

# Pathways for Glycoprotein Degradation in Mammalian Cells

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## **Abstract**

In mammalian cells, N-glycoproteins comprise the bulk of secretory cargo. N-glycosylation is tightly linked to the mechanisms of co- and post-translational folding and quality control in the endoplasmic reticulum (ER). Aberrant glycoproteins must be cleared from the secretory pathway. Several mechanisms for glycoprotein degradation exist, of which ER-associated degradation (ERAD) is most well-studied. The latter involves retrotranslocation of misfolded glycoproteins into the cytosol, followed by proteasomal destruction of the polypeptide. N-linked oligosaccharides are removed from glycoproteins during degradation to form free oligosaccharides (FOS). FOS analysis has been employed to gain a global, rather than single protein-centred view of the pathways of ERAD and the mechanisms of its regulation. It was established that convergent ERAD pathways, distinct in the site of deglycosylation, as well as sensitivity to a number of modulators, exist in the cell. It was further demonstrated that the pathway leading to the production of ER-localised FOS is linked to retrograde Golgi-to-ER transport and is masked by the activity of Golgi endomannosidase. Investigating the involvement on the latter in ERAD has uncovered an endomannosidase-mediated pre-degradative glycoprotein processing route and showed that endomannosidase can rescue a fraction of glycoproteins from degradation following inhibition of ER glucosidases. Further investigation of endomannosidase activity and specificity in different cell lines showed that the ability of the enzyme to process truncated monoglucosylated oligosaccharides appears to be necessary and sufficient for evolutionary conservation in mammals. Overall, the data presented have demonstrated on the global level the connections between the pathways for glycoprotein degradation and processing in the Golgi apparatus, underlining the complexity of biosynthetic regulation in the secretory pathway.

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Dedicated to the memory of my grandfather, Yuri Nikolaevich Kukushkin.

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# List of abbreviations

|           |                                                                         |            |                                                                        |
|-----------|-------------------------------------------------------------------------|------------|------------------------------------------------------------------------|
| 2-AA      | 2-anthranilic acid                                                      |            | acid                                                                   |
| A1AT      | $\alpha$ 1-antitrypsin                                                  | ENGase     | endo- $\beta$ -N-acetylglucosaminidase                                 |
| AP        | adaptor primer                                                          | ER         | endoplasmic reticulum                                                  |
| ATP       | adenosine triphosphate                                                  | ERAD       | endoplasmic reticulum-associated degradation                           |
| BHK-21    | bovine hamster kidney-21                                                | ERGIC      | endoplasmic reticulum/Golgi intermediate compartment                   |
| BiP/GRP78 | immunoglobulin heavy chain binding protein/glucose-regulated protein 74 | ERQC       | endoplasmic reticulum quality control compartment                      |
| BVDV      | bovine viral diarrhoea virus                                            | FCS        | foetal calf serum                                                      |
| CFTR      | cystic fibrosis transmembrane conductance regulator                     | FOS        | free oligosaccharide(s)                                                |
| CHO-K1    | Chinese hamster ovary-K1                                                | GANAB      | neutral $\alpha$ -glucosidase AB (ER glucosidase II $\alpha$ -subunit) |
| CHPV      | Chandipura virus                                                        | GAPDH      | glyceraldehyde 3-phosphate dehydrogenase                               |
| CNX       | calnexin                                                                | GDP        | guanidine diphosphate                                                  |
| COPII     | coating protein II                                                      | GFP        | green fluorescent protein                                              |
| CPY       | carboxypeptidase Y                                                      | Glc (G)    | glucose                                                                |
| CRT       | calreticulin                                                            | GlcNAc (N) | N-acetylglucosamine                                                    |
| CST       | castanospermine                                                         | GPI        | Glucosylphosphatidylinositol                                           |
| DAPI      | 4', 6-diamidino-2-phenylindole                                          | GM130      | Golgi matrix protein of 130 kDa                                        |
| DEPC      | diethylpyrocarbonate                                                    | GRP94      | glucose-regulated protein 94                                           |
| DMEM      | Dulbecco's Modified Eagle Medium                                        | GTP        | guanidine triphosphate                                                 |
| DMJ       | 1-deoxymannojirimycin                                                   | GU         | glucose unit                                                           |
| DNA       | deoxyribonucleic acid                                                   | HA         | haemagglutinin                                                         |
| DTT       | dithiothreitol                                                          | HBV        | hepatitis B virus                                                      |
| ECL       | enhanced chemiluminescence                                              | HCV        | hepatitis C virus                                                      |
| EDEM      | endoplasmic reticulum degradation-enhancing mannosidase-like protein    | HEK-293    | human embryonic kidney 293                                             |
| EDTA      | ethylenediaminetetraacetic                                              | HIV        | human immunodeficiency                                                 |

|              |                                                                                                                   |          |                                                           |
|--------------|-------------------------------------------------------------------------------------------------------------------|----------|-----------------------------------------------------------|
|              | virus                                                                                                             | OS-9     | osteosarcoma-9                                            |
| HL60         | human promyelocytic leukemia-60                                                                                   | OST      | oligosaccharyltransferase                                 |
| HPLC         | high-performance liquid chromatography                                                                            | PBS      | phosphate-buffered saline                                 |
| HRP          | horseradish peroxidase                                                                                            | PCR      | polymerase chain reaction                                 |
| IMAGE        | Integrated Molecular Analysis of Genomes and their Expression Consortium                                          | PDI      | protein disulphide isomerase                              |
|              |                                                                                                                   | PEG      | polyethyleneglycol                                        |
|              |                                                                                                                   | PEI      | polyethyleneimine                                         |
|              |                                                                                                                   | PGC      | porous graphitised carbon                                 |
| IPTG         | isopropylthiogalactoside                                                                                          | PNGase   | peptide:N-glycanase                                       |
| LB           | lysogeny broth                                                                                                    | PVDF     | polyvinylidene fluoride                                   |
| MALDI-TOF    | matrix-assisted laser desorption/ionization-time of flight                                                        | RACE     | rapid amplification of cDNA ends                          |
|              |                                                                                                                   | RER      | rough endoplasmic reticulum                               |
| Man (M)      | mannose                                                                                                           | RNA      | ribonucleic acid                                          |
| MAN1A1       | Golgi mannosidase IA                                                                                              | RPM      | revolutions per minute                                    |
| MAN1A2       | Golgi mannosidase IB                                                                                              | SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| MAN1B1       | ER mannosidase I                                                                                                  |          |                                                           |
| MAN1C1       | Golgi mannosidase IC                                                                                              |          |                                                           |
| MAN2A1       | Golgi mannosidase II                                                                                              | SEC      | size exclusion chromatography                             |
| MAN2C1 (NAM) | cytosolic mannosidase (neutral $\alpha$ -mannosidase)                                                             | SEM      | standard error of the mean                                |
| MANEA        | endomannosidase                                                                                                   | SRP      | signal recognition particle                               |
| MDBK         | Madin-Darby bovine kidney                                                                                         | SW       | swainsonine                                               |
| MDCK         | Madin-Darby canine kidney                                                                                         | TFA      | trifluoroacetic acid                                      |
| MTS/PMS      | (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)/phenazine methosulfate | TG       | thapsigargin                                              |
|              |                                                                                                                   | TX100    | Triton X-100                                              |
|              |                                                                                                                   | TX114    | Triton X-114                                              |
|              |                                                                                                                   | UBP      | ubiquitin-binding protein                                 |
|              |                                                                                                                   | UDP      | uridine diphosphate                                       |
| MWCO         | molecular weight cut-off                                                                                          | UGGT     | UDP-glucose:glycoprotein glucosyltransferase              |
| NAP-DNJ      | N-(6'-(4"-azido-2"-nitrophenylamino)hexyl)-1-deoxynojirimycin                                                     | UPR      | unfolded protein response                                 |
| NB-DNJ       | N-butyldeoxynojirimycin                                                                                           | UTR      | untranslated region                                       |
| NHK          | $\alpha$ 1-antitrypsin, <i>null</i> -Hong Kong variant                                                            | VIP36    | vesicular integral membrane protein 36                    |
| OD           | optical density                                                                                                   | VIPL     | vesicular integral membrane protein-like                  |
| ORF          | open reading frame                                                                                                | YOS9     | yeast osteosarcoma-9.                                     |

# Chapter 1. General introduction

## N-linked glycosylation

### Overview

Glycosylation is the most common post-translational modification of proteins found in nature (Varki et al. 1999). It is ubiquitous throughout eukaryotic domain and has been more recently identified in bacteria and archaea. Addition of carbohydrate moieties to a protein molecule can have a dramatic impact on its structure and function, and can play a wide variety of roles *in vivo*, greatly expanding the functional capacity of a polypeptide. More than half of all eukaryotic protein species are predicted to be glycosylated (Apweiler et al. 1999).

Much of our knowledge of the processes involved in protein glycosylation stems from studies carried out in yeast (Herscovics et al. 1993). The ease of genetic manipulation combined with a high degree of conservation of the mechanisms of glycosylation provided an invaluable insight into the early stages of post-translational processing of glycoproteins, as well as quality control, ER-associated degradation and secretion.

In eukaryotic cells, the three major types of glycosylation are N-linked, O-linked and GPI-anchored. O-linked glycosylation typically involves the sequential addition of monosaccharides onto the hydroxyl residue of the serine or threonine side chains. Glucosylphosphatidylinositol (GPI) is a complex glycolipid transferred to the C-terminus of a

protein, acting as a tether (“anchor”) associating soluble proteins with the lipid bilayer. N-linked glycosylation, constituting approx. 90% of total protein glycosylation in eukaryotes (Apweiler et al. 1999), involves an en bloc transfer of a conserved presynthesised oligosaccharide onto the nitrogen atom of asparagine side chains (Parodi et al. 1972).

Asparagine-linked oligosaccharides play a variety of roles, serving as regulators in the biosynthetic pathway of glycoprotein folding, maturation and subcellular distribution, and themselves having functions in immunity, cell signaling, adhesion etc. (Larkin et al. 2011). Additionally, N-glycosylation alters the physical properties of a protein molecule by modifying its primary structure with a bulky, highly hydrophilic moiety. This factor is especially important in light of the fact that N-glycosylation occurs cotranslationally, before the nascent polypeptide can assume its native conformation. Addition of a glycan can therefore be a critical determinant for the correct folding of glycoproteins both at the physical/chemical level (by increasing hydrophilicity of the surface regions of the polypeptide) and at the level of molecular biology (by regulating quality control and binding of ER chaperones). The present work has been centered on the regulatory functions of N-linked oligosaccharides, employing the intricate connections between N-glycosylation, protein secretion and degradation both as tools and as objects of study.

## **Biosynthesis of the core oligosaccharide**

Biosynthesis of the conserved oligosaccharide subsequently transferred onto asparagine side chains of newly synthesised proteins begins with a sequential addition of monosaccharide residues onto a dolichol phosphate molecule in the membrane of the endoplasmic reticulum (Huffaker et al. 1983). UDP-GlcNAc and GDP-Man serve as precursors for the first stage of the biosynthesis, in which the addition of monosaccharide residues takes place at the cytosolic face of the membrane. Two  $\beta$ 1,4-linked GlcNAc residues are transferred first, followed by mannosylation of the resulting disaccharide. The first branching point is established at this

stage by further  $\alpha$ 1,3- and  $\alpha$ 1,6-mannosylation of the first mannose residue, followed by the addition of two  $\alpha$ 1,2-linked mannoses (“C” arm, see Fig. 1.1)

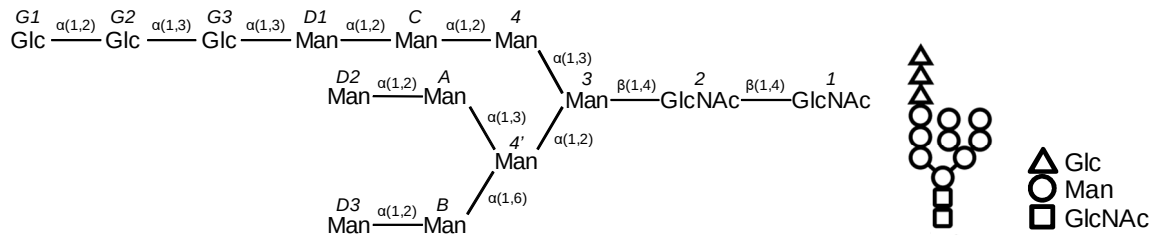


Fig. 1.1. **Structure of  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ .** Left: structural diagram showing monosaccharide nomenclature used throughout the text, and linkage types (reducing end to the right). Right: schematic diagram of the same glycan (reducing end downwards).

