting hypoglycemia, he does have occasional hypoglycemic symptoms postexercise. All subjects gave informed consent.

Screening of GCK Gene and Identification of Mutations—Genomic DNA was extracted from peripheral leukocytes. The 10 exons of the pancreatic beta cell isoform of the *GCK* gene were amplified by PCR (primer sequences are available upon request). Unidirectional sequencing was performed using universal M13 primers and Big Dye Terminator Cycler Sequencing kit v3.1 (Applied Biosystems) according to the manufacturer's instructions. Reactions were analyzed on an ABI 3730 capillary sequencer (Applied Biosystems), and sequences were compared with the reference sequence (NM_000162) using Mutation Surveyor v3.24 (SoftGenetics). Mutation testing was undertaken in family members to establish co-segregation and in 400 normal chromosomes to establish mutation frequency.

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Protein Extraction—Two preparations of each mutant glutathione *S*-transferase-tagged human pancreatic glucokinase were prepared as described previously following heterologous expression in *Escherichia coli* (42). Extracted enzyme purities were determined using the Agilent 230 Protein kit (Agilent Technologies UK Ltd.) with a cutoff for purity of ≥95%. Protein concentration was calculated using the Bio-Rad Bradford reagent assay (BioRad Laboratories, Ltd.). Recombinant FLAG-tagged human glucokinase regulatory protein (GKRP; gene name, *GCKR*) was also prepared using previously described protocols (43, 44).

Glucose-dependent Kinetic Assays—GCK activity was determined spectrophotometrically using glucose-6-phosphate dehydrogenase-linked assays as described previously (42). In these assays, GCK activity was observed at 30 °C, pH 7.4 over a 0–100 mM glucose concentration range with ATP in excess. As the mutants V389D, V389R, and V389P had greatly reduced affinity for glucose *versus* wild-type enzyme, the glucose $S_{0.5}$ was instead determined from assays using 0–600 mM glucose.

Briefly, the reaction mixture was prepared on ice and contained 7.5 mM $MgCl_2$, 187.5 mM KCl, 125 mM HEPES buffer, 1.25 mM $NADP^+$, 6.25 mM ATP, 2.5 mM DTT, 0.13% BSA, and 66 units of glucose-6-phosphate dehydrogenase. The activity of 10 milliunits of GCK was observed over the following glucose concentration range for 5 min: 100.00, 50.00, 25.00, 12.50, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, and 0.00 mM. One milliunit of GCK was defined as that which converted 1 nmol of substrate/min.

ATP-dependent Assays—These assays were conducted as for the glucose-dependent assays, although GCK activity was instead measured over a 0–5 mM ATP concentration range with glucose in excess. The altered reaction mixture contained 18.75 mM glucose and no ATP. Ten milliunits of GCK were assayed in the presence of 5.00, 4.00, 3.00, 2.50, 2.00, 1.50, 1.00, 0.75, 0.50, 0.25, 0.10, and 0.00 mM ATP.

Glucose $S_{0.5}$, n_H, and turnover number (K_{cat}) values were calculated using the Hill equation, whereas the affinity for ATP (ATP_{K_m}) was derived from the Michaelis-Menten equation. All data fits were performed using Kaleidagraph v3.5 (Synergy Software).

GKRP Competitive Inhibition Assays—GKRP-mediated inhibition of GCK activity was determined spectrophotometrically using glucose-6-phosphate dehydrogenase-linked assays based on published protocols (43, 44). The assays were carried out at 5 mM glucose, 37 °C, pH 7.1.

The number of GKRP units used per assay was 0.5, 1.0, and 1.5 with 1 GKRP unit inhibiting 10 milliunits of GCK by 50% under standard assay conditions. The reaction mixture contained 25 mM HEPES buffer, 0.5 mM $NADP^+$, 1 mM ATP, 2 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, 3.8 μM BSA, and 4 units of glucose-6-phosphate dehydrogenase. The reaction rate was recorded over 5 min as before. Data were presented as "percentage of GCK activity," which was normalized to that obtained with no GKRP, and IC_{50} values (half-maximal inhibitory concentration) were also calculated from these data.

GKA-mediated Activation Assays—These assays were performed as described previously in the presence of 10 milliunits of GCK and saturating GKA (0–60 μM) and glucose (0–100 mM) concentrations (45). The reaction mixture was the same as for the glucose-dependent assays but also contained RO0281675 (resuspended in DMSO) at the following concentrations: 60.00, 27.00, 9.00, 3.00, 1.00, 0.30, and 0.00 μM. Data were expressed as "-fold activation," which described the ratio $K_{cat}/S_{0.5}$ determined in the presence and absence of saturating levels of RO0281675 (60 μM). The GKA half-maximal effect concentration (EC_{50}) was also calculated and described the drug concentration that gave GCK activity 50% between baseline and the maximal responses.

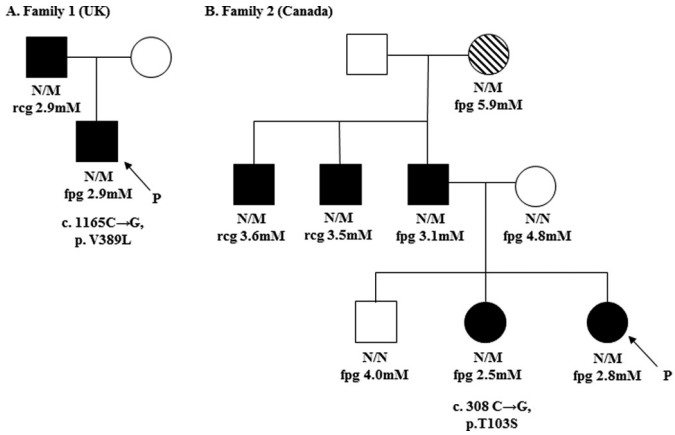


FIGURE 1. **Pedigrees for HH families 1 and 2 (*A* and *B*, respectively).** Males are shown as *squares*, and females are shown as *circles*. *Filled symbols* show individuals with hypoglycemia, *open symbols* show normoglycemic individuals, and *hatched symbols* show individuals with T2D. Fpg or random capillary glucose (*rcg*) levels and mutation status for each tested individual are shown *under* the *symbols* (*N/M* denotes family members heterozygous for mutation, and *N/N* denotes those who do not carry the mutation). *P* denotes the probands.

Calculation of Relative Activity Index (RAI) and GSIS Threshold—RAI values allow the direct comparison of mutants with wild-type enzyme and take into account all GCK intrinsic kinetic parameters ($S_{0.5}$, ATP_{K_m}, n_H, and K_{cat}). The relationship between the RAI for a heterozygous mutant and the GSIS threshold can also be calculated mathematically. Details of all equations used have been published previously (7, 46, 47).

Structural Analysis—Crystal structures for the closed and super-open conformations of GCK (40) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank. Molecular modeling was carried out using PyMOL v0.99 (Schrödinger, LLC).

RESULTS

Novel GCK Mutations in Two Families with HH—Fig. 1, *A* and *B*, show the pedigrees for the HH probands identified in the UK and Canada, respectively. Direct sequencing of *GCK* in the proband of family 1 identified a novel heterozygous mutation, V389L (c.1165C→G, p.Val-389 → Leu). Family studies established that the mutation was inherited from the proband's affected father.

The proband from family 2 was also heterozygous for a novel mutation, T103S (c.308C→G, p.Thr-103 → Ser). Family studies established that the mutation was also present in the affected sister, father, two paternal uncles, and paternal grandmother, with all but the latter exhibiting fasting hypoglycemia. The paternal grandmother was diagnosed with T2D at age 75 years, although notably she is of Metis descent (a First Nation Canadian population at high risk of developing diabetes (48)).

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TABLE 1

Kinetic data for wild-type and four naturally occurring mutant GCKs

Data are shown as means ± S.E. for $n = 18$ experiments and are representative of two preparations of wild-type and each mutant protein.