Following the addition of these first seven monosaccharides, the dolichol-PP-heptasaccharide is flipped to the luminal face of the ER membrane by an ATP-dependent, bi-directional flippase (Helenius et al. 2002). The lipid-linked oligosaccharide is then further modified with four mannose (“A” and “B” arms, see Fig. 1.1) and three glucose residues, which are transferred onto the heptasaccharide using Dol-P-Man and Dol-P-Glc as precursors, respectively. The latter have to be synthesised on the cytosolic face of the membrane and then flipped into the lumen by a mechanism that remains unclear. The described biosynthetic pathway ultimately results in the production of Dol-PP- $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ . The core oligosaccharide is widely conserved among eukaryotes and constitutes the form in which carbohydrates are transferred onto the asparagine side chains of newly synthesised glycoproteins (Lehle et al. 2006).

## Translocation and N-glycosylation of proteins in the endoplasmic reticulum

The endoplasmic reticulum is the port of entry of proteins into the secretory pathway. The biosynthesis of proteins destined for extracellular export or various locations within the endomembrane/cell membrane systems, takes place on ribosomes associated with the surface of the endoplasmic reticulum. mRNA translation begins in the cytosol, followed by

recognition of an ER targeting signal (typically a region of 11—27 amino acids consisting of a central hydrophobic core flanked with positively charged residues) at the N-terminal end of the emerging polypeptide by the signal recognition particle (SRP) (Martoglio et al. 1998). SRP then binds to the ribosome, causing it to be translocated and bind to the Sec61 pore complex within the ER membrane. Protein synthesis can then proceed, with the polypeptide penetrating the ER membrane through the pore formed by Sec61. The signal sequence is usually cleaved by signal peptidase at the luminal face of the ER membrane (Fewell et al. 2009). The classic SRP-mediated pathway of translocation is not universal. It is evident that at least two SRP-independent pathways may contribute to the translocation of secreted proteins into the ER. Firstly, ribosomes engaged in secreted protein synthesis can remain docked to the ER membrane after the completion of protein synthesis. At the same time, mRNA molecules themselves are thought to contain ER localisation signals. When an mRNA encoding a secreted protein associates with a pre-docked ribosome, protein synthesis can initiate directly on the ER surface, followed by insertion of the preprotein into the ER membrane/lumen (Potter et al. 2002; Seiser et al. 2000; Potter et al. 2000). Secondly, some yeast and short mammalian preproteins have been shown to be translocated into the ER post-translationally in a partially unfolded state (Zimmermann et al. 1988; Rothblatt et al. 1989).

Translocation of the preprotein into the endoplasmic reticulum is assisted by ER chaperones which also prevent aggregation and maintain the unfolded state of a newly synthesised polypeptide. In particular, immunoglobulin heavy chain-binding protein (BiP) in complex with its membrane counterpart Sec63p have been shown to bind to the emerging unfolded proteins in the ER lumen, facilitating and “driving” translocation (Simon et al. 1992; Matlack et al. 1999; Liebermeister et al. 2001, Alder et al. 2005).

Following the formation of Dol-PP-Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub>, the core oligosaccharide is cotranslationally transferred onto the asparagine side chains of the proteins synthesised on the RER-associated ribosomes. The transfer occurs at the luminal side of the ER membrane and

is mediated by oligosaccharyltransferase (OST). OST was first purified from mammalian tissues and yeast cells by detergent extraction and characterised as an enzyme catalysing the transfer of Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> onto peptides (Sharma et al. 1981; Das et al. 1980; Ronin 1980). The acceptor substrate requirement for the enzyme was shown to be the -Asn-X-Ser/Thr- motif, where X is any naturally occurring amino acid except proline (Hart et al. 1979; Welply et al. 1983). The carboxyamido group of the asparagine side chain acts as a nucleophile and Dol-PP as a leaving group in the glycosylation reaction. The complete Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> lipid-linked oligosaccharide in general appears to be a more effective substrate than its truncated forms, e.g. deglycosylated glycans (reviewed in Hubbard et al. 1981). However, shorter oligosaccharyl-lipids, including Dol-PP-GlcNAc<sub>2</sub>, are able to serve as substrates for asparagine-linked glycosylation as well (Sharma et al. 1981; Chen et al. 1977).

## **Processing by glucosidases, and the calnexin/calreticulin cycle**

Newly synthesised glycoproteins in the endoplasmic reticulum bear three glucose residues on the C-arm of the core asparagine-linked glycan. These residues are subject to removal by ER-resident glucosidases I and II (reviewed in Kornfeld et al. 1985). The series of trimming reactions begins with the  $\alpha$ 1,2-specific membrane-bound glucosidase I removing the terminal glucose residue, resulting in a diglycosylated glycan, followed by glucosidase II-mediated cleavage of the first  $\alpha$ 1,3-linked glucose residue. These first two processing reactions occur rapidly and immediately after the glycosylation of asparagine residues by OST, and enable the substrate glycoprotein to bind to calnexin (CNX), a membrane-bound ER chaperone, or calreticulin (CRT), its soluble homologue.

CNX/CRT are lectin-chaperones specific towards monoglucosylated asparagine-linked glycans. Although the lectin specificities for the two chaperones appear to be the same, the set of glycoproteins interacting with either CNX or CRT only partially overlap (reviewed in

Caramelo et al. 2008). This is attributed in part to the membrane-bound/soluble status of CNX and CRT, respectively (Hebert et al. 1997), but additional factors are likely to be involved, since in most cases documented CRT fails to fully compensate for CNX when functional CNX is depleted (Molinari et al. 2004; Pieren et al. 2005). The apparent influence of viral infection on the cross-specificity of CNX and CRT is of particular interest. When CNX-deficient fibroblasts were infected with vesicular stomatitis virus, the viral G protein, which in normal cells only interacts with CNX, was shown to bind to CRT. However, following transfection of the same G protein, the interaction with CRT in CNX-deficient cells was not detected (Pieren et al. 2005).

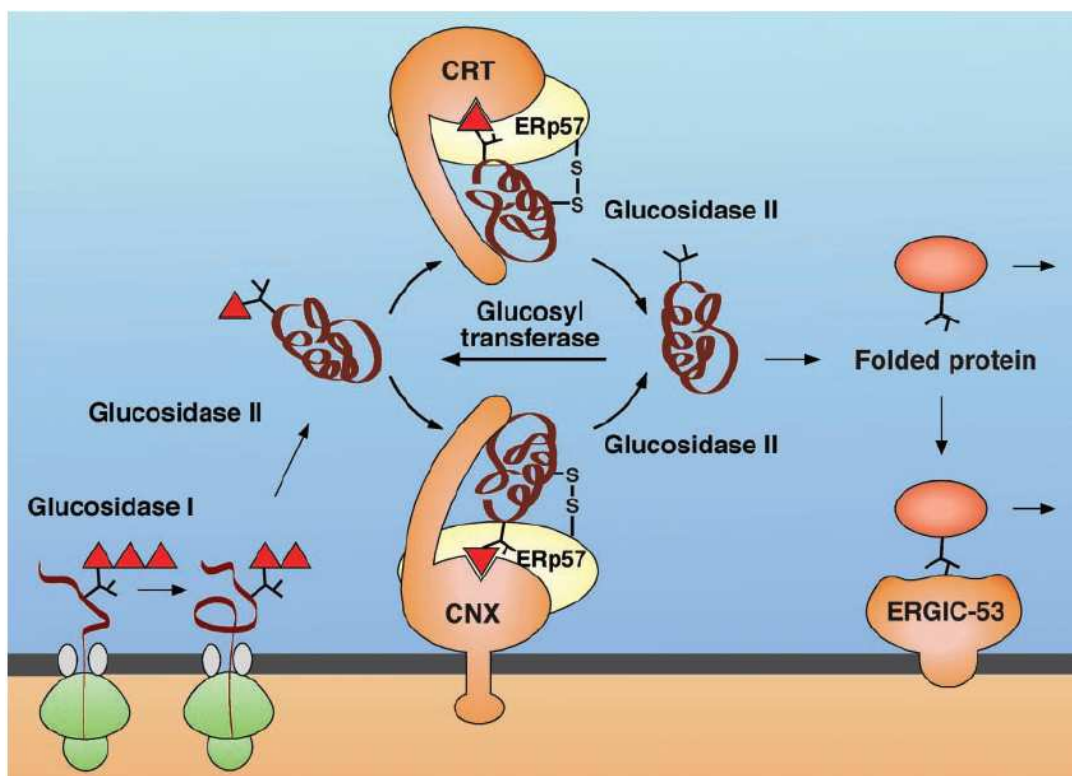


Fig. 1.2. **The calnexin-calreticulin cycle.** Nascent glycoproteins are acted upon by glucosidases I and II to yield a monoglucosylated asparagine-linked oligosaccharide. The glycan then assists binding of the glycoprotein to CNX/CRT, which in turn recruit a protein disulphide isomerase, ERp57. Following removal of the last glucose residue by glucosidase II, the glycan is released from CNX/CRT, but can be reglucosylated by the folding-sensitive UGGT, allowing for re-entry into the folding cycle. Correctly folded proteins are no longer retained in the ER and can be anterogradely transported either in a receptor-assisted manner, or by bulk flow. Adapted from Helenius et al., 2001.

Following binding to substrate glycoproteins, CNX/CRT assist their folding by preventing

aggregation in the crowded environment of the ER, and also by promoting disulphide bond formation mediated by ERp57 oxidoreductase, which is recruited to the CNX/CRT-glycoprotein complex. Additionally, lectin-glycoprotein association prevents Golgi exit of folding intermediates. Although binding of most substrates to CNX/CRT appears to be exclusively glycan-mediated, there is evidence to suggest that both factors may act as classic chaperones, i.e. associate with proteins independently of their N-glycosylation or in the presence of glucosidase inhibitors (Danilczyk et al. 2001; Mizrachi et al. 2004; Wanamaker et al. 2005; Ihara et al. 1999; Saito et al. 1999). The binding period is determined by the lag between the trimming of glucose residues 2 and 3 by ER glucosidase II, which is the rate-limiting step in the deglycosylation process (Daniels et al. 2003; Deprez et al. 2005; Wang et al. 2005), allowing for an increased half-life of the monoglucosylated form of a glycoprotein in the ER and therefore increasing the time period in which it can acquire the native conformation. Following the removal of the last  $\alpha$ 1,3-linked glucose residue, the substrate glycoprotein is released from CNX/CRT (Hebert et al. 1995). It then binds to UDP-glucose:glycoprotein glucosyltransferase (UGGT), a key enzyme in the quality control pathway. UGGT acts as a folding sensor, identifying traits of a misfolded protein such as exposed hydrophobic patches (Sousa et al. 1995; Totani et al. 2009). If such traits are found, the asparagine-linked glycan on the substrate glycoprotein is reglucosylated by UGGT, allowing for an additional round of binding to CNX/CRT. If the substrate molecule is not reglucosylated by UGGT, it becomes subject to COPII-mediated transport to the Golgi apparatus for further processing and secretion. Reglucosylation is required for certain slow-folding glycoproteins (Pearse et al. 2008), but most proteins are able to complete folding in one round of association with CNX/CRT (Solda et al. 2007).

The repeated rounds of folding-deglycosylation-reglucosylation are termed the CNX/CRT cycle. It constitutes the central pathway involved in ER quality control in eukaryotic cells. The cycle does not exist in *Saccharomyces cerevisiae*, although in *S. pombe* a CNX-like

molecule is present (reviewed in Parodi 1999). As evident from the molecular mechanism of the CNX/CRT cycle, the key enzymes involved in the quality control pathway are glucosidase II and UGGT. Working in tandem, they confer the “folding status tag” onto the substrate glycoprotein by employing the “glucose code” (G=2: nascent newly synthesised glycoprotein; G=1: folding in progress; G=0: folding terminated) (Jakob et al. 1998). The concept of such glycan-based code could be further expanded to include other glycan forms and their corresponding interactors, i.e. lectins and enzymes (Aebi et al., 2010; Fig. 1.3).

Glucosidase II is an enzymatically unique enzyme, essentially catalysing two independent reactions (hydrolysis of the  $\text{I}\alpha\text{1—3Glc}$  and  $\text{Glc}\alpha\text{1—3Man}$  bonds) at different rates, enabling and then terminating the association of folding intermediates with CNX/CRT. A soluble heterodimer, the enzyme consists of a larger  $\alpha$ -subunit, displaying homology to the hydrolase 31 family and proposed to possess the catalytic activity of the enzyme, and the  $\beta$ -subunit, originally identified as a substrate of protein kinase C (D'Alessio 1999; Sakai et al. 1989). Subsequent studies have proposed the role for the  $\beta$ -subunit in ER retention of the catalytic subunit (Trombetta et al. 1996), as well as increasing the stability (Pelletier et al. 2000) and conferring substrate specificity (Watanabe et al. 2009).

The importance of UGGT in quality control is determined by its remarkable ability to selectively glucosylate misfolded/incompletely folded glycoproteins, as well as multimeric complexes that have failed to achieve the required quaternary structure. This is achieved by the association of UGGT with hydrophobic patches of amino acid side chains normally buried within the correctly folded globule (Sousa et al. 1995; Totani et al. 2009). The recognition is mediated by the large N-terminal region of UGGT (approx. 80% of the molecule). The C-terminal region displays homology with other glycosyltransferases and is believed to be responsible for the  $\text{Ca}^{2+}$ -dependent glucosylation reaction.

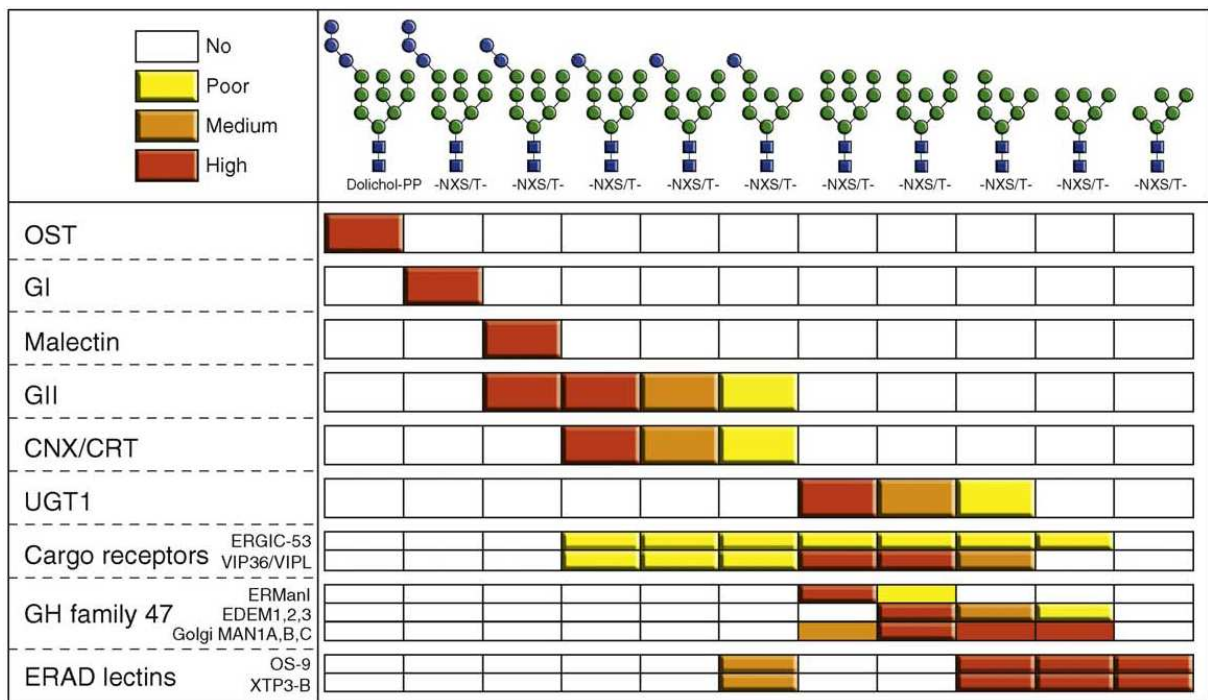


Fig. 1.3. **Oligosaccharide preferences of various ER-resident enzymes and lectins.** A colour code shows arbitrary strength of affinities/activities of the given sugar modifying/binding protein (from white (no affinity/activity) to red (higher affinity/activity)). Adapted from Aebi et al., 2010.

As previously established *in vitro* and more recently demonstrated in yeast, glucosidase II displays decreased activity against demannosylated substrates (Stigliano et al. 2011). However, in the case of UGGT, the only requirement for the glycan substrate appears to be the availability of the acceptor D1 mannose residue, with no preference for extensively demannosylated glycans (Stigliano et al. 2011). It is therefore tempting to speculate that progressive demannosylation of substrate glycoproteins leads to increased half-lives of their monoglucosylated forms, perhaps increasing the chances of slowly folding intermediates to acquire native conformation after the “default” time window of CNX/CRT binding appears to be insufficient. However, progressive time-dependent demannosylation has also been implicated in the eventual removal of the misfolded substrate from the CNX/CRT cycle and targeting of the glycoprotein for ER-associated degradation.

For most proteins, the CNX/CRT cycle ensures correct folding of the substrate glycoproteins exiting the endoplasmic reticulum. However, genetic mutations, translational errors or various

cellular stress conditions can cause certain proteins to become terminally misfolded. Given the extremely high protein concentration in the ER (>300 mg/ml), essentially creating an aggregate-prone environment in a compartment specialised for protein folding, it is vital that such terminally defective proteins are disposed of promptly. Their degradation occurs via several mechanisms. The focus of the current work has been drawn to ER-associated degradation (ERAD), which constitutes the major, specialised pathway for the disposal of terminally misfolded proteins accumulating in the lumen of the endoplasmic reticulum. Additional mechanisms have been described. Autophagy appears to be one of the major non-proteasomal pathways for degradation of misfolded ER proteins (Kruse et al. 2006a; Kruse et al. 2006b). “Overflow degradation” via the secretory pathway/vacuole (Kruse et al. 2006c; Spear et al. 2003) occurring when ERAD is saturated, has been demonstrated in yeast. A similar pathway based on rerouting of incompletely assembled multimeric complexes into the lysosome has been shown to operate under normal conditions in eukaryotes, exemplified by rerouting of T-cell receptors lacking the limiting  $\zeta$ -subunit into the lysosome via the secretory pathway (Minami et al. 1987). Several other cases of proteasome-independent proteolysis have been demonstrated and are discussed in more detail below (reviewed in Schmitz 2004).

Processing by glucosidases and the CNX/CRT cycle, commencing cotranslationally or immediately after protein biosynthesis and glycosylation by OST, ensure ER retention of folding intermediates before they acquire the native conformation. Folded, deglycosylated glycoproteins no longer associate with the quality control machinery, allowing for their export into post-ER compartments.

## **ER-Golgi transport**

While a fraction of ER-targeted proteins ultimately reside in the ER, most are exported via vesicular transport to the ER-Golgi intermediate compartment (ERGIC) and the Golgi apparatus (reviewed in Dancourt et al. 2010). Although evidence of non-conventional protein

export exists (Reiterer et al. 2008), the majority of secretory proteins reported in the literature appear to exit the ER in a coat protein complex II (COPII)-dependent manner. COPII budding machinery is organised at specialised ER exit sites (Orci et al. 1991; Bannykh et al. 1996; Rossanese et al. 1999) and consists of two principal protein complexes, Sec23-Sec24 and Sec13-Sec31. Formation of a COPII-coated vesicle *in vitro* requires the presence of these two complexes together with an additional initiation factor, the Sar1p GTPase (Matsuoka et al. 1998). Sec24p is known to contain several substrate recognition sites that bind to a diverse set of sorting signals on transmembrane proteins (Miller et al. 2003). However, soluble luminal cargo proteins cannot be recognised by the COPII subunits directly. Export of such proteins can occur via one of the two mechanisms: receptor-mediated (active) or bulk-flow (passive), depending on the secretory protein and cell type (reviewed in Barlowe 2003). The former depends on transmembrane adaptor proteins that provide the linkage to the COPII machinery. The latter relies on simple diffusion of secretory proteins into the COPII vesicles.

Several sorting receptors have been identified in the ER-Golgi export pathway. ERGIC53 is an ER/Golgi intermediate compartment-localised mannose-specific lectin interacting with both high-mannose type N-linked carbohydrates and protein motifs of cargo proteins (Appenzeller-Herzog et al. 2005; Itin et al. 1996). Other examples of lectins involved in ER-Golgi transport include VIP36 and VIPL. Interestingly, these two receptors display strong affinity for deglycosylated forms of high-mannose type glycans (Kamiya et al. 2008; Fig. 1.3), suggesting that glucose trimming might not only be required for the release of glycoproteins from the CNX/CRT cycle, but can also directly contribute to their ER-Golgi trafficking. Other cargo receptors in the ER-Golgi pathway include proteins of the p24 family and multispinning transmembrane proteins of the Erv group (reviewed in Dancourt et al. 2010). Deletion of receptors from either of the aforementioned groups typically results in the accumulation of specific secretory cargo in the ER, whereas traffic of other cargo proteins remains unaffected.

Whether cargo receptors themselves participate in the quality control of secretory proteins by selectively associating with correctly folded cargo is not completely understood. Several studies indicate preferential binding of ERGIC53 (Appenzeller-Herzog et al. 2005) and receptors of the Erv family (Otte et al. 2004; Dancourt et al. 2009) to proteins in the native conformation. However, it is also evident that for chimeric misfolded proteins, a COPII export signal can “override” ER retention mechanisms, whether the latter are primarily determined by the cargo receptors or the ER-resident quality control machinery (Kincaid et al. 2007). It is therefore likely that folding-dependent ER retention is not ensured by one particular mechanism but rather depends on a balance of factors which could vary between individual secretory proteins.

Multiple rounds of transfer between the ER and Golgi require a continuous supply of proteins (COPII and associated factors, receptors, chaperones) and lipids. A robust system of COPI-dependent retrograde vesicular transport exists to mediate retrieval of the machinery required for COPII traffic. COPI vesicles are assembled in the Golgi/ERGIC membranes when a membrane-bound GTPase Arf1 recruits seven subunits of the coatamer (Ostermann et al. 1993). COPI subunits recognise defined retrograde sorting signals on transmembrane proteins such as the dilysine motif (Cosson et al. 1994). As in the case with COPII transport, additional receptors are involved in cargo sorting for COPI-mediated transport. Soluble as well as some type II transmembrane proteins terminating in the short peptide KDEL (or related sequences) are recognised by the KDEL receptor (Semenza et al. 1990; Lewis et al. 1990). Rer1p is another factor of retrograde retrieval, recognising sorting motifs in the transmembrane domain of specific cargo (Nishikawa et al. 1993; Sato et al. 2001).

COPI-mediated retrograde transport is not only important for the retrieval of the anterograde machinery. It also maintains ER homeostasis by preventing secretion of luminal ER-resident proteins exiting the compartment by bulk flow (reviewed in Pelham 1996). For example, mutations in the KDEL receptor cause ER proteins such as BiP to be secreted. Additionally,

cycling between the ER and Golgi has been demonstrated for misfolded proteins, and several Golgi factors have been suggested to influence ER-associated degradation. The role of post-ER compartments and retrograde transport in ERAD and quality control are discussed below.

## **Processing of N-glycans in the Golgi apparatus**

The deglycosylated structure of the asparagine-linked glycan remaining after trimming by glucosidases in the endoplasmic reticulum is subject to the action of several ER- and Golgi-localised mannosidases (Taylor et al. 2006). The role of ER mannosidase I (MAN1B1) in “productive” demannosylation (as opposed to demannosylation leading to ERAD targeting – discussed below) is not entirely clear. However, it is likely that the enzyme does to some extent participate in the trimming of N-glycans on correctly folded glycoproteins as well as misfolded ones. Nevertheless, the bulk of demannosylation occurs following transport of folded glycoproteins into the Golgi apparatus, where the biosynthetic glycosyltransferase-mediated reactions resulting in a great variety of N-linked glycans also occur (reviewed in Kornfeld et al. 1985). As a general rule, some or all of the four  $\alpha$ 1,2-linked mannose residues are removed by cis-Golgi localised mannosidases IA (MAN1A1), IB (MAN1A2) and IC (MAN1C1). In some cases, N-linked glycans remain in this state and are termed high-mannose oligosaccharides. In other cases, they are processed further into complex or hybrid structures. Complex glycans are built on a core of three mannose and two N-acetylglucosamine residues. Two additional  $\alpha$ 1,3- and  $\alpha$ 1,6-linked mannose residues (branches A and B) are removed by Golgi mannosidase II (MAN2A1). The resulting core is typically modified by the addition of one or more GlcNAc residues to each arm, followed by elongation with galactose and N-acetylneuraminic acid, resulting in bi-, tri- or tetra-antennary glycans (in rarer cases, structures containing five or more branches are synthesised). Hybrid structures are generated when one of the arms of the core glycan is incompletely processed by Golgi mannosidases. In this case, the fully demannosylated arm is processed into a complex

form, while the other one remains in the high-mannose state.