GCK	$S_{0.5}$	n_H	ATP_{Km}	K_{cat}	RAI	GSIS threshold	Yield	Activity/mg
	mM		mM	s^{-1}		mM	mg	
Wild type	7.5 ± 0.1	1.6 ± <0.1	0.4 ± <0.1	62.9 ± 1.2	1.0	5.0	17.7 ± 0.7	6.6 ± 0.5
V389L	3.5 ± 0.1	1.6 ± <0.1	0.5 ± <0.1	67.8 ± 1.6	6.0	2.9	20.3 ± 1.8	11.3 ± 0.8
V389D	78.6 ± 4.5	1.5 ± <0.1	1.5 ± 0.1	10.3 ± 1.1	<0.1	7.1	0.9 ± 0.3	0.16 ± 0.1
T103S	3.3 ± 0.1	1.6 ± <0.1	0.4 ± <0.1	59.2 ± 1.7	8.4	2.9	9.5 ± <0.1	11.4 ± 2.2
T103I	24.9 ± 0.6	1.7 ± <0.1	0.6 ± <0.1	68.1 ± 0.1	<0.1	6.7	12.5 ± 0.5	11.2 ± 0.5

TABLE 2

Kinetic data for wild type and a range of amino acid substitutions at positions 103 and 389

Data are shown as mean ± S.E. for $n = 6$ experiments and are representative of two preparations of wild-type protein and one preparation of each mutant protein.

GCK	Hydro-	$S_{0.5}$	n_H	ATP_{Km}	K_{cat}	RAI	GSIS threshold
		mM		mM	s^{-1}		mM
Wild type	Phobe	7.5 ± 0.1	1.6 ± <0.1	0.4 ± <0.1	62.9 ± 1.2	1.0	5.0
V389L	Phobe	3.5 ± 0.1	1.6 ± <0.1	0.5 ± <0.1	67.8 ± 1.6	6.0	2.9
V389C	Phobe	8.3 ± 0.3	1.5 ± <0.1	0.5 ± <0.1	58.5 ± 2.4	0.8	5.0
V389S	Phobe	23.9 ± 2.5	1.3 ± <0.1	0.7 ± <0.1	39.9 ± 2.1	<0.1	6.5
V389P	Phobe	39.4 ± 0.6	1.8 ± <0.1	0.6 ± <0.1	31.3 ± 0.3	<0.1	6.8
V389D	Phile	78.6 ± 4.5	1.5 ± <0.1	1.5 ± 0.1	10.3 ± 1.1	<0.1	7.1
V389R	Phile	68.8 ± 0.8	1.6 ± <0.1	0.5 ± <0.1	42.3 ± 0.4	<0.1	6.8
T103S	Phobe	3.3 ± 0.1	1.6 ± <0.1	0.4 ± <0.1	59.2 ± 1.7	8.4	2.9
T103P	Phobe	6.0 ± 0.1	1.9 ± <0.1	0.6 ± <0.1	51.9 ± 0.7	1.2	4.7
T103Y	Phobe	9.1 ± 1.0	1.3 ± 0.1	0.4 ± <0.1	39.0 ± 0.9	0.9	5.5
T103C	Phobe	11.2 ± 0.2	1.5 ± <0.1	0.5 ± <0.1	52.3 ± 1.4	0.3	5.7
T103I	Phobe	24.9 ± 0.6	1.7 ± <0.1	0.6 ± <0.1	68.1 ± 0.1	<0.1	6.7
T103N	Phile	5.8 ± 0.1	1.6 ± <0.1	0.5 ± <0.1	51.5 ± 1.1	1.2	4.5
T103K	Phile	7.5 ± 0.3	1.3 ± <0.1	0.4 ± <0.1	25.6 ± 0.7	0.9	5.6

Neither variant was identified in >400 normal chromosomes. Mutations in other known HH genes (*ABCC8*, *KCNJ11*, and *HNF4A*) were also excluded.

Kinetic Analyses of Recombinant Human Wild-type and Mutant GCKs—Table 1 shows the kinetic characteristics of wild-type, V389L, V389D, T103S, and T103I GCKs as determined using *in vitro* glucose-6-phosphate dehydrogenase-linked assays. Both the V389L and T103S HH mutations have an increased RAI when compared with wild type (6.0 and 8.4, respectively, *versus* 1.0). This was largely driven by an increased affinity for glucose demonstrated by reduced $S_{0.5}$ values (3.5 ± 0.1 and 3.3 ± 0.1 mM, respectively, *versus* 7.5 ± 0.1 mM). The ATP_{K_m}, n_H, and K_{cat} values for these HH mutations were similar to those obtained with wild-type enzyme. Conversely, the MODY mutations V389D and T103I had greatly reduced RAI values (both <0.1 *versus* 1.0 for wild type). Again, this effect was largely driven by a decreased affinity for glucose ($S_{0.5}$, 78.6 ± 4.5 mM for V389D and 24.9 ± 0.6 mM for T103I). However, with the V389D mutant, there was also a reduced affinity for the second substrate ATP when compared with wild type (ATP_{K_m}, 1.5 ± 0.1 *versus* 0.4 ± <0.1 mM) and turnover number (K_{cat}, 10.3 ± 1.1 *versus* 62.9 ± 1.2 s^{-1}). It was also of note that the yield of V389D recombinant protein was greatly reduced compared with the other enzymes (only 0.9 ± 0.3 mg).

Table 2 shows the results of kinetic assays characterizing a range of amino acid substitutions designed to further probe the physiochemical requirements of positions 389 and 103. There is a clear preference for hydrophobic residues at position 389 with hydrophilic substitutions having a deleterious effect on protein function. Substitutions at residue 103 result in a spectrum of effects on enzyme function with activating, near-normal, and inactivating kinetics.

In Silico Calculation of GSIS Thresholds—Table 1 shows the calculated GSIS thresholds for patients with the four individual mutations. Both HH mutations have a predicted GSIS threshold of 2.9 mM, which accurately mirrors the fpg levels of 2.9 and 2.8 mM observed in the V389L and T103S probands, respectively. Calculated GSIS thresholds of 7.1 and 6.7 mM for the V389D and T103I mutations, respectively, are in line with the expected fpg levels in patients with MODY mutations (~5.5–8.0 mM) (5). The severely deleterious effect of the V389D mutation on enzyme function is not reflected in the predicted fpg level due to compensation by the wild-type allele (49).

Response to Physiological Hepatic Inhibitor GKRP—Fig. 2 shows the results of inhibition assays where 10 milliunits of GCK (wild type, V389L, T103S, and T103I) were incubated with 0.5, 1.0, and 1.5 units of GKRP. V389D was not included in these experiments as its activity per mg was so low that to see measurable inhibition by regulatory protein inappropriately large quantities of the enzyme would be needed. As expected, the IC_{50} value for wild type was 1.1 ± 0.1 units of GKRP; the HH mutant V389L yielded identical results. GKRP was less able to inhibit mutant T103S and T103I activity with IC_{50} values of 1.5 ± <0.1 and 4.7 ± 0.5 GKRP units, respectively.

Activation by Small Molecular GKA RO0281675—Table 3 shows -fold activation of each enzyme in terms of $K_{cat}/S_{0.5}$ in the presence of 60 μM GKA. The MODY mutation T103I showed the greatest -fold increase in activity, roughly 6 times that of the wild-type enzyme (90.9 ± 4.0 *versus* 14.4 ± 0.3, respectively). The V389D MODY mutation also demonstrated a high -fold increase in activity of 33.9 ± 2.1. Unsurprisingly, both HH mutations were activated to a lesser degree with -fold

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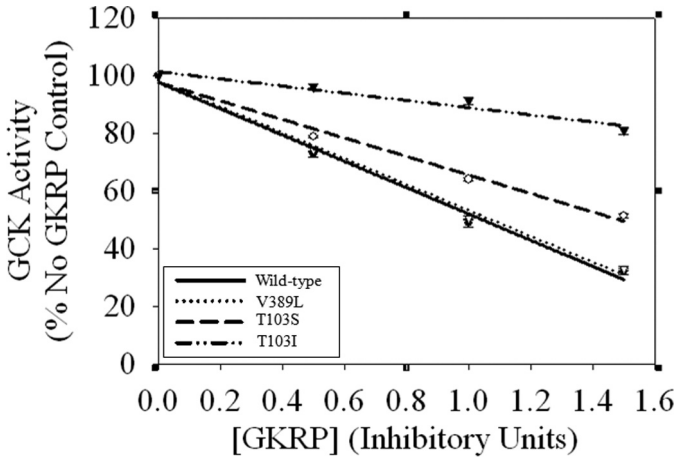


FIGURE 2. **Wild-type, V389L, T103S, and T103I GCKs incubated with 0.5, 1.0, and 1.5 units of GKRP.** GCK activity was normalized to a control in which the reaction conditions were identical, but GKRP was absent. IC_{50} values were calculated using these data and defined as the half-maximal inhibitory concentration of GKRP in each experiment. Data are shown as means ± S.E. for $n = 6$ experiments and are representative of two preparations of wild-type and each mutant protein.