Many variations of the described processing reactions occur in cells, resulting in a great diversity of the end N-glycans. It is estimated that there may be as many as 1000 different types of asparagine-linked oligosaccharides on mammalian glycoproteins (Kornfeld et al. 1985). Moreover, many proteins are heavily glucosylated, bearing 10 or more different N-glycans. In most cases, each individual glycoprotein exists as a heterogeneous population of common polypeptides bearing different glycans. These glycoprotein variants are termed glycoforms. The final glycosylation pattern of each particular protein is determined in part by the primary structure of the polypeptide itself, and in part by the expressing species/tissue/cell type. Whereas in extreme cases specific glycosylation sites can require a strictly defined glycan, for other proteins a general requirement for a particular type of glycan (e.g., bi-antennary or hybrid) is more common.

Removal of glucose residues from the C arm of the nascent N-linked oligosaccharide (see Fig. 1.1) is critical for the oligosaccharide maturation process. Inhibition of ER glucosidases by castanospermine (CST) results in the escape of triglucosylated glycoproteins from the ER. In this case, the processing of asparagine-linked glycans is limited to the removal of  $\alpha$ 1,2-linked mannose residues on the A and B arms of the oligosaccharides (Gross et al. 1986). However, in mammalian cells this is generally overcome by an alternative deglycosylation route that operates in the ERGIC and Golgi. This pathway is mediated by endo- $\alpha$ -mannosidase (MANEA) (Anumula et al. 1983; Lubas et al. 1987; Lubas et al. 1988; Moore et al. 1990). Endomannosidase cleaves the bond between the D1 and C mannose residues, removing the glucosyl cap together with the glucose-substituted mannose. It is capable of processing glucosylated oligosaccharides with one to three glucose residues. In the absence of glucosidase inhibitors, endomannosidase is believed to provide a backup deglycosylation mechanism for glycoproteins that escape the ER without complete processing by glucosidases (Weng et al. 1996).

The role of N-glycan processing of in the Golgi apparatus is not limited to the biosynthetic pathway. There is some evidence that shuttling of misfolded proteins between ER and Golgi routinely occurs in eukaryotic cells and in some cases precedes ER-associated degradation (Hsu et al. 1991; Hammond et al. 1994; Yamamoto et al. 2001). Processing by cis-Golgi-localised mannosidases is known to influence ERAD (Hosokawa et al. 2007), and multiple authors have suggested that Golgi enzymes might play an important role in glycoprotein disposal. Post-ER processing of ERAD substrates has been addressed in the current work, and the implications for the involvement of the Golgi apparatus in the degradation of glycoproteins are discussed in more detail below.

# ER-associated degradation

## Targeting of glycoproteins for ER-associated degradation

ERAD targeting is a complex part of quality control in the endoplasmic reticulum. The process is better understood for glycoproteins, but in multiple instances non-glycosylated misfolded proteins have been shown to utilise the same factors *en route* to degradation. It is however clear that N-linked glycosylation is an important determinant of ERAD, providing a flexible structural substrate for manipulation of the rates and pathways of glycoprotein disposal.

### ***ER mannosidase I/EDM model***

The original model of targeting glycoproteins for ERAD attributed the major role in the process to ER mannosidase I (MAN1B1) (reviewed in Spiro 2004). The latter is an  $\alpha$ 1,2-specific membrane-bound enzyme, which displays a substrate preference for the D2 mannose residue (Bischoff et al. 1986). It was generally accepted that the slow action of ER mannosidase I provided the ultimate “time trigger” for the CNX/CRT cycle, i.e. the signal required to terminate the futile folding attempts and transfer the misfolded glycoprotein to the ERAD machinery (reviewed in Spiro 2004). Overexpression of the enzyme led to an increased rate of degradation of  $\alpha$ 1-antitrypsin null-Hong Kong variant (NHK), a terminally misfolded mutant version of  $\alpha$ 1-antitrypsin commonly employed to study ERAD, and even led to degradation of wild-type  $\alpha$ 1-antitrypsin (Wu et al. 2003; Hosokawa et al. 2003b). On the other hand, treatment with kifunensine, an inhibitor of ER mannosidase I, as well as deoxynojirimycin, a more broad mannosidase inhibitor, were shown to decrease the rates of ERAD (Yang et al. 1998; Su et al. 1993; Liu et al. 1999; Liu et al. 1997; Hosokawa et al. 2003b; Tokunaga et al. 2000), although, interestingly, the ability of mannosidase inhibitors to

increase half-lives of misfolded proteins appeared to be substrate-dependent (Yang et al. 1998; Mancini et al. 2003). Similarly, deletion of ER mannosidase I in yeast slowed down the degradation of mutant carboxypeptidase Y (CPY\*) (Knop et al. 1996).

At least two models of the exact mechanism of ER mannosidase I-mediated ERAD enhancement have been proposed. Liu et al. suggested that demannosylation enhances interaction between the misfolded protein and CNX/CRT by increasing the half-life of the monoglucosylated form of the N-glycan (Liu et al. 1999). Interaction with CNX/CRT was shown in this work to be required for proteasomal degradation of mutated  $\alpha$ 1-antitrypsin, as well as for the dependence of its degradation on mannosidase activity. However, in other instances, CNX/CRT interaction protected non-mutant proteins from ERAD (Moore et al. 1993; Hebert et al. 1996; Kearse et al. 1994), and the mannosidase-dependent ERAD enhancement appeared to be uncoupled from the interaction with the chaperones (Wang et al. 2000).

The second model, which was until recently accepted by most authors in the field, involved a specific glycan signal generated by ER mannosidase I and subsequently recognised by an ER lectin distinct from CNX/CRT (Jakob et al. 1998). EDEM factors (ER degradation enhancing mannosidase-like protein), a group of ER lectins carrying the family 47 glycosidase domain, were proposed to act as such receptors (reviewed in Spiro 2004).

EDEM was first identified as a membrane-bound factor upregulated during ER stress, binding to misfolded glycoproteins and accelerating their degradation, while itself not possessing enzymatic activity as evidenced by *in vitro* experiments (Hosokawa et al. 2001). siRNA-mediated depletion of endogenous EDEM had the effect opposite of that observed following its overexpression (Molinari et al. 2003). EDEM-mediated acceleration of glycoprotein degradation was prevented by inhibiting ER mannosidase I with kifunensine. A yeast homologue of EDEM, Htm1p, was identified independently and proposed to possess a similar

function (Nakatsukasa et al. 2001; Jakob et al. 2001). Based on the kifunensine dependence of EDEM-stimulated ERAD acceleration, as well as the mannosidase homology of the protein, EDEM was postulated to be the receptor for ER mannosidase I-trimmed oligosaccharides, namely M8 isomer B.

Several mechanistic studies explored the involvement of EDEM in the CNX/CRT cycle. Overexpression of EDEM was shown to decrease the half-life of the glucosylated form of a mutated glycoprotein (this effect was reversed following EDEM knockdown), which essentially correlated with the period of interaction between the glycoprotein and CNX. Quite strikingly, glycoproteins undergoing productive folding were not affected (Molinari et al. 2003). A separate report pointed to a physical interaction between CNX and EDEM and provided evidence for direct transfer of misfolded substrates from the former to the latter (Oda et al. 2003). Taken together, the studies by Molinari et al. and Oda et al. suggested that, by binding to calnexin and associating with both sugar and misfolded protein determinants, EDEM sequesters misfolded ERAD substrates from the CNX/CRT cycle, preventing their reglucosylation by UGGT. Trimming by ER mannosidase I favoured the interaction of a protein with EDEM since, according to Molinari et al., demannosylated glycans are poor substrates for UGGT.

The model in which EDEM served as the link between glycan processing and protein degradation through the ubiquitin-proteasome system, became widely accepted. The nature of EDEM specificity for misfolded proteins, however, was not entirely clear. It has been shown that EDEM selectively binds misfolded substrates and prevents them from aggregation, suggesting a chaperone function (Hosokawa et al. 2006). In an elegant interpretation, the authors suggest that binding of misfolded proteins to EDEM may parallel that of productively folded ones to calnexin, maintaining “retrotranslocation competence” of proteins destined for degradation and preventing their release into the ER lumen in an intrinsically unstable free form.

Two homologues of the originally identified EDEM1 were demonstrated to be present in mammalian cells: EDEM2 (Mast et al. 2005; Olivari et al. 2005) and EDEM3 (Hirao et al. 2006). The effect of EDEM2 on glycoprotein degradation appeared to be highly similar to that of EDEM1 (originally termed EDEM). As in the case of EDEM1, EDEM2 was induced upon ER stress and following overexpression, accelerated degradation of mutant glycoproteins but not unglycosylated substrates. Similarly to EDEM1, EDEM2 did not show any enzymatic activity *in vitro*. Surprisingly though, in the report by Olivari et al. both EDEM1 and EDEM2 were identified as soluble luminal proteins in HEK293 cells, which contradicts the original work carried out in Cos cells (Hosokawa et al. 2001). Likewise, EDEM3 was shown to be an ER luminal protein accelerating NHK ERAD in a proteasome-dependent manner (Hirao et al. 2006). However, in contrast to EDEM1 and EDEM2, EDEM3 enhanced mannose trimming of NHK and also total cellular glycoproteins, an activity dependent on the integrity of the mannose homology domain. Demonstration of the enzymatic activity of EDEM3 led the authors to propose that the mechanism of ERAD acceleration by the lectin is different from that of its homologues.

### ***Extensive demannosylation/OS-9 model***

The demonstration of enzyme activity displayed by one of the EDEM homologues was one of the first reports in the field to challenge the ER mannosidase I-centric model of ERAD targeting, where EDEM acted as an enzymatically inert acceptor of the specific M8B signal generated by the slow-acting ER mannosidase I on the proteins remaining bound to CNX/CRT after a series of futile folding attempts. A series of new developments in the studies on glycoprotein disposal have gradually revised and expanded the initial scheme proposed for ERAD targeting (reviewed in Lederkremer 2009).

- 1) Even prior to the report on EDEM3, several studies indicated that ERAD substrates can be trimmed to M5—M6 prior to degradation (Hosokawa et al. 2003a; Frenkel et

- al. 2003). The extent of such trimming did not correlate with ER retention alone but appeared to be specific for proteins that were subject to degradation (Frenkel et al. 2003).
- 2) Lack of D2 mannose (i.e., M8 isomer B) was shown to be insufficient for ERAD in yeast (Quan et al. 2008; Clerc et al. 2009) and mammalian cells (Ermonval 2001). In the study by Ermonval, following transfer of a truncated (M5) oligosaccharide to proteins in mutant cells, the removal of the D1 mannose residue was demonstrated to still be required for ERAD.
  - 3) ERAD substrates were shown to be targeted to a specific location within the ER (Kamhi-Nesher et al. 2001), which the authors viewed as a membrane-bound sub-ER compartment. The transport of substrates to this region preceded the final stages of mannose trimming, and mannosidase inhibition caused an accumulation of ERAD substrates in a similar location but in a punctate pattern (Frenkel et al. 2003). CNX and CRT (but not UGGT) were also shown to be transported to the same sub-ER region, termed ER quality control compartment (ERQC) by Lederkremer and colleagues (reviewed in Lederkremer, 2009).
  - 4) Accumulation of ER Man I in ERQC allows for a high local concentration of the enzyme and enables extensive mannose trimming (Avezov et al. 2008). Additionally, EDEM3 was shown to possess enzymatic activity (Hirao et al. 2006), and furthermore Htm1, the yeast homologue of EDEM1, was implicated in the removal of the D3 mannose residue from glycoproteins (Quan et al. 2008, Clerc et al. 2009), an activity that, at least in some cases, is required for ERAD (see below). Mammalian EDEM1 itself was later shown to trim the D3 residue from misfolded proteins (Hosokawa et al. 2010). There is also evidence of the involvement of Golgi enzymes in the processing of misfolded proteins, which is discussed below.

- 5) The discovery of Yos9p (in yeast) and OS-9/XTP3-B (in mammalian cells), ER lectins required for ERAD, and the investigation of their lectin affinities led to the proposal of a glycan signal different from the previously postulated M8B. Namely, Yos9p (Quan et al. 2008) and OS-9 (Hosokawa et al. 2009, Mikami et al. 2010) appear to be selective for glycans with exposed  $\alpha$ 1,6-linked mannose residues (see Fig. 1.3).
- 6) Yos9p was found to associate with Hrd3p and Hrd1p, the components of the ubiquitin-proteasome machinery in the ER membrane (Denic et al. 2006; Gauss et al. 2006). In mammalian cells, this is paralleled by the interaction between OS-9/XTP3-B and the complex containing SEL1L (homologue of Hrd3p) and HRD1 (homologue of Hrd1p) (Christianson et al. 2008). The discovery of these complexes in yeast and mammalian cells has provided the first direct link between the glycan-mediated ER quality control and the ubiquitin-proteasome system.