TABLE 3

Data obtained from assaying wild-type and the mutant GCKs in presence of 60 μM RO0281675

-Fold activation was calculated by dividing K_{cat} by the $S_{0.5}$ value at this drug concentration, and EC_{50} is the concentration of drug that gives a GCK activity 50% between base-line and the maximal responses. Data are shown as mean ± S.E. for $n = 6$ experiments and are representative of two preparations of wild-type and each mutant protein.

GCK	-Fold activation ($K_{cat}/S_{0.5}$)	EC_{50}
		μM
Wild type	14.4 ± 0.3	5.8 ± 1.9
V389L	7.4 ± 1.3	3.9 ± 0.1
V389D	33.9 ± 2.1	34.6 ± <0.1
T103S	5.7 ± 0.5	2.6 ± 1.3
T103I	90.9 ± 4.0	12.8 ± 4.2

increases in activity of 5.7 ± 0.5 for T103S and 7.4 ± 1.3 for V389L.

Structural Modeling: Thr-103 but Not Val-389 Maps to Allosteric Activator Site—Fig. 3*A* shows the location of residues Thr-103 and Val-389 within the closed conformation of human GCK (40). Thr-103 lies within the allosteric activator site, specifically within β-strand 4 of the small domain, a region where the majority of other activating mutations are reported (Fig. 3*B*). However, this residue does not form direct contacts with bound GKAs, and so T103S can be described as a group 2 activating mutation (41). Surprisingly, residue Val-389 is located far from the allosteric activator site in α-helix 11 of the large domain. Not only is this distal from the site of GKA binding, but it is also far from the only other group 3 activating mutation, M197I (14, 41), highlighting a novel regulatory region within the enzyme.

DISCUSSION

The current unmet medical need for more effective T2D treatments capable of sustainably lowering glycemia to desirable levels means the quest for novel therapeutic targets is both a timely and important one (2–4). The fundamental role of GCK in glucose homeostasis (specifically within the beta cell and liver) (26, 27) makes it an interesting and well established drug target. Our functional characterization of two novel GCK-HH activating mutations and the corresponding MODY mutations at the same amino acid residues provides further insight into important regulatory regions within GCK, more specifically sites where the functional balance can sway in either direction.

Intrinsic kinetic parameters for wild-type and the four mutant GCKs measured via spectrophotometric *in vitro* assays revealed that the increased activity index of both HH mutations (V389L and T103S) was largely driven by an increased affinity for glucose (Table 1), a result observed with previously reported

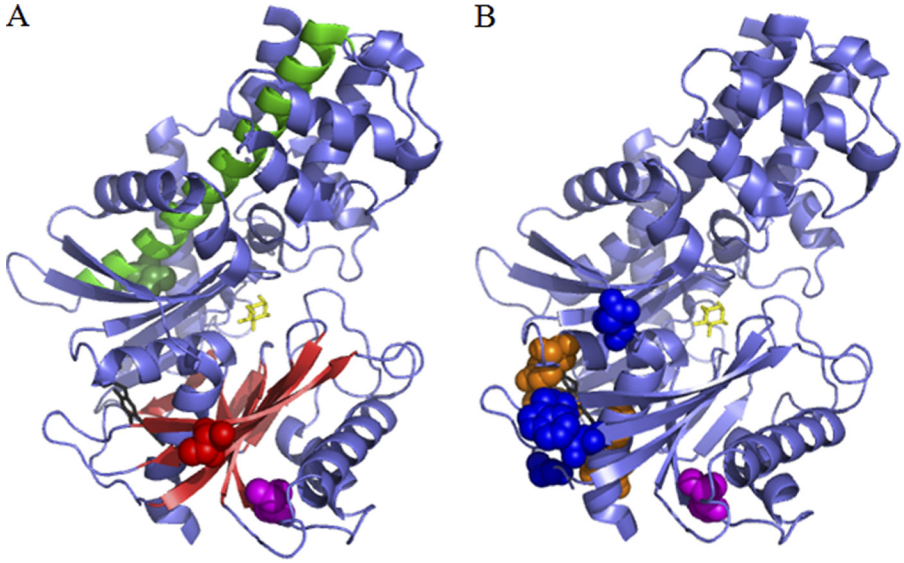


FIGURE 3. *A* shows the positions of residues Thr-103 (*red*) and Val-389 (*green*) within the crystal structure of closed human GCK (40). Thr-103 maps to the allosteric activator site close to where GKAs (*black*) bind, specifically within β-strand 4 of the small domain β-sheet (*red*). Val-389 (*green*) is both distal from this site and residue Met-197 (*magenta*) (14) and instead maps to α-helix 11 (*green*) within the large domain. *B* shows the position of all other naturally occurring activating mutations reported. Group 1 mutations (those in the allosteric activator site making direct contact with GKA) are shown in *orange*, group 2 mutations (those in the allosteric activator site but not contacting GKA) are in *blue*, and group 3 mutations (residues greater than 15 Å from the allosteric activator site) are in *magenta* (41). The binding sites of the GKA "compound A" (*black*) and glucose (*yellow*) are also shown in both figures (40).

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naturally occurring GCK-HH mutations (5, 7, 9–11, 13–15). The reduced activity index of the GCK-MODY mutant T103I followed this theme; however, the substitution of an aspartate for a valine at residue 389 (V389D) had disastrous consequences with regard to enzyme function with affinity for the two substrates glucose and ATP decreased and a substantially reduced overall rate of catalysis. This deleterious effect on GCK function was also mirrored by the protein yield in both recombinant V389D protein preparations. This suggests that not only does V389D directly alter GCK activity, but protein stability as a whole may be reduced. The result of these kinetic characterizations provides a potential explanation for the pathogenesis behind these four mutations as they correspond to both an elevated or diminished capacity to bind and turn over glucose (V389L/T103S and V389D/T103I, respectively).

As GCK activity is fundamental to glucose metabolism in both the beta cell and hepatocyte (26, 27), it is subject to a number of different transcriptional (50) and post-translational regulatory mechanisms (5, 51–58). Therefore, the pathogenic mechanism behind a GCK mutation may not just lie at the level of altered enzyme kinetics. This has been shown with mutations that co-segregate with hyperglycemia in MODY families but whose kinetics are near-normal or even mildly activating. Two such examples are V62M (5, 45, 59) and G72R (5, 59, 60). Kinetic analysis revealed both mutations to be mildly activating and to have only marginal thermolability *versus* wild-type enzyme and the previously reported instability mutant E300K (5, 47, 61, 62). Further investigation revealed that both mutants exhibited defective binding of the endogenous hepatic inhibitor GKRP and a small molecular GKA (5, 34, 45, 59). As well as its direct inhibitory role, GKRP is thought to paradoxically bind and maintain a "reserve pool" of GCK in the hepatocyte nucleus that may be released rapidly upon postprandial increases in glucose (63). Additionally, small molecular GKAs are thought to bind at the same allosteric site as an as yet unidentified endogenous GCK regulator within the beta cell (34). Therefore, it was postulated that loss of these interactions upon GCK mutation could have physiologically pathogenic consequences, a result that was confirmed in Min6 cells where both the V62M and G72R mutants showed reduced catalytic and protein stability compared with wild-type enzyme (59). *In vitro* assays studying the interaction of GCK mutants with both GKRP and GKAs can therefore provide insight into whether the pathogenic mechanism behind a particular mutation lies at levels in addition to directly altered kinetics. Studies performed by Heredia *et al.* (64) investigated the regulation of a number of naturally occurring GCK activating mutants by GKRP. Their data showed differences in GKRP binding affinity between the mutants that was proposed to reflect the mutational mechanism. For example, mutations that enhanced the interaction between glucose and the apoenzyme had the lowest GKRP affinity (as these substitutions stabilized a more compact structure in which the GKRP-binding site was altered), whereas those that enhanced the glucose-bound GCK conformational change demonstrated only slightly reduced GKRP affinity *versus* wild-type enzyme (64).

The results of the GKRP inhibition assays performed in this study (Fig. 2) show that GKRP was able to inhibit wild-type GCK and the V389L mutant equally. However, GKRP-mediated inhibition of both Thr-103 mutants was less effective compared with the wild-type enzyme with the binding to mutant T103I affected the most. This is consistent with data suggesting that the interaction of GKRP with GCK occurs at leucine-asparagine motifs within the hinge region of the enzyme (65), a site that maps next to the allosteric activator site (within which Thr-103 is found). Additionally, these data suggest that the T103S activating mutation may directly increase glucose binding to the apoenzyme, whereas V389L may enhance the conformational change from the inactive to active structure once glucose is bound (64). As GKRP is specific to the liver and not expressed within the beta cell (43, 66, 67), it is difficult to postulate the precise consequence of a reduced GKRP binding affinity on overall GCK activity, particularly as the ability to bind this inhibitor contributes to but is not a determinant of clinical phenotype (5, 14, 45, 59, 60, 64). As GKRP binds T103S less effectively, it could be assumed that there is less inhibition of GCK activity in the liver, thus leading to an increase in hepatocyte glycolytic flux (43, 68), which would contribute to the hypoglycemia of this HH patient. Equally, the greatly decreased GKRP affinity observed with T103I may mean that there are markedly reduced hepatic nuclear reserves of this mutant enzyme, consequently hindering postprandial glucose clearance by the liver (63), contributing to the hyperglycemia in this MODY patient.

Table 3 shows the results of the GKA RO0281675-mediated stimulation of wild-type and the four GCK mutants. Interestingly, all four mutants responded to the drug, suggesting that not even the particularly deleterious V389D substitution resulted in an enzyme that could not be converted into the active conformation. The MODY mutants V389D and T103I showed the greatest -fold increase in activity. This is perhaps a reflection of the reduced activity of the MODY mutants prior to stimulation, a hypothesis supported by both HH mutants in this study that showed the smallest -fold increases in activity and also by the previous findings of Grimsby and Grippo (10, 69) who demonstrated that the V455M GCK activating mutant was resistant to GKA-mediated stimulation. The much greater response of the inactivating T103I mutant *versus* that of V389D may be explained by the physiochemical properties of these substituted residues. Both threonine and isoleucine are hydrophobic, meaning it may be easier to "force" GCK into the closed active structure using small molecular activators, whereas the V389D mutant has a hydrophilic residue (aspartate) being pushed into a hydrophobic pocket normally occupied by valine. This is also reflected in V389D having the highest EC_{50} value of the four mutants.

Molecular modeling of these mutations within the GCK crystal structure (40) allows the mechanisms behind their pathogenesis to be further probed. Kamata *et al.* (40) proposed a three-GCK conformation model that explained both the sigmoidal binding curve and cooperativity with respect to glucose. The transition between functional states requires a global conformational change and the breaking and reforming of numerous intramolecular bonds (40), both of which are easily affected by the physiochemical properties of substituted residues at sites critical to function.

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Val-389 is situated near the C-terminal end of α-helix 11. It is surrounded by hydrophobic residues Leu-386, Ile-390, Met-393, Met-41, Tyr-234, Trp-257, and Ala-232. The observation that certain amino acid substitutions at residue 389 may increase GCK activity identifies this structural position and α-helix 11 as a novel site regulating GCK function, one that is distal from both the allosteric activator site and the only other reported naturally occurring group 3 activating mutation, M197I (14, 40, 41). Residue 389 lies within a solvent-excluded region of the molecule, a location suited by hydrophobic valine and the even more hydrophobic leucine but not by hydrophilic aspartate. Table 2 shows the kinetic parameters measured for a series of mutations at position 389. These data showed that all hydrophilic substitutions drastically reduced GCK activity, whereas all hydrophobic replacements except serine gave activating or near-normal kinetics (except the substitution of proline (P), which may break this α-helix). This finding is similar to that observed with the other group 3 activating mutant, M197I. This residue is also located within a hydrophobic pocket where the introduction of hydrophilic residues is deleterious to GCK function (14).

The mutational mechanisms behind the substitutions at residue 103 are much harder to explain than those at residue 389. Thr-103 is a surface residue; however, it is unclear how a conservative mutation such as the replacement of a threonine with a serine would affect GCK activity. Mutating the threonine to an aliphatic isoleucine puts a non-polar hydrophobic residue in a solvent-exposed environment, which may account for the inactivating kinetics of T103I. A series of amino acid substitutions were carried out at this position to see how GCK activity was altered. Table 2 shows these mutants to demonstrate a spectrum of kinetic properties. T103S was the only activating mutation observed. The mutants T103N, T103K, T103P, and T103Y all demonstrate near-normal kinetics, meaning that either (i) these mutations would not be pathogenic or (ii) their mutational mechanism would not lie at the level of altered glucose catalysis (59). Importantly, only one of these mutations (T103N) has been identified in a GCK-MODY patient (70). Finally, the mutants T103I and T103C had a reduced activity index, reflecting the potential intolerance of an aliphatic side chain in this solvent-exposed region.

In summary, the newly identified importance of α-helix 11 suggests that further activating mutations are likely to exist and that they may not necessarily map to the allosteric activator site. Our results build upon earlier work by Pal and Miller (41) who preemptively identified further activating mutations by genetic screening as well as that by Sayed *et al.*(14) who characterized the first activating mutation outside of the allosteric activator site. This study highlights the importance of the continued study of naturally occurring *GCK* activating mutations as detailed kinetic analysis at these residues may enable the fine tuning of GKAs for the treatment of diabetes.

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The P446L variant in *GCKR* associated with fasting plasma glucose and triglyceride levels exerts its effect through increased glucokinase activity in liver

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Genome-wide association studies have identified a number of signals for both Type 2 Diabetes and related quantitative traits. For the majority of loci, the transition from association signal to mutational mechanism has been difficult to establish. Glucokinase (GCK) regulates glucose storage and disposal in the liver where its activity is regulated by glucokinase regulatory protein (GKRP; gene name *GCKR*). Fructose-6 and fructose-1 phosphate (F6P and F1P) enhance or reduce GKRP-mediated inhibition, respectively. A common *GCKR* variant (P446L) is reproducibly associated with triglyceride and fasting plasma glucose levels in the general population. The aim of this study was to determine the mutational mechanism responsible for this genetic association. Recombinant human GCK and both human wild-type (WT) and P446L-GKRP proteins were generated. GCK kinetic activity was observed spectrophotometrically using an $NADP^+$-coupled assay. WT and P446L-GKRP-mediated inhibition of GCK activity and subsequent regulation by phosphate esters were determined. Assays matched for GKRP activity demonstrated no difference in dose-dependent inhibition of GCK activity or F1P-mediated regulation. However, the response to physiologically relevant F6P levels was significantly attenuated with P446L-GKRP ($n = 18$; $P \leq 0.03$). Experiments using equimolar concentrations of both regulatory proteins confirmed these findings ($n = 9$; $P < 0.001$). In conclusion, P446L-GKRP has reduced regulation by physiological concentrations of F6P, resulting indirectly in increased GCK activity. Altered GCK regulation in liver is predicted to enhance glycolytic flux, promoting hepatic glucose metabolism and elevating concentrations of malonyl-CoA, a substrate for *de novo* lipogenesis, providing a mutational mechanism for the reported association of this variant with raised triglycerides and lower glucose levels.