Yos9p is a product of the YOS9 gene, the yeast homologue of OS-9, a gene found in amplified chromosomal DNA from human osteosarcoma cells (Su et al. 1996). Yos9p, as well as its mammalian homologues OS-9 and XTP3-B, share the mannose-6-phosphate receptor homology (MRH) domain (Whyte et al. 2001; Munro 2001). Multiple publications have demonstrated the requirement for Yos9p in the degradation of glycoproteins with luminal defects (ERAD-L) in yeast (Buschhorn et al. 2004; Bhamidipati et al. 2005; Kim et al. 2005; Szathmary et al. 2005). An intact MRH domain was required for the degradation of glycoproteins (Bhamidipati et al. 2005). However, although degradation of non-glycosylated proteins was not affected by the deletion of Yos9p, the lectin was found to bind misfolded proteins independently of their glycosylation and the integrity of the MRH domain (Bhamidipati et al. 2005). It is unclear what function, if any, does such binding serve.

Two principal findings elevated the assumed role of Yos9p and OS-9/XTP3-B to being the key determinants in glycoprotein ERAD in yeast and mammalian cells, respectively. Firstly,

the lectins were shown to be part of the membrane-bound complexes of Hrd3p-Hrd1p in yeast (Denic et al. 2006; Gauss et al. 2006) and SEL1L-HRD1 in mammalian cells (Christianson et al. 2008), suggesting a direct connection between Yos9p/OS-9/XTP3-B-mediated glycan recognition and subsequent ubiquitination and degradation of misfolded proteins. Secondly, the investigation of the lectin properties of Yos9p revealed a specificity for  $\alpha$ 1,6-linked mannose residues. In particular, it has been demonstrated that the removal of the D3 mannose is critical for the degradation of certain misfolded glycoproteins in yeast (Quan et al. 2008; Clerc et al. 2009). Similar specificity was reported for OS-9 (Hosokawa et al. 2009; Mikami et al. 2010). The cleavage of mannose D3 in yeast has been shown to be dependent on Htm1p, a yeast homologue of mammalian EDEM factors. Indeed, prior studies have shown that deletion of Htm1p decreases the interaction between Yos9p and CPY\* (Szathmary et al. 2005). In human cells, overexpression of ER mannosidase I but not EDEM3 reduced the dependence of NHK degradation on OS-9, suggesting that processing by the former enzyme may represent a separate pathway for glycoprotein degradation. Importantly, the observed dependence of ERAD on OS-9 (as evidenced by siRNA-mediated knockdown experiments) following ER mannosidase I overexpression was partially restored after a 2-hour chase, supporting the fact that the enzyme is capable of more extensive demannosylation than the removal of a single D2 mannose residue (Hosokawa et al. 2009).

If the removal of mannose D3 is the primary glycan signal required for ERAD, what enzymes are responsible for generating this signal? Several candidates have been identified. In particular, the newly discovered enzymatic activity of EDEM proteins (see above) is likely to contribute to the process in mammalian cells in the same way as Htm1p does in yeast. ER mannosidase I, previously believed to only cleave a single D2 mannose, has been later shown to undergo compartmentalisation in the ERQC, allowing for very high local concentrations and extensive trimming to M5—M6. Such trimming would remove the substrate glycoproteins from the reglucosylation cycles, but also expose an  $\alpha$ 1,6-linked mannose which

could be recognised by OS-9/XTP3-B. Indeed, substrate association with XTP3-B (but not EDEM1) was inhibited by kifunensine or knockdown of ER mannosidase I (Groisman et al. 2010). Finally, Golgi mannosidases IA, IB and IC are known to specifically trim the D3 mannose, making them attractive candidates for the “marking” of glycoproteins for disposal.

### ***Recognition of misfolded domains***

Although the glycan-based signal for ERAD has been demonstrated to be required for glycoprotein degradation in multiple studies, it is clear that the quality control system must also recognise polypeptide-based signals, i.e. misfolded domains (Xie et al. 2009). Firstly, this is the only option for the disposal of non-glycosylated misfolded proteins. Secondly, aberrant domains can be located within the ER membrane or in the cytosolically exposed parts of the polypeptide. Proteins bearing such non-lumenal lesions must also be degraded (ERAD-M and ERAD-C: see below). Multiple branches of ER quality control must therefore cooperate to ensure correct folding of all types of secreted proteins. The connection between glycan- and protein-based recognition of misfolded glycoproteins is not entirely understood. One important question is whether an exclusively glycan-based signal could be sufficient for ER-associated degradation. Although manipulations such as overexpression of ER mannosidase I or inhibition of ER glucosidases can lead to ERAD of non-mutant glycoproteins, they are likely to cause such effects through preventing entry or prematurely terminating the CNX/CRT cycle and therefore compromising substrate folding. Another question concerns the mechanisms of folding-based quality control underlying the generation of the glycan-based ERAD signal discussed in the previous section.

In a recently proposed model for yeast quality control, enzymatic activity of Htm1p is controlled by its interaction with the protein disulphide isomerase Pdi1p (Gauss et al., 2011). In this model, Htm1p demannosylates substrate glycoproteins only in complex with Pdi1p, thus generating an ERAD signal. While Pdi1p assists folding of substrate glycoproteins

irrespective of their glycosylation status, stochastic interaction of Htm1p with Pdi1p favours marking of slow-folding proteins for ERAD. Whether or not a similar model could be applicable to higher eukaryotes utilising different folding mechanisms (i.e., a functional CNX/CRT cycle), is insofar unknown.

Interactions with misfolded protein domains have been demonstrated for the proteins of downstream degradation machinery. In particular, Hrd3p, a key component of the Hrd1 ubiquitin ligase complex, was shown to form a luminal quality control scaffold by interacting with Yos9p responsible for the glycan-based substrate recognition, but also by binding to misfolded proteins directly (Denic et al. 2006; Gauss et al. 2006). Hrd3p has been known for some time to be essential for the stability of the Hrd1p (Gardner et al. 2000), the core component of the HRD1 complex involved in the retrotranslocation and ubiquitination of substrate molecules, but the precise role of Hrd3p in protein degradation remained unclear (Plempner et al. 1999). Its importance as a link between the luminal quality control and the ubiquitin-proteasome systems has been revealed by establishing its interaction with Yos9p. However, binding of misfolded proteins to Hrd3p did not depend on their glycosylation or lectin association (Gauss et al. 2006; Stanley et al. 2011). Taken together, these findings suggested that a bipartite signal is required for the degradation of luminal ERAD glycoprotein substrates (Bhamidipati et al., 2005). The primary signal involves demannosylation of the B branch of N-linked oligosaccharides (in the case of Htm1p, this could be triggered by its association with Pdi1p as shown by Gauss et al.) which then promotes binding of the substrate to Yos9p (Cormier et al. 2009; Groisman et al. 2010). The lectin delivers the glycoprotein to Hrd3p, which selectively bind misfolded proteins and trigger their retrotranslocation and ubiquitination through the association with the Hrd1p ubiquitin ligase.

In mammalian cells, SEL1L was identified as a homologue of Hrd3p upregulated during ER stress (Kaneko et al. 2003) and required for protein dislocation during ERAD (Mueller et al. 2006). SEL1L is believed to integrate glycan-based recognition into the downstream

degradation pathways by interacting with OS-9/XTP3-B (Christianson et al. 2008) in a manner similar to that observed in yeast for Hrd3p/Yos9p. However, no evidence for direct interactions of SEL1L with misfolded substrates is available to date.

The ubiquitin ligase protein complexes are attractive candidates for triggering ERAD of misfolded proteins, whether or not they require glycan-based targeting to precede the final “commitment” stage. The yeast HRD complex is known to associate with both ERAD substrates and stable proteins, but only mediate ubiquitination of the former (Gardner et al. 2001). It is therefore conceivable that the ubiquitination machinery acts as a “gatekeeper”, making the final assessment of the substrate's folding status prior to its degradation. Additionally, in mammalian cells luminal chaperones BiP and GRP94 have been implicated in delivering misfolded proteins to the Hrd1 complex in association with OS-9/XTP3-B (Christianson et al. 2008), as well as in the disposal of non-glycosylated substrates (Skowronek 1998; Okuda-Shimizu et al. 2007). Similarly, in yeast cells, Kar2p, a homologue of BiP, was shown to bind to the Yos9p/Hrd3p/Hrd1p complex, suggesting a role in substrate delivery to the degradation machinery (Denic et al. 2006). Finally, chaperones of the ERdj family have been demonstrated to facilitate degradation of both glycosylated (Nishikawa et al. 2001; Ushioda et al. 2008) and non-glycosylated misfolded proteins (Dong et al. 2008; reviewed in Otero et al. 2010).

## **Late stages of ERAD: the ubiquitin-proteasome system**

The ultimate outcome of ER-associated degradation is the proteasome-mediated disposal of polypeptides. For this, proteins are subjected to ubiquitination through a system of ubiquitin-conjugating enzymes and ubiquitin ligases. Although proteolysis of various substrates occurs independently of their origin and localisation, the process of marking proteins with ubiquitin chains can occur via a number of mechanisms mediated by distinct membrane-associated complexes. There appear to exist at least three general pathways of ERAD, the employment

of which depends on the physical location of a misfolded domain. These pathways are best characterised in yeast and are termed ERAD-L (proteins with misfolded luminal domains, including all soluble luminal proteins), ERAD-M (proteins with misfolded transmembrane domains) and ERAD-C (proteins with misfolded cytosolic domains) (Huyer et al. 2004; Vashist et al. 2004; Carvalho et al. 2006). For glycoproteins, oligosaccharide-based sorting of misfolded glycoproteins from folding intermediates (see above) precedes the stage of “commitment”, when the substrate molecule is subjected to retrotranslocation and ubiquitination. Little is understood about the mechanism of selection of one degradation pathway over others. It has been suggested that a series of checkpoints directing misfolded proteins into distinct degradation pathways operates within the secretory pathway (Vashist et al. 2004). The checkpoints comprise the lumenally localised quality control system required for the surveillance of glycoproteins, as well as systems detecting cytosolic and membrane lesions on membrane proteins. These checkpoints are likely to act sequentially (Vashist et al. 2004) and extend beyond the ER. Indeed, for some misfolded proteins in yeast (typically luminal) ER-to-Golgi transport is required for degradation (Caldwell et al. 2001, Vashist et al. 2004). The system of checkpoints in the secretory pathway has been proposed to reach as far as the plasma membrane, as exemplified by the retrieval of misfolded proteins from the yeast plasmalemma for intracellular disposal (reviewed in Arvan et al. 2002).

An investigation of the factors involved in the degradation of substrates with various topology revealed that the convergent ERAD pathways are defined by distinct ubiquitin-ligase complexes (Carvalho et al. 2006). Ubiquitination is a universal eukaryotic pathway required for the destruction of proteins by the 26S proteasome. It involves tagging of protein substrates with ubiquitin, a small cytosolic polypeptide capable of forming linear or branched chains. Three enzymatic activities are required for ubiquitination: E1 (ubiquitin-activating), E2 (ubiquitin-conjugating or carrier) and the key activity, E3 ubiquitin ligase. In yeast, E3 ligase Hrd1p is the central component in both ERAD-L and ERAD-M. In the former case, substrate

degradation requires a near stoichiometric complex of Hrd1p with Hrd3p, Der1p and Usa1p (Carvalho et al. 2006). Limited data on ERAD-M suggest that proteins with intramembrane defects require Hrd1p and Hrd3p, but not the other components of the Hrd1 core complex. Targeting could either be assisted by Yos9p or other loosely associated factors, or occurring directly via interaction of the misfolded protein with Hrd1p. The requirement for additional factors by ERAD-L substrates could reflect the need for a more elaborate mechanism of retrotranslocation. The nature of the protein-conducting channel in ERAD-L has been a subject of some debate. This role has been originally attributed to the Sec61p channel which mediates cotranslational insertion of polypeptides into the ER lumen (Wiertz et al. 1996, Scott et al. 2008; Willer et al. 2008). However, some studies have disputed the involvement of Sec61p in retrotranslocation and have proposed alternative factors to mediate ER-to-cytosol transport of misfolded proteins. Der1p and Usa1p are attractive candidates for a role in the process because they are essential for ERAD-L (Carvalho et al. 2006). Participation of the mammalian Der1p ortholog Derlin-1 in ERAD (Ye et al. 2004, Lilley et al. 2004, Wahlman et al. 2007) supports the notion that Der1p/Derlin-1 is an important component mediating retrotranslocation. More recent data have even suggested that Hrd1p itself may form a protein channel capable of dislocating substrates (Carvalho et al 2010).