INTRODUCTION

A recent genome-wide association (GWA) scan identified *GCKR* as a potential locus for modulating triglyceride and fasting plasma glucose (fpg) levels (1). *GCKR* encodes glucokinase regulatory protein (GKRP), and following fine mapping attributed the signal to the common non-synonymous SNP rs1260326 (c.1403 C > T, p.P446L, MAF 34%) (2). Subsequent population-based studies have replicated the association of this variant with triglyceride and fpg levels. Individuals homozygous for the risk allele (L) have on average a 0.15 mmol/l increase in triglyceride and a

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0.06 mmol/l reduction in fpg levels compared with individuals with two copies of the wild-type (WT) allele (P) (2–4). The mutational mechanism behind this genetic association is currently unknown. However, unlike many signals arising from GWA studies, there is a non-synonymous SNP in a strong biological candidate gene driving the genetic association, thus facilitating functional studies. Even with such tractable variants, identifying the mutational mechanism for common risk alleles presents a challenge due to their small physiological effects. It is therefore essential that the 'correct' assays are selected and performed if these small differences in protein function are to be observed.

Glucokinase (GCK) is a key regulator of glucose storage and disposal in the liver. GKRP regulates GCK activity competitively with respect to the substrate glucose (5,6). GKRP action is in turn controlled by the phosphate esters fructose 6- and fructose 1-phosphate (F6P and F1P), which compete with each other for binding, and enhance or inhibit the action of the regulatory protein, respectively (7–9).

There is some controversy in the literature as to whether GKRP also regulates GCK in pancreatic β-cells (10–12). The vast majority of studies state that *GCKR* is not expressed in rodent β-cells (11,12). However, there is evidence from one study that an alternatively spliced *GCKR* variant is expressed in the β-cells of rodents and represents the major isoform in this tissue (10). To date, no studies have been reported which investigate *GCKR* expression in human pancreatic islets.

Biological evidence to support the association of these phenotypes with the *GCKR* SNP comes from both cellular and rodent models (13,14). Counter intuitively, Slosberg *et al*. over expressed *GCKR* in the human liver cell line HepG2 and showed an increase in GCK activity and expression at the protein level. From this, they suggested that GKRP as well as inhibiting GCK activity, also has a paradoxical role in extending GCK half-life by binding to and stabilizing the enzyme, thus protecting it from degradation (14). This finding is supported by data from both homo- and heterozygous *gkrp* knockout mice which display a marked reduction in *gck* expression and reduced GCK enzymatic activity under saturating glucose concentrations (11). At least one study has also shown that *in vivo* over-expression of *gck* in rat hepatocytes led to a drop in plasma glucose levels with a corresponding increase in circulating triglycerides (13). Therefore, findings from both groups suggested that (i) GKRP expression has a direct affect on GCK activity and (ii) there is a link between GCK activity and plasma glucose/circulating triglyceride levels.

A number of studies investigating the interaction of GCK and its regulatory protein have been reported but due to technical difficulties in purifying human recombinant GKRP, all of these have used the rat isoform (9,15,16). One of these studies investigated the effect of rodent P446L-GKRP on GCK activity and reported no differences in the inhibitory ability of the variant protein or its response to phosphate esters compared with WT (9). However, despite their high sequence homology (~88%), there are important differences in the regulation of rat and human GKRP (7,17). First, human regulatory protein inhibits GCK even in the absence of F6P, an affect not observed with the same concentration of rat GKRP. Secondly, human regulatory protein was shown to have a higher affinity for this phosphate ester compared with the rodent protein (7).

The aims of this study were therefore, first, to investigate the affect of both human WT and P446L-GKRP on human GCK activity and secondly, to determine whether their regulation by phosphate esters was altered. Finally, we aimed to establish whether *GCKR* is expressed in human pancreatic islets, and if so, to look at this expression relative to that of *GCK*.

RESULTS

WT and P446L-GKRP both inhibit GCK activity

To assess the inhibitory function of our purified recombinant human WT and P446L-GKRP proteins following heterologous expression in *Escherichia coli*, one unit of each regulatory protein was used in assays to study competitive inhibition of GCK activity over a glucose concentration range (0–100 mM). Figure 1A demonstrates that GCK activity is inhibited with both GKRPs (each data point being plotted as a percentage of the negative control in which GKRP was absent and glucose concentration was 100 mM), establishing that both WT and P446L proteins are functional. These results also show no difference between the ability of WT and variant regulatory protein to inhibit GCK in the absence of the phosphate esters F1P and F6P.

To further compare GKRP activity, competitive inhibition assays were repeated at fixed concentrations of both GKRP proteins ($n = 3$) as shown in Figure 1B (100 nM GKRP) and C (150 nM GKRP). These data also demonstrate no difference between the two regulatory proteins at 100 nM. However, at 150 nM, P446L-GKRP is less inhibitory than WT at concentrations of 25 mM glucose and above ($P \leq 0.03$). Ki (inhibitor dissociation constants) were calculated from Dixon plots of 1/GCK activity versus [GKRP] for the equimolar concentration experiments (Supplementary Material, Fig. S1). The Ki values did not significantly differ between the two regulatory proteins (24.0 ± 40.7 nM for WT versus 23.1 ± 40.8 nM for P446L-GKRP; mean ± SEM; $P = 1$).

Our data are consistent with GKRP acting as a competitive inhibitor of GCK activity (18). The $Kcat_{GCK}$ (maximal specific activity of GCK) remained unchanged in the presence of 0–150 nM regulatory protein (51.8 ± 1.6 to 51.9 ± 6.9 for WT versus 46.8 ± 0.7 to 53.4 ± 3.6 for P446L-GKRP; $n = 3$), and the $S_{0.5}$ (glucose concentration needed for half maximal rate) was raised with increasing concentrations of both GKRPs (8.7 ± 0.2 to 16.8 ± 1.3 for WT versus 8.0 ± 0.3 to 18.2 ± 1.1 for P446L-GKRP).

F1P increases GCK activity with both WT and P446L-GKRP

Data obtained using comparable activities of regulatory protein demonstrated the expected reduction in GKRP-mediated inhibition of GCK activity across a F1P concentration range of 0–500 μM (Fig. 2A). This GKRP-mediated inhibition was decreased by the same extent for both WT and P446L-GKRP (53.7 ± 1.1 to 92.8 ± 1.2% GCK activity for

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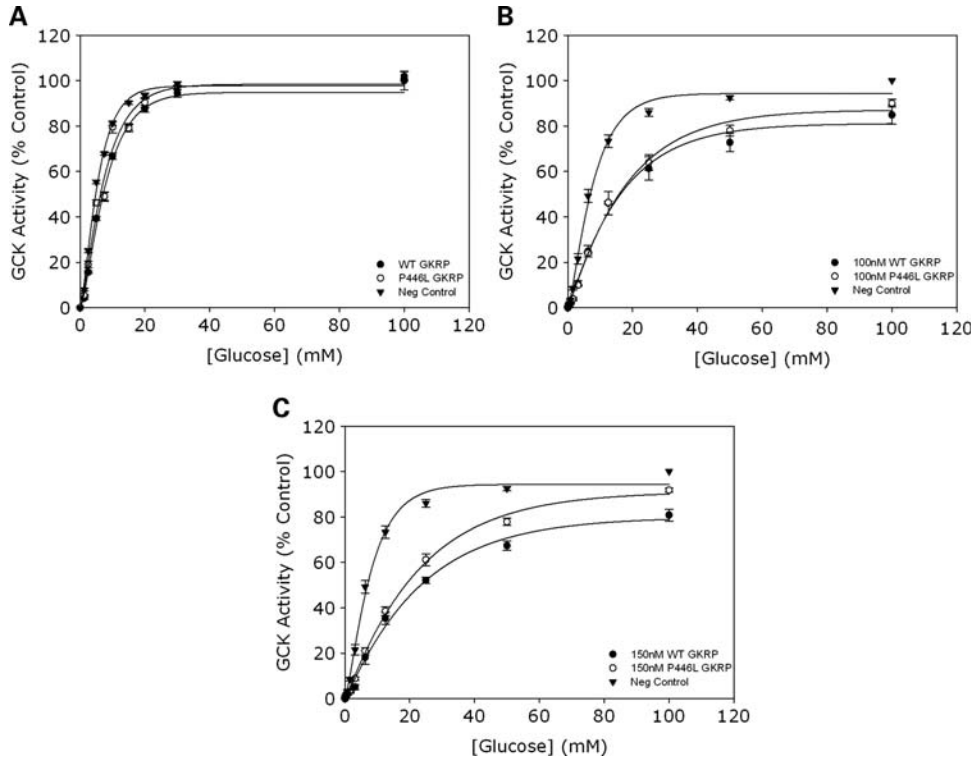