In contrast to ERAD-L and ERAD-M, the ERAD-C pathway in yeast uses a different ubiquitin ligase, Doa10p, which can also polyubiquitinate cytosolic and nuclear substrates. The three pathways converge at the Cdc48p ATPase complex which provides the energy required for retrotranslocation (Ye et al. 2001; Jarosch et al. 2002; Rabinovich et al. 2002). A similar role has been attributed to p97/VCP, a mammalian homologue of Cdc48p (Ye et al. 2001; Rabinovich et al. 2002). The mammalian p97/VCP complex consists of the core p97 AAA-ATPase and its heterodimeric ubiquitin-binding substrate-recruiting cofactor NPL4/UFD1 (Meyer et al. 2002; Ye et al. 2003). In yeast, Cdc48p interacts with Npl4p/Ufd1p (Hitchcock et al. 2001; Bays et al. 2001), homologues of mammalian NPL4/UFD1. Additional

interaction of Cdc48p with Ubx2p, a transmembrane protein linking the ATPase complex to the ubiquitin ligase complexes, has been demonstrated in yeast (Schuberth et al. 2005; Neuber et al. 2005; Wilson et al. 2006) (Fig. 1.4).

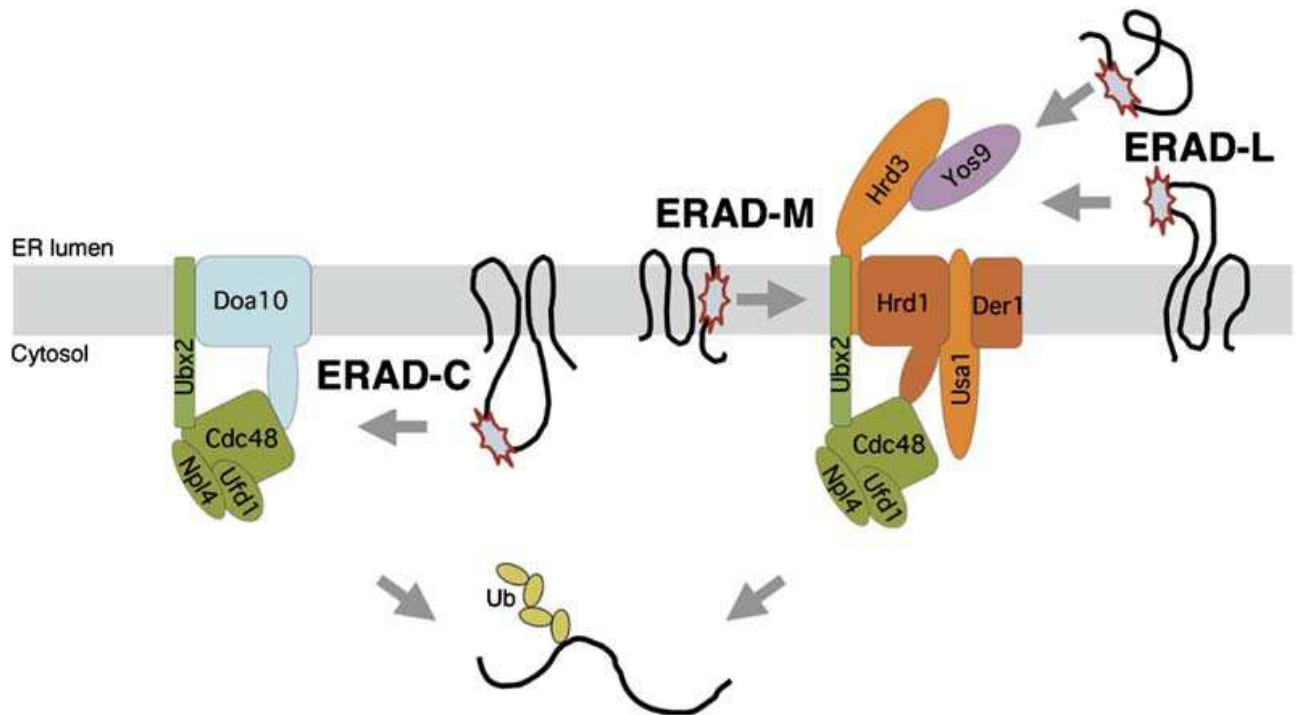


Fig. 1.4. **Convergent pathways of ER-associated degradation.** The scheme shows the ubiquitin-ligase complexes involved in the ERAD-L, -M, and -C pathways. Components in orange and green belong to the Hrd1p core and Cdc48p ATPase complexes, respectively. Stars show the location of the misfolded domain of a substrate. Ub is ubiquitin. Adapted from Carvalho et al. 2006.

Amongst the variety of cofactors and interaction factors of the Cdc48 ATPase complex, one is of particular interest for the current study, namely peptide:N-glycanase (PNGase, Png1p). The latter hydrolyses the  $\beta$ -aspartylglycosamine bond of N-linked glycoproteins/glycopeptides (Suzuki et al. 1993; Suzuki et al. 1994; Kitajima et al. 1995; Romisch 1997), leading to a deglycosylated polypeptide and a GlcNAc<sub>2</sub>-terminating free oligosaccharide. Soon after the discovery of PNGase in mammals, deglycosylation of glycoproteins was linked to their retrotranslocation from the ER as part of a sequence of events ultimately leading to proteasomal destruction – a process later termed ER-associated degradation (Wiertz et al. 1996). PNGase is currently hypothesised to be a part of a “glycoprotein degradation complex”

(reviewed in Suzuki 2007) which in mammalian cells links the ubiquitin ligase complex, the p97 complex, PNGase and several other factors including the putative retrotranslocation channel Derlin-1 (Katiyar et al. 2005) with the 26S proteasome, allowing for an entire sequence of reactions required for glycoprotein degradation (retrotranslocation, ubiquitination, deubiquitination, deglycosylation, protein hydrolysis) to occur at a single cellular location (Suzuki 2003). Deglycosylation is generally thought to occur prior to proteasomal degradation, although the reverse has been demonstrated as well (Kario et al. 2008). Proteasomal inhibition uncouples the two events and results in a transient accumulation of deglycosylated intermediates in the cytosol (Wiertz et al. 1996). Furthermore, following siRNA-mediated knockdown of PNGase, dislocated N-linked glycoproteins have been detected in the cytosol (Blom et al. 2004). Dislocation of glycoproteins to the cytosol is therefore not dependent on deglycosylation.

Following retrotranslocation and ubiquitination of misfolded proteins from the ER to the cytosol, the ubiquitin signal is recognised by the proteasome. Tetraubiquitin appears to be the minimum signal for efficient proteasome targeting (Thrower et al. 2000). Recognition is mediated by the subunits of the 19S regulator, Rpn10 and Rpn13. Other subunits mediate interaction with deubiquitinating enzymes such as UBP (reviewed in Jung et al. 2009). The 26S proteasome then catalyses the hydrolysis of the deubiquitinated polypeptide in an ATP-dependent manner.

## **Golgi involvement in glycoprotein degradation and quality control**

It has been known for some time that quality control in yeast is not restricted to the endoplasmic reticulum. Post-ER compartments mediate various mechanisms for identifying and degrading aberrant proteins in the secretory pathway. While some of these mechanisms rely on the retrieval of misfolded substrates back to the ER, followed by ER-associated degradation (Vashist et al. 2001, Hirayama et al. 2010), others employ alternative degradation

pathways such as rerouting of proteins into the vacuole (Chang et al. 1995; Hong et al. 1996; Li et al. 1999). In some cases, anterograde traffic is required for efficient degradation (Caldwell et al. 2001; Vashist et al. 2001). In particular, ER-to-Golgi transport has been shown to be essential for ERAD-L, i.e. degradation of proteins with luminal defects, whereas ERAD-C was independent of anterograde traffic (Vashist et al. 2001). When determinants of both ERAD-L and ERAD-C were fused together in a single substrate, the chimeric protein was efficiently degraded in cells defective in ER export. This observation suggested a system of sequential checkpoints to monitor protein folding (Vashist et al. 2004). In this model, ER-localised quality control machinery is incapable of identifying proteins with lumenally oriented misfolded regions. It can, however, target membrane proteins with cytosolically localised aberrations for degradation. In a somewhat contradictory report, it has been suggested that when misfolded proteins carry ER export signals, the cargo receptors compete with chaperones for the substrates (Kincaid et al. 2007) and therefore stimulate their transport to the Golgi apparatus, where they become subject to additional quality control checkpoints. In the latter study, luminal proteins were efficiently degraded without a requirement for anterograde transport, and their exposure to the hypothetical Golgi-localised quality control factors was therefore concluded to be merely a result of a competition between export and degradation systems in the ER.

Although the importance of transport between the ER and Golgi for protein degradation in yeast is clear, the exact mechanisms of traffic and, even more importantly, the nature of the post-ER quality control remain obscure. The latter appears to be capable of identifying conformational features distinct from those recognised by the ER-localised quality control (Wang et al. 2010). This, together with the fact that some of the proteins degraded via the vacuolar pathway can be functionally restored on the cell membrane through vacuolar sorting mutants, has led some authors to propose that Golgi-based quality control is more stringent than ER-based surveillance and sensitive to subtle structural aberrations (Li et al. 1999; Wang

et al. 2010).

Whether similar mechanisms operate in mammalian cells is controversial. It is not unlikely that with the dramatically increased complexity of quality control and stress response in the endoplasmic reticulum, the need for additional conformational surveillance has become redundant (Arvan et al. 2002). However, the effect of the Golgi apparatus on protein degradation has been demonstrated in multiple cases. As in yeast, in certain cases this is exemplified by retrograde retrieval of misfolded substrates to the ER, followed by ER-associated degradation (Hosokawa et al. 2007, Pan et al. 2011). Early indications also suggested rerouting of incompletely assembled/matured proteins at the post-Golgi level (Minami et al. 1987; Armstrong et al. 1990).

Retrieval of ERAD substrates from the Golgi apparatus has been proposed to be mediated by the KDEL receptor (Yamamoto et al. 2001). However, the process does not appear to be limited to ER-resident proteins. Additional cargo receptors have been implicated in the retrieval of secretory proteins. VIP36 has a characteristically Golgi substrate binding pH optimum and localisation and could therefore participate in the retrograde transport of misfolded proteins to the ER (Kamiya et al. 2008). Glycan-dependent binding to VIP36 (see Fig. 1.3) followed by Golgi-to-ER translocation has indeed been recently demonstrated for  $\alpha$ 1-antitrypsin (Reiterer et al. 2010). A similar role has been proposed for Bap31, a cargo receptor present in both ER and post-ER compartments (Ladasky et al. 2006). In general, binding of cargo receptors to glycans appears to be strongest for deglycosylated but not extensively demannosylated forms, as opposed to the binding of ER-lectins which display a preference for highly demannosylated glycans (see Fig. 1.3; reviewed in Aebersold et al., 2010). This trend highlights the importance of the “glycocode” in differential surveying of glycoproteins and targeting substrate molecules to their fates. Retrieval of misfolded proteins can also occur indirectly via interactions with molecular chaperones such as BiP/GRP78, which bear ER retention signals and can cycle between the ER and cis-Golgi (Hammond et al.

1994).

Amongst Golgi-resident enzymes that could potentially participate in ERAD, attention has been drawn primarily to mannosidases. Demannosylation of N-linked carbohydrates is a well-known factor of glycoprotein degradation in the ER (see above). The role of the early-Golgi class I mannosidases showing increased specificity for D1 and D3 mannose residues (Lal et al. 1998) is of particular interest in view of the recent reports on glycan-mediated ERAD targeting. Since the removal of the D3 mannose is suggested to be critical for glycoprotein ERAD, Golgi mannosidases are attractive candidates for participation in the hypothetical pathway of post-ER quality control. Additionally, trimming of the acceptor D1 mannose followed by retrograde transport to the ER would prevent the glycoprotein from reassociating with CNX/CRT. Indeed, overexpression of Golgi mannosidases IA, IB and IC has been demonstrated to accelerate ERAD of a misfolded luminal substrate,  $\alpha$ 1-antitrypsin NHK variant (Hosokawa et al. 2007). Interestingly, early reports suggested the involvement of Man9-mannosidase, which was initially believed to be an ER-resident enzyme, in extensive demannosylation of substrate glycoproteins (Roth et al. 1990; Bause et al. 1992; Bieberich et al. 1997). However, this enzyme was later shown to be identical to Golgi mannosidase IA (Lal et al. 1994; Bieberich et al. 1997).