Figure 1. (**A**) Competitive inhibition of GCK using equivalent activities of WT and P446L-GKRP ($n = 24$). Competitive inhibition of 10 m U/ml GCK by one GKRP unit (WT=black circles, P446L=white circles, negative control=black triangles) was observed over a glucose concentration range of 0–100 mM. Each data point was plotted as a percentage of the negative control in which GKRP was absent and glucose concentration equaled 100 mM. Statistical analysis revealed no difference between the intrinsic inhibitory capacity of WT and P446L-GKRP. (**B** and **C**) Competitive inhibition of GCK using equimolar concentrations of WT and P446L-GKRP ($n = 3$). GCK activity was assayed in the presence of 0, 100 and 150 nM WT (black circles) and P446L (white circles) GKRP, over a glucose concentration range of 0–100 mM. Each data point was plotted as a percentage of the negative control in which GKRP was absent (black triangles) and glucose concentration equaled 100 mM. At 100 nM GKRP, there was statistically no difference between the responses of the two regulatory proteins (although it was noted that the variance of this data was larger than seen for other experiments and $n = 3$). At 150 nM GKRP, statistical analysis showed the intrinsic inhibitory capacity of P446L-GKRP to be significantly lower than that of the WT regulatory protein at [glucose] over 25 mM ($P \leq 0.03$).

WT versus 55.7 ± 1.5 to 93.6 ± 1.4% GCK activity with P446L; means $\pm$ SEM; $n = 18$; $P > 0.1$).

Experiments using fixed concentrations of both WT and variant regulatory protein confirmed that there was no difference in F1P-mediated inhibition of the two GKRPs at concentrations up to and including 500 μM of phosphate ester (92.5 ± 2.6% for WT versus 90.1 ± 1.8% for P446L; $n = 9$; $P = 0.2$), despite the initial difference in intrinsic inhibitory capacity at 0 μM F1P (Fig. 2B).

It has been suggested previously that F1P causes an increase in GCK activity by promoting dissociation of the GKRP:GCK complex (7). Therefore the 'apparent' inhibitor dissociation constant ($Ki_{apparent}$) would be expected to increase upon addition of F1P, as regulatory protein dissociation from the enzyme is promoted. In our studies, $Ki_{apparent}$ from Dixon plots of 1/GCK activity versus [F1P] reflected this. Also, these calculated $Ki_{apparent}$ values did not significantly differ between the two regulatory proteins when using either comparable activity (24.5 ± 2.0 μM for WT versus 31.2 ± 4.3 μM for P446L-GKRP; $P = 0.1$) or equimolar concentrations (54.0 ± 10.8 μM for WT versus 46.3 ± 4.2 μM for P446L-GKRP; $P = 0.5$), suggesting a similar dissociation of both regulatory proteins from GCK in response to F1P. This was also corroborated by observing the [F1P] that caused reversal of GCK inhibition by half (i.e. 75% enzyme activity), which was ~15 μM F1P for both GKRPs in the matched activity experiments, and ~15 and 20 μM F1P for P446L and WT-GKRP, respectively, in the comparable concentration experiments.

Differences in F6P-mediated regulation of P446L-GKRP compared with WT

GCK activity was observed over 0–500 μM F6P in the presence of either equal activities or equivalent concentrations of both GKRPs (Fig. 3A and B, respectively). It is well established that F6P-mediated regulation of GKRP leads to enhanced GCK inhibition, and hence a corresponding reduction in observed enzyme activity (7,9,18). This was seen with both WT and P446L-GKRP when using the same activity of regulatory protein, although the reduction in GCK activity was significantly less over the physiologically relevant 25–500 μM F6P concentration range with the variant protein (31.9 ± 2.6 to 23.3 ± 1.0% GCK activity versus 28.5 ± 2.8 to 21.2 ± 0.8% GCK activity for WT; means $\pm$ SEM; $n = 18$; $P = 0.04$, 0.0001, 0.006 and 0.03 for 25, 50, 100 and 500 μM F6P, respectively) (Fig. 3A).

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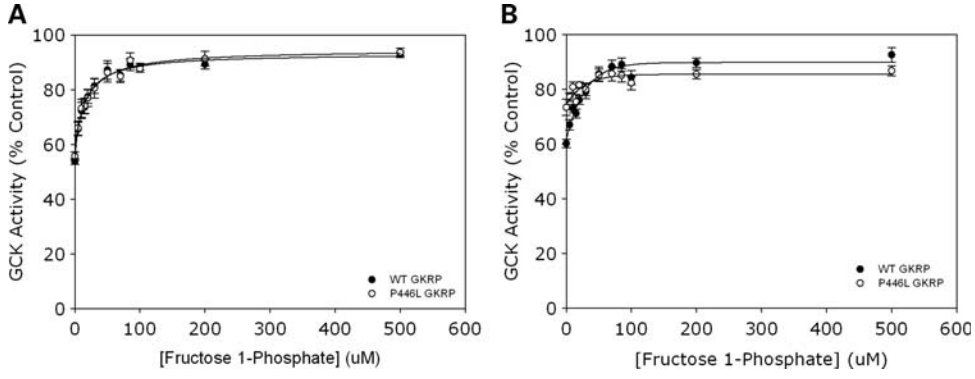


Figure 2. (**A** and **B**) F1P negatively modulates both WT and P446L-GKRP-mediated inhibition of GCK, leading indirectly to an increase in enzyme activity. Inhibition of 10 m U/ml GCK by (A) one GKRP unit, 5 mM glucose ($n = 18$) and (B) 100 nM GKRP, 10 mM glucose ($n = 3$) was observed over 0–500 μM F1P (black circles=WT, white circles=P446L). GCK activity was plotted as a percentage of that obtained in the absence of either regulatory protein. Statistical analysis revealed no difference in response of WT and P446L-GKRP to F1P up to and including 500 μM F1P ($P \geq 0.1$). $Ki_{apparent}$ from Dixon plots of [F1P] versus 1/GCK activity did not significantly differ between the two regulatory proteins (A) 31.2 ± 4.3 μM for WT versus 24.9 ± 2.0 μM for P446L-GKRP, (B) 54.0 ± 10.8 μM for WT versus 46.3 ± 4.2 μM for P446L-GKRP; mean ± SEM; $P \geq 0.1$.