## **Effect of glucosidase inhibition on ERAD**

Inhibition of ER  $\alpha$ -glucosidases is a technique widely used both in research and in therapy (reviewed in Dwek et al. 2002), and is of particular importance for the present work. Glucosidase inhibition leads to the failure of glycoproteins to enter the calnexin/calreticulin cycle, which leads to increased levels of protein misfolding, ER stress and ERAD (Pahl et al. 1995). Additionally, impaired deglucosylation may hinder the post-ER processing of N-linked oligosaccharides (Karlsson et al. 1993). However, in most cells endomannosidase provides a backup deglucosylation pathway, which compensates for the glucosidase blockage and

ensures the trimming of the  $\alpha$ 1,2-linked mannose residues D1 (by removing it *en bloc* with the glucosyl cap) and C (by exposing it to the action of Golgi mannosidases I) (Karaivanova et al. 1998). Endomannosidase-mediated deglucosylation, however, does not allow for the glycoprotein to reassociate with CNX/CRT since D1 mannose is required for reglucosylation by UGGT and hence for the interaction with the lectin-chaperones.

The ultimate impact of glucosidase inhibition on the secreted glycoproteins therefore primarily involves defective folding rather than defective glycosylation. The degree of misfolding correlates with the dose-dependent antiviral effect of glucosidase inhibitors (Jordan et al. 2002). N-butyldeoxynojirimycin, as well as other glucosidase inhibitors, has been shown to reduce infectivity of HIV, HBV, HCV, its surrogate model BVDV and other viruses dependent on the host folding and quality control pathways (Dwek et al. 2002). Evasion of the CNX/CRT cycle can also be beneficial for certain proteins such as  $\Delta$ F508-CFTR, the mutated version of the cystic fibrosis transmembrane conductance regulator, which is targeted for degradation in normal conditions, but escapes in a functional form following glucosidase inhibition (Norez et al. 2006).

The precise effect of glucosidase inhibition on the rates and mechanisms of ERAD is not entirely clear. Castanospermine (CST) was shown to promote/accelerate degradation of otherwise secreted (Moore et al. 1993) as well as misfolded proteins (Liu et al. 1999). In the case of otherwise correctly folded glycoproteins, some are affected, whereas others are not (Kearse et al. 1994). In the case of proteins that are predisposed for ERAD, pre-treatment with CST (but not treatment during the chase, when initial deglucosylation to a G1 form has already occurred) has been shown in some cases to promote an alternative proteasome-independent mechanism of degradation (Liu et al. 1999). It is noteworthy that the degradation of NHK- $\alpha$ 1-antitrypsin is in normal conditions dependent on EDEM. However, following CST treatment, EDEM-mediated acceleration of NHK ERAD was abrogated (Oda et al. 2003). At the same time, the kinetics of NHK disposal without EDEM overexpression were

unaffected. It is therefore unlikely that EDEM did not accelerate ERAD because of its own dependence of CNX/CRT cycle for folding. In the latter case an ERAD-inhibitory effect similar to that observed following EDEM knockdown would be expected. Taken together, these data point to a possible switch to an alternative, EDEM- and potentially proteasome-independent degradation mechanism.

It is unclear whether the degradation following glucosidase blockage is mannosidase-dependent. It appears that the importance of ER mannosidase I for the degradation of fully glucosylated glycoproteins may also be substrate-specific. Proteins that are in normal conditions folded correctly but degraded following CST treatment (survival=default) are not affected by mannosidase inhibitors (Weng et al. 1993). However, if the protein is misfolded due to a genetic mutation or otherwise prone to degradation in normal conditions (ERAD=default), this degradation is affected by kifunensine/DMJ, but generally not CST (Wang et al. 2000; Tokunaga et al. 2000).

## **Free oligosaccharides as biomarkers of glycoprotein degradation**

Neutral free oligosaccharides have been identified previously as biomarkers of ER-associated degradation (Moore et al. 1994; Mellor et al. 2004; Alonzi et al. 2008; Hirayama et al. 2010). Free oligosaccharides can be released from lipid-linked intermediates as well as glycoproteins undergoing ERAD (Chantret et al. 2008; Fig. 1.5). These species in normal conditions are transported into the cytosol using a putative transporter which is sensitive to inhibition by benzyl-mannose (Moore 1998, Duvet et al. 2004). However, in normal conditions the majority of FOS is glycoprotein-derived (Duvet et al. 2004, Alonzi et al. 2008). Cytosolic FOS are generated by the action of PNGase as part of the sequence of events leading to proteasomal destruction of retrotranslocated glycoproteins. Following their release into the cytosol, FOS undergo a series of trimming reactions catalysed by cytosolic enzymes endo- $\beta$ -N-acetylglucosaminidase (ENGase) and the neutral cytosolic  $\alpha$ -mannosidase (NAM, MAN2C1)

(Kmićek et al. 1995; Cacan et al. 1996; Duvet et al. 1998). The former rapidly trims the outermost GlcNAc residue from GlcNAc<sub>2</sub>-terminating oligosaccharides. The cytosolic mannosidase progressively demannosylates FOS, predominantly resulting in Man<sub>5</sub>GlcNAc<sub>1</sub> form retaining an intact C arm and therefore distinct from the isomer produced by ER- and Golgi-resident class I mannosidases. It is important to note that due to the fast action of ENGase, in the steady state cytosolically located FOS are almost exclusively GlcNAc<sub>1</sub>-terminating. Oligosaccharide structures such as Man<sub>5</sub>, Man<sub>6</sub> and to a lesser extent larger FOS can then access the lysosome via a so far unknown mechanism, where they are subjected to further hydrolysis (Saint-Pol et al. 1997; Saint-Pol et al. 1999).

There is also evidence of an alternative source of FOS. Several studies suggest that a proportion of free glycans can be released from glycoproteins in the lumen of the ER, possibly by means of a luminal peptide:N-glycanase (Weng et al. 1997; Loo et al. 1998; Fayadat et al. 2000; Spiro et al. 2001, Duvet et al. 2004). Whether or not this pathway of FOS production is linked to an alternative route for glycoprotein degradation, remains to be established. In normal conditions glycoprotein-derived luminal FOS are evidently transported into the cytosol in a manner similar to lipid-derived FOS and enter the same catabolic pathways as those utilised to process cytosolic FOS (Moore et al. 1995; Moore 1998, Duvet et al. 2004).

Glucosidase inhibition has a dual effect on the quantity and quality of FOS. Firstly, due to the abrogation of the normal glycosylation-assisted folding and quality control, the overall rates of protein misfolding and degradation/ER stress increase in the cell (See above, and Pahl et al. 1995, Branza-Nichita et al. 2001). Secondly, since deglycosylation of N-linked glycans is compromised, glucose residues are not removed from the glycoproteins undergoing ERAD, resulting in the production of glucosylated FOS (Fig. 1.5). The latter are processed by ENGase and NAM, but fail to get transported to the lysosome (Moore et al. 1994). The catabolism of glucosylated FOS is therefore arrested at Glc<sub>1–3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub>, which are

slowly processed to Glc<sub>1</sub>–<sub>3</sub>Man<sub>4</sub>GlcNAc<sub>1</sub> (Alonzi et al. 2008). Additionally, luminal FOS cannot exit the ER in the glucosylated form (Moore et al. 1995; Haga et al. 2009).

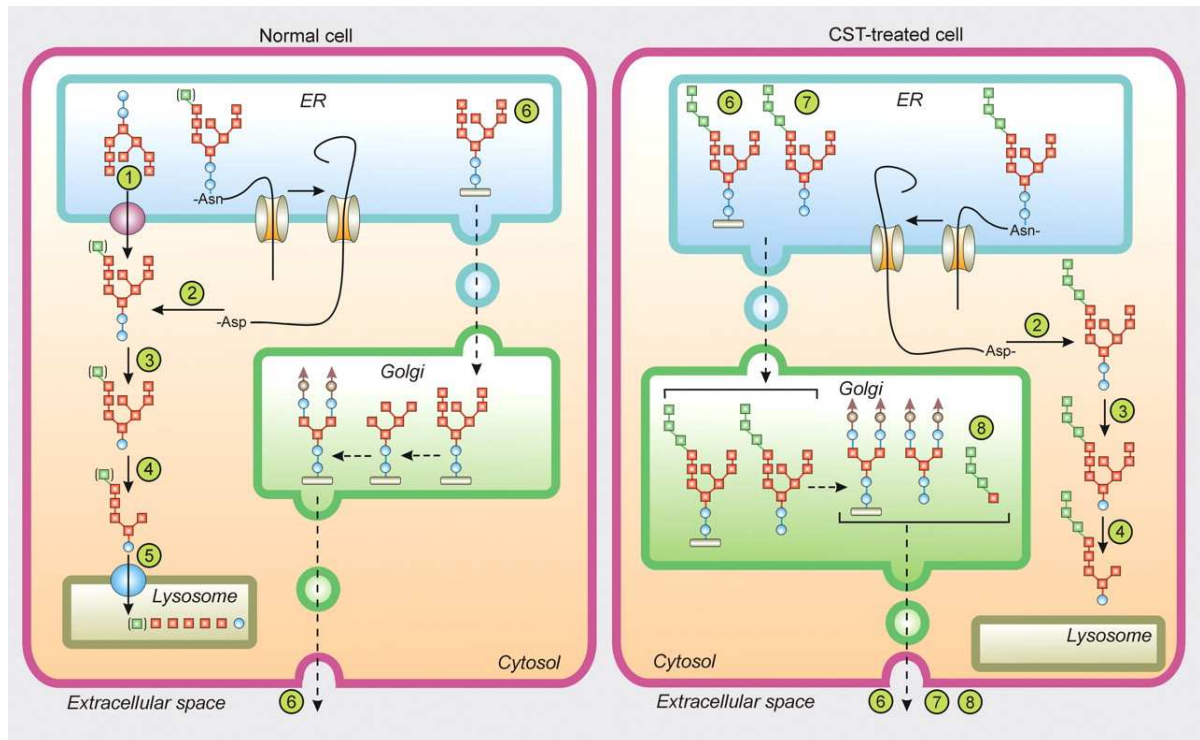


**Fig. 1.5. Models for the subcellular trafficking of FOS.** Normal cells: (1) Lipid-derived GlcNAc<sub>2</sub>-terminating FOS that are generated in the lumen of the ER are deglucosylated by ER glucosidases I and II before being transported into the cytosol. (2) Similar structures (sometimes monoglucosylated) are generated in the cytosol by a PNGase acting on misfolded glycoproteins. All the resulting cytosolic GlcNAc<sub>2</sub>-terminating FOS are sequentially trimmed by an ENGase (3) and cytosolic mannosidase (4) to yield Man<sub>5</sub>GlcNAc<sub>1</sub>. (5) This structure is transported into lysosomes where it is hydrolyzed into Man and GlcNAc residues. (6) Correctly folded glycoproteins are transported to the Golgi apparatus by vesicular transport where they are modified by hydrolases and glycosyltransferases to yield glycoproteins bearing complex-type N-glycans. (6) These glycoproteins are then targeted to their final destinations including the extracellular space. CST (or NB-DNJ)-treated cells: (7) CST inhibits ER glucosidases, and triglucosylated lipid-derived GlcNAc<sub>2</sub>-terminating FOS accumulate in the ER because they are not substrates for the ER-to-cytosol transport machinery, and, along with glycoproteins bearing triglucosylated N-glycans, are transported to the Golgi apparatus. (8) Here, both types of structure are deglucosylated (cleavage of Glc<sub>3</sub>Man<sub>1</sub>) by Golgi endomannosidase before undergoing normal maturation and secretion. In glucosidase-inhibited cells glycoproteins (6), GlcNAc<sub>2</sub>-terminating FOS (7), and endomannosidase-generated Glc<sub>3</sub>Man<sub>1</sub> (8) are secreted via the classical secretory pathway. In addition, triglucosylated GlcNAc<sub>1</sub>-terminating FOS accumulate in the cytosol of CST-treated cells because although steps (2), (3), and (4) occur normally, the triglucosylated FOS are not efficiently transported into lysosomes. The major FOS species accumulating in the cytosol in this case is Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub>. Adapted from Chantret et al. 2008.

Distinct populations of FOS can be stabilised by employing various glycosidase inhibitors. Glucosidase inhibition arrests degradation of FOS in the lumen and the cytosol while maintaining the separation of the two pools. Blockage of the cytosolic mannosidase stabilises the GlcNAc<sub>1</sub>-terminating, oligomannosidic group of FOS, and impairs their access to the lysosome (Butters et al. 2009). Finally, inhibition of lysosomal mannosidases arrests the catabolism of FOS on the late stages, leading to an accumulation of small (Man<sub>2-4</sub>) glycans. FOS analysis has been employed for the evaluation of ERAD in mammalian and yeast cells, both in physiological conditions and in combination with glucosidase or mannosidase inhibition.