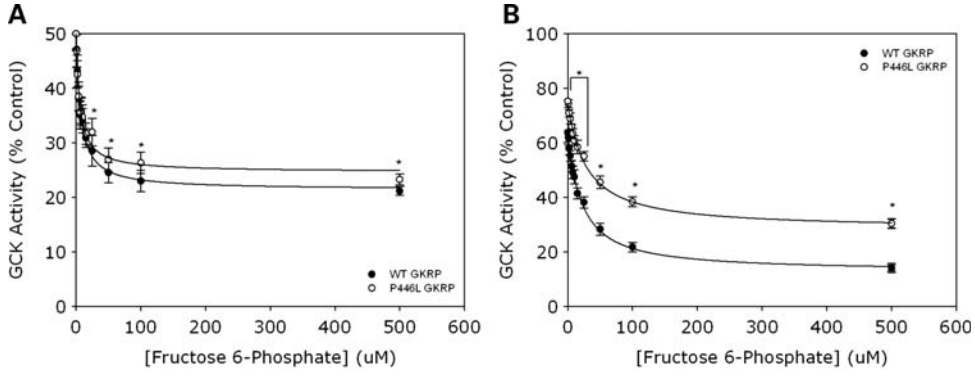


Figure 3. (**A** and **B**) F6P-mediated regulation of P446L-GKRP is significantly diminished compared with WT regulatory protein. Inhibition of 10 m U/ml GCK by (A) one unit GKRP, 5 mM glucose ($n = 18$) and (B) 100 nM GKRP, 10 mM glucose ($n = 3$) was observed over 0–500 μM F6P (black circles=WT, white circles=P446L). GCK activity was plotted as a percentage of that obtained in the absence of regulatory protein. Statistical analysis showed there to be a significant difference between the response of WT and P446L-GKRP (A) over the physiologically relevant range 25–500 μM F6P (31.9 ± 2.6 to 23.3 ± 1.0% GCK activity for P446L versus 28.5 ± 2.8 to 21.2 ± 0.8% GCK activity for WT; means ± SEM; $P = 0.04$, 0.0001, 0.006 and 0.03 for 25, 50, 100 and 500 μM F6P, respectively) and (B) over the entire 0–500 μM F6P range ($P < 0.001$). Dixon plots of [F6P] versus 1/GCK activity showed $Ki_{apparent}$ was also significantly different between WT and variant regulatory protein in both (A) the comparable activity (31.0 ± 4.4 versus 21.1 ± 2.7 μM, respectively; $P = 0.05$) and (B) equimolar concentration experiments (16.6 ± 1.7 versus 9.2 ± 1.0 μM, respectively; $P < 0.001$).

These results were confirmed in experiments using the same concentration of both regulatory proteins, as there was a significant difference between the response of WT and P446L-GKRP over the entire F6P concentration range (63.7 ± 2.6 to 14.1 ± 1.2% versus 71.9 ± 1.4 to 33.8 ± 3.1%, respectively; $n = 9$; $P < 0.001$) (Fig. 3B).

Increasing F6P concentration has been shown in this study to enhance GKRP-mediated inhibition of GCK activity, and thus it would be expected that $Ki_{apparent}$ would be decreased as a result of reduced dissociation of the inhibitor GKRP from GCK (i.e. 'tighter' enzyme:regulatory protein binding). This was reflected in our data, as Dixon plots of [F6P] versus 1/GCK activity showed a decrease in $Ki_{apparent}$ upon addition of F6P. $Ki_{apparent}$ was also significantly different between WT and P446L-GKRP for both the equal activity (31.0 ± 4.4 μM versus 21.1 ± 2.7 μM, respectively; $P = 0.5$) and equimolar concentration experiments (16.6 ± 1.7 μM versus 9.2 ± 1.0 μM, respectively; $P < 0.001$). The IC_{50} (concentration of F6P needed to inhibit enzyme activity by 50%) was also significantly different between WT and P446L-GKRP; 23.8 versus 35.0 μM, respectively, for matched activity experiments ($P = 0.01$), and 25.0 versus 56.3 μM, respectively, for equal concentration experiments ($P < 0.001$), reflecting a decreased affinity of the variant regulatory protein for F6P in both experiments.

GCKR is highly expressed relative to *GCK* in human liver, with very low levels in human pancreatic islets

Owing to the association of P446L-GKRP with fpg levels, we investigated whether the observed modulation in the P446L-GKRP response to physiologically relevant F6P concentrations could have consequent metabolic affects in human pancreatic islets as well as human liver. To address this question, we quantified *GCKR* and *GCK* mRNA levels in appropriate human tissues. Quantitative reverse

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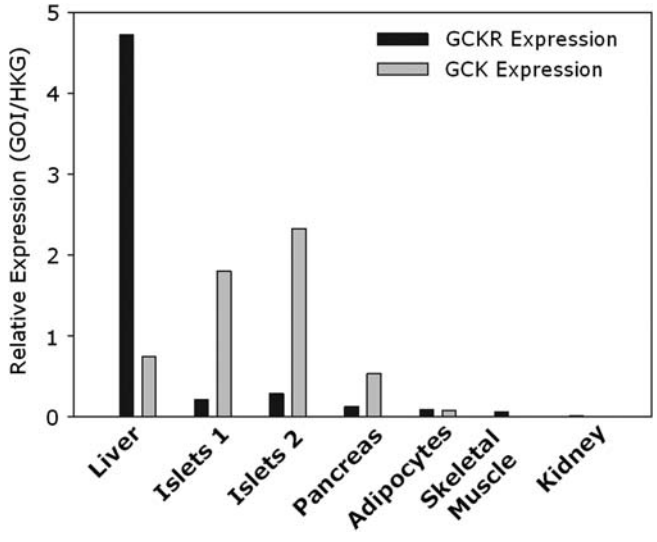


Figure 4. *GCKR* and *GCK* expression in human liver, pancreas, isolated islets, adipocytes, skeletal muscle and kidney. One microgram RNA was converted to cDNA via RT–PCR and expression of the human *GCKR* and *GCK* genes observed via Taqman gene expression analysis. Liver ($n = 1$), islets ($n = 2$), pancreas ($n=1$) and adipocytes ($n = 1$) were studied (as well as appropriate negative controls). These data show *GCKR* to be highly expressed in molar excess in the liver compared with *GCK*. However, in the pancreas and islets, *GCK* levels are much higher than that of the regulatory protein. In adipocytes, skeletal muscle and kidney both genes are only present at negligible levels.

transcription–polymerase chain reaction (qRT–PCR) analysis of liver ($n = 1$), islets ($n = 2$), whole pancreas ($n = 1$) and adipocytes ($n = 1$) demonstrated that *GCKR* mRNA is expressed in molar excess in human liver compared with *GCK*, thus supporting a functional role for the regulatory protein in the hepatocyte (19). Conversely, although *GCKR* is expressed at very low levels in human islets, whole pancreas and adipocytes (only a tenth of that seen in liver), the much higher relative expression of *GCK* suggests regulation of this enzyme in these tissues is largely not GKRP mediated (Fig. 4).

DISCUSSION

In this study, we have performed the first functional characterization of the human P446L-*GCKR* variant, which has been reproducibly associated with an average 0.15 mmol/l increase in triglyceride and a 0.06 mmol/l decrease in fpg levels in the general population (1–4). Our data demonstrate both human WT and variant regulatory protein to be functional and able to inhibit GCK activity in a dose-dependent manner. Both GKRPs were also equally sensitive to F1P-mediated regulation, which is consistent with that shown previously using rodent proteins (16). However, our analyses have revealed a previously unappreciated change in F6P-mediated modulation of the P446L regulatory protein at physiologically relevant concentrations of this phosphate ester. These results differ from those reported in earlier rodent studies which showed no differences between the two proteins and confirm the important differences in the properties of human GKRP compared with rodent homologues (7).

The differing responses of the P446L-variant regulatory protein to F1P and F6P may be explained by looking at the binding site of these molecules. The crystal structure of GKRP has not been solved; however, it does have homology with other proteins of known crystal structure, such as the isomerase domain of bacterial glucosamine 6-phosphate synthase (GlmS), which catalyses the conversion of F6P into either glucose 6-phosphate or glucosamine 6-phosphate (9,20–22). This isomerase domain consists of two sugar isomerase subdomains (23), and analysis of the GlmS crystal structure (21) and functional studies on rat GKRP mutants (9) suggests a single substrate binding site lies between the interface of these two sub-domains. It has also been suggested that GKRP adopts different structural conformations in order to accommodate either F1P or F6P at this single binding site, and only one of these (that favoring F6P-binding) can complex with GCK (9,24). Residue 446 lies between two sequence motifs thought to be involved directly in binding of phosphate esters. As previously reported, mutating certain residues outside of these motifs can still affect binding (and hence regulation of GKRP activity) (9). Also, residue 446 is a conserved proline across human, rat and *Xenopus laevis* regulatory protein, and all bar the latter are responsive to both F1P and F6P (7,9). Therefore, we propose that residue 446 may not play a direct role in the binding of phosphate esters, but may be important structurally within GKRP. This amino acid substitution could then either reduce the ability of the regulatory protein to adopt the F6P-binding conformation or hinder the F6P–GKRP complex from binding to and inhibiting GCK.

Our data show F6P-mediated regulation of P446L-GKRP to be significantly attenuated compared with WT over a physiological range of F6P concentrations, which ultimately leads to a reduction in GCK-inhibition by the variant regulatory protein. This is predicted to increase glycolytic flux and hence glucose uptake by the liver. This enhanced rate of glycolysis (as is seen with GCK over-expression in rats) may raise levels of other liver metabolites such as malonyl-CoA, increasing triglyceride levels via two mechanisms; first by acting as a substrate for *de novo* lipogenesis and secondly by inhibiting carnitine-palmitoyl transferase-1 (thus blocking fatty acid oxidation) (13). This perturbation of hepatic metabolism could account for the reduced glucose and raised triglycerides seen in L446-GKRP individuals. However, the very low *GCKR* mRNA levels detected in human islets (compared with human liver) suggest that GKRP has only a very minor (if any) functional role in the regulation of GCK in the pancreas. Consequently, pancreatic GCK is unlikely to be affected in individuals with this variant regulatory protein confirming that the phenotype observed is driven by a hepatic effect.

Broader implications of these findings regard the alteration of GCK activity to treat diabetes. Aberrant GCK function has been implicated in both monogenic forms of diabetes and in the variation of fpg levels within the general population, and as such it is a well-established therapeutic target (25,26). The initial genetic association data concerning the P446L-*GCKR* variant (1–4) supported by our functional studies confirm that changing the regulation of GCK activity solely in the liver could alter other hepatic metabolic pathways, thus resulting in an unfavorable lipid profile. This has

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already been shown in one study in rodents, where over-expressing hepatic *gck* lowers plasma glucose as well as raising triglyceride levels (13). However, a number of other studies have shown that hepatic over-expression of *gck* does not always result in an increase in triglycerides, suggesting that the degree of increased *gck* activity is critical (27,28). As such, potential new therapies targeting GKRP in the liver should be approached with caution.

In conclusion, our functional studies demonstrate that altered F6P-mediated GKRP regulation in carriers of the L446 allele explains the association between the common P446L-*GCKR* variant and higher triglyceride and lower fpg levels in several human populations. This work demonstrates that with suitable functional studies it is possible to establish mutational mechanisms for common variants of modest effect size identified by GWA studies.

MATERIALS AND METHODS

Site-directed mutagenesis

p-FLAG CTC vector (Sigma Aldrich Company Ltd, Gillingham, UK) containing human GKRP coding sequence was kindly supplied by Katy Brocklehurst (7). The P446L mutation was introduced via PCR-based site-directed mutagenesis using *Pfu*-polymerase (Promega, Southampton, UK) and primers shown in Supplementary Material, Table S1 (Operon Biotechnologies GmbH, Cologne, Germany). Mutant and WT plasmids were then used to transfect BL21 competent cells (Stratagene Europe, Amsterdam, The Netherlands), and DNA obtained using QIAprep Spin Miniprep Kit (Qiagen Ltd, Crawley, UK). Mutant and WT sequences were confirmed via direct sequencing using primers in Supplementary Material, Table S2.

Protein extraction

Glutathione *S*-transferase-tagged human pancreatic GCK was prepared as described previously (29). The pancreatic isoform was used as it has the same affinity for GKRP as liver GCK (30). The extracted enzyme was pure and at a concentration of 1.2 mg/ml, as determined by the Agilent 230 Protein kit (Agilent Technologies UK Ltd, Stockport, UK) and Bio-Rad Bradford reagent assay (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), respectively. Three preparations each of FLAG-tagged human WT and P446L-GKRP were extracted based on a protocol previously described but without the use of a DEAE-sepharose ion-exchange resin (7). The extracted regulatory proteins were confirmed as being greater than 95% pure via Agilent analysis, and concentrations quantified as before (average 0.19 mg/ml for WT and 0.12 mg/ml for P446L-GKRP; $n = 3$). Storage buffer [0.7 M glycerol, 0.2 M KCl, 0.06 M Tris–HCl (pH 8), 5 mM dithiothreitol (DTT), 0.06 M glucose (GCK preparation only); all Sigma Aldrich Company Ltd] was added to recombinant GCK and GKRP in 1:1 volume ratio prior to snap-freezing and storage at -80°C. For initial regulatory protein preparations ($n = 1$), storage buffer including glucose was added to samples. As this affected competitive inhibition assays, storage buffer was changed using Vivaspin20 columns containing diafiltration cups (Sartorius Mechatronics, Epsom, UK) to one in which glucose was absent.

GKRP assays

GKRP inhibition of GCK activity was determined spectrophotometrically using glucose 6-phosphate dehydrogenase (G6PDH)-linked assays (Sigma Aldrich Ltd). Experiments using both comparable activity/mg (amount of WT and P446L-GKRP used in each experiment inhibited 10 m U/ml GCK by the same extent at 5 mM glucose in the absence of the phosphate esters) (7) and equimolar concentrations (either 0, 50, 100 or 150 mM of both regulatory proteins used per assay) (9) were conducted. Assays were based on previously described protocols (7,29,30) but with the following modifications. Competitive inhibition assays were at 37°C, pH 7.1, and contained 2 mM $MgCl_2$, 3.8 μM bovine serum albumin, 25 mM KCl, 25 mM HEPES, 0.5 mM $NADP^+$, 1 mM ATP, 1 mM DTT, 0–100 mM glucose, 4 U/ml G6PDH, 10 m U/ml GCK and 1 U GKRP. All assay reagents (except GCK and GKRP which were generated in-house) were obtained from Sigma Aldrich Ltd. One m-unit of GCK was defined as that which converts 1 nmol substrate per minute, whereas one GKRP unit was defined as that which inhibits 10 m U/ml GCK by 50% under the standard assay conditions defined above (7). On average at 5 mM glucose, one GKRP unit resulted from 15.87 μg/ml of WT and 14.05 μg/ml of P446L-GKRP ($n = 3$ independent protein preparations). One GKRP unit of both WT and variant P446L regulatory protein were subsequently used per equimolar activity assay.

F1P and F6P assays were performed using the same assay conditions as for competitive inhibition experiments, but only 5 mM glucose (7). Both phosphate esters were purchased from Sigma Aldrich Ltd.

Ki (inhibitor dissociation constants) were determined from Dixon plots of 1/GCK activity versus [GKRP] as previously described (7) for the competitive inhibition assays. Plots of 1/GCK activity versus [F6P] or [F1P] were also used to determine apparent inhibitor dissociation constants, '$Ki_{apparent}$', from these experiments. As the phosphate esters are secondary regulators of GCK activity (act via the inhibitor GKRP), Dixon plots provided an insight into dissociation of the phosphate esters, GKRP and GCK, but could not be used to distinguish specifically between the individual components of these interactions.

Gene expression analysis

GCKR and *GCK* expressions were observed via qRT–PCR in human liver ($n = 1$), human islets ($n = 2$), human pancreas ($n = 1$) and human adipocytes ($n = 1$). All samples were part of a commercially available human RNA tissue panel (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France), except for the human islets which were collected as part of a previous study at Oxford University which had been conducted with full ethical consent. RNA samples were initially treated with DNase I (Applied Biosystems, Warrington, UK) to remove all genomic contamination. This was followed by random primed first-strand cDNA synthesis using 1 μg of each RNA sample and a High Capacity cDNA

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Reverse Transcription Kit (Applied Biosystems). Reactions in which the enzyme was absent were also carried out to produce negative controls for the final qRT–PCR. cDNA was diluted 1/100 and 4 μl used in a total reaction volume of 10 μl [5.5 μl of Taqman gene expression master mix and 0.5 μl of the appropriate Taqman gene expression assay (both Applied Biosystems)] and each sample run in triplicate. *GCKR* and *GCK* expressions were made relative to three house-keeping genes (*HPRT*, *B2M* and *ACTB*), and normalized to the 1/100 dilution of the standard curve (for each gene-specific assay).

Statistical analysis

Paired two-tail *t*-tests were used for statistical analysis of all assay data, with a cut-off value for statistical significance of 0.05.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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