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ER-associated degradation is an essential component of quality control in the eukaryotic secretory pathway. For glycoproteins that constitute the majority of secretory cargo, the degradation pathways involve removal of the N-linked glycan moieties, a process tightly linked to retrotranslocation and disposal of the polypeptide parts of degrading molecules. Deglycosylation results in the production of free oligosaccharides in the lumen of the ER and cytosol. Detailed studies of FOS trafficking, as well as quantitative and qualitative analysis of FOS themselves, provide valuable insight into glycoprotein quality control and ERAD targeting. In this work, FOS analysis has been employed to investigate the cellular pathways for glycoprotein degradation, as well as to identify potential participants of novel ERAD pathways.

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# **Chapter 2. Accumulation of lumenal free oligosaccharides suggests alternative glycoprotein degradation**

## **Introduction**

Previous studies have underlined the value of free oligosaccharide (FOS) analysis in cell culture as a robust method for quantitatively assessing the cellular potency of various glucosidase inhibitors (Mellor et al. 2004; Alonzi et al. 2008; Rawlings et al. 2009). Impaired processing by ER glucosidases results in the inability of substrate glycoproteins to interact with CNX/CRT (Kearse et al. 1994; Hebert et al. 1995). Perturbation of the normal cellular pathways of folding and quality control results in increased protein misfolding (Jordan et al. 2002, reviewed in Schröder et al. 2005) and, as a result, in the induction of UPR, leading to the up-regulation of ER chaperones and ERAD factors (Travers et al., 2000; reviewed in McCracken et al. 2000 and Schröder et al. 2005). FOS could be considered a byproduct of ERAD (see Chapter 1). Following treatment with the glucosidase inhibitors, the steady-state levels of glucosylated FOS are representative of the degree of ER glucosidase inhibition (Alonzi et al. 2008). Since degradation of glucosylated FOS is impaired, they are relatively stable in the cell for at least 24 h periods. High levels of glucosidase inhibition result in the production of mainly triglucosylated FOS, together with some partially deglucosylated Glc<sub>2</sub> and Glc<sub>1</sub>-FOS (Alonzi et al. 2008). At lower concentrations some inhibitors cause the accumulation of high-

er levels of Glc<sub>1</sub>- rather than Glc<sub>3</sub>-FOS, which is thought to be due to preferential inhibition of the second glucosidase II-mediated deglycosylation reaction (Lee et al., unpublished). In the cytosol, these FOS are processed by ENGase and NAM to yield Glc<sub>1–3</sub>Man<sub>4–5</sub>GlcNAc<sub>1</sub>, in most cases representing the bulk of the steady-state FOS in glucosidase-inhibited cells.

FOS profiles of cells treated with high concentrations of N-butyldeoxynojirimycin, an  $\alpha$ -glucosidase inhibitor, are in most cases highly consistent between various cell lines and organisms. In the FOS studies, HL60, a human cell line, was used as a reference point. Despite the general consistency of NB-DNJ-treated cellular FOS profiles, in certain cases they were found to be distinctively different from the “consensus” HL60 profile. For example, NB-DNJ-treated Chinese hamster ovary (CHO-K1) and Madin-Darby bovine kidney (MDBK) cells, aside from accumulating a set of FOS similar to those detected in HL60 cells, were noted to additionally produce high levels of a glycan which was shown to be an oligosaccharide with the structure Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>. (Alonzi et al., unpublished, and this work). Several other GlcNAc<sub>2</sub>-terminating FOS were detected alongside with the major peak. Since the detected FOS represented the whole pool of glycans accumulating over 24 hours, while the cytosolic processing by ENGase occurs rapidly following deglycosylation (Kmićik et al. 1995, Cacan et al. 1996), it was unlikely that the presence of GlcNAc<sub>2</sub>-terminating species was merely due to incomplete removal of the reducing terminal GlcNAc residue. It was therefore hypothesised that these FOS represented a novel stable population of free glycans, detectable in some cell lines.

The aim of this part of the project was to elucidate the origin and the properties of stably GlcNAc<sub>2</sub>-terminating free oligosaccharides present in CHO-K1 and MDBK cells following glucosidase inhibition and mainly represented by Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>, and to investigate their relationship to the known pathways of glycoprotein degradation in mammalian cells.

# Materials and methods

## Materials

Tissue culture media were from Gibco/Invitrogen or Sigma. Cell lines were from ATCC. AnalaR and HPLC grade solvents were from VWR International. Restriction, ligation and PCR enzymes were from NEB UK. Water was Milli-Q grade. pLEXm and pOTB7-MAN1B1 vectors were a gift from Dr. Chris Scanlan (Oxford Glycobiology Institute). Polyethylenimine was from Polysciences, Inc. NB-DNJ was provided by Celltech. YB148 was provided by Dr. Yves Bleriot (Universite de Poitier, France). pcDNA3-NAM was a gift from Prof. Pirkko Heikinheimo and Elina Kuokkanen (University of Helsinki, Finland). pcDNA3- $\alpha$ 1AT and pcDNA3.1(-)-EDEM1 were a gift from Dr. John Christianson (University of Oxford, UK). CHPV was a gift from Dr. Anthony Marriott (University of Warwick, UK). All other reagents were from Sigma, unless otherwise stated.

## Plasmid construction

ER mannosidase I (MAN1B1) ORF was subcloned into pLEXm by digesting pOTB7-MAN1B1 and the target vector with EcoRI and XhoI, followed by PCR purification of both reaction mixtures, which were then mixed, ligated, and transformed into XL10-Gold E. Coli cells (Stratagene). pLEXm-transformed clones were selected on ampicillin-containing agar since pOTB7 does not confer ampicillin resistance.

## Cell culture

MDBK, CHO-K1, HL60 and HEK-293T cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cultures were maintained in DMEM supplemented with foetal calf serum (10%), penicillin (100 µg/ml) and streptomycin (100 U/ml).

## Toxicity assay

Cell proliferation was assayed using the CellTiter 96 AQueous kit (Promega, UK), according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at the density of 2000 cells/well and incubated overnight. Media were then replaced with drug-containing media, and cells were grown for the desired time periods. To assay cell viability, MTS/PMS mixture was added to the wells. Cell-free medium was used as a blank. Plates were then incubated in standard tissue culture conditions for 4 h, and absorbance was measured at 490 nm using an ELISA plate reader.

## Antibodies

The following antibodies were used: goat polyclonal anti- $\alpha$ 1-antitrypsin, biotin conjugate (Abcam), goat polyclonal anti-NAM (Santa Cruz Biotechnology), mouse polyclonal anti- $\beta$ -actin (Abcam), mouse monoclonal to BiP/GRP78 (BD Biosciences), mouse monoclonal anti-GAPDH (Sigma). Goat anti-mouse IgG (Abcam) and mouse anti-goat IgG (Santa Cruz Biotechnology) HRP conjugates were used as secondary antibodies for immunoblotting.

## Transfection of plasmids

Conditions were optimised for both cell lines to ensure highest transfection efficiencies. Cells were seeded into 6-well plates at the densities of  $0.3 \times 10^6$  cells/well (CHO-K1) or  $0.5 \times 10^6$  cells/well (293T). The following day, plasmids were transfected using polyethylenimine (PEI) for CHO-K1 or Fugene 6 (Roche) for HEK-293T cells. For CHO-K1, media were replaced with 4 ml serum-free DMEM. PEI was mixed with DNA (3  $\mu$ g DNA per well) at the P:N ratio of 1:14 in a NaCl solution (150 mM) and incubated at room temperature for 10 min. The resulting mix (400  $\mu$ l) was added dropwise into the wells. Cells were then incubated for 4 h at 37°C, after which foetal calf serum was added to the wells up to 10%. Compound treatment was started in fresh medium following an overnight incubation. For  $\alpha$ 1-antitrypsin ( $\alpha$ 1AT) expression, cells were grown in 225-cm<sup>2</sup> flasks, and the protocol was adjusted proportionally. In this case, media were not replaced post-transfection, and compound treatment was started immediately in serum-free medium. For transfection of HEK-293T cells, Fugene 6 was mixed with DNA in serum-free medium (4  $\mu$ l reagent, 1  $\mu$ g DNA per well, total volume 100  $\mu$ l), incubated for 45 minutes at room temperature and added dropwise to the cells. Treatment was started next day by adding the compound from a concentrated stock solution.

## Free oligosaccharide extraction

FOS were extracted from cells and analysed as described previously (Alonzi et al. 2008). Following transfection and/or compound treatment, cells were washed with PBS, scraped and centrifuged. Cell pellets were dounce homogenized in 1 ml water. Cell homogenates were passed through a mixed bed ion exchange column (400  $\mu$ l AG3-X4 / 200  $\mu$ l AG50W-X12), pre-equilibrated with 5 $\times$ 1 ml water. The flow-through was collected with 4 $\times$ 1 ml water washes. The resulting material was dried under vacuum and resuspended in 30  $\mu$ l water.

## **Carbohydrate fluorescent labeling**

Free oligosaccharides were labeled with 2-anthranilic acid (2-AA) as described previously (Neville et al. 2004). 1 ml acetonitrile/water (97:3, v/v) was added and the samples. Labeled oligosaccharides were purified using Spe-ed Amide-2 affinity chromatography cartridges (Applied Separations, UK). The columns were pre-equilibrated with acetonitrile (1 ml), water (1 ml), and acetonitrile again (1 ml). Samples were applied and let pass through the columns by gravity flow, followed by a acetonitrile/water (95:5, v/v) wash (2×1 ml). Samples were eluted with water (2×0.75 ml).

## **Concanavalin A affinity chromatography**

Labeled free oligosaccharides were purified by affinity chromatography using Concanavalin A-Sepharose 4B resin (100 µl). Columns were pre-equilibrated with water (2×1 ml), followed by 1 ml solution containing MgCl<sub>2</sub>, MnCl<sub>2</sub> and CaCl<sub>2</sub> (1 mM each), and finally 2×1 ml 50 mM Tris-HCl buffer, pH 7.2. Samples in 50 mM Tris-HCl buffer, pH 7.2 were applied by gravity flow, followed by a 2×1 ml 50 mM Tris-HCl buffer, pH 7.2 wash. Labeled, purified FOS were then eluted using 2×1 ml hot (70°C) 0.5 M methyl- $\alpha$ ,D-mannopyranoside. A 50 µl aliquot was taken from the resulting solution for HPLC analysis.

## **PGC (porous graphitised carbon) chromatography**

Where required (specifically for protein-linked glycan analysis), 2-AA labeled samples were concentrated following removal of residual unconjugated 2-AA and other contaminants by PGC chromatography as described previously (Alonzi et al. 2008). 1 ml (25 mg) PGC columns (Thermo Electron) were pre-equilibrated with 1 ml of methanol, followed by 1 ml of water, 1 ml of acetonitrile containing 0.1% TFA (trifluoroacetic acid) and finally 2×1 ml of

water. After sample loading, the columns were washed with 2×1 ml of water before the oligosaccharides were eluted with 2 ml of 50% acetonitrile containing 0.1% TFA. Samples were then lyophilised and resuspended in the required amount of water.

## **Carbohydrate analysis by HPLC**

Normal-phase high-performance liquid chromatography (NP-HPLC) was used to separate the purified 2-AA labeled FOS according to a previously published method (Neville et al. 2004). A 4.6×250 mm TSK Gel Amide-80 column (Sigma, UK) was installed in a Waters Alliance 2695 separations module. An in-line Waters 474 fluorescence detector set at  $Ex_{\lambda}=360$  nm and  $Em_{\lambda}=425$  nm was used. Data were processed using Empower software. Glucose unit (GU) values were determined by interpolation from a standard curve obtained by analyzing a 2-AA labeled glucose oligomer standard (partial hydrolysate of dextran).

## **Virus purification**

BHK-21, CHO-K1 or MDBK cells were infected with Chandipura virus (CHPV) in serum-free medium. 4 h post-infection, media were replaced with complete media supplemented with FCS and the appropriate compounds. Viral media were collected following incubation for 24 h and clarified twice by low-speed centrifugation (600g, 10 min). Viral particles were precipitated by adding 1/3 volume of a buffer containing PEG-6000 (final concentration 6%, w/v) and NaCl (final concentration 0.5 M) and incubating the media overnight at 4°C without agitation, followed by centrifugation (2000g, 15 min). The pellets were resuspended in 5 ml PBS and sonicated to disrupt clumps (Branson sonifier, set. 1, 50% duty cycle, 5 bursts, on ice), then centrifuged again (2000g, 15 min). Supernatants were loaded on a cushion of 30%/60% (w/v) sucrose in PBS and centrifuged at 100,000g for 1 h. Virus bands were col-

lected and diluted >10-fold with PBS, filtered through a 0.22  $\mu\text{m}$  filter to remove residual clumps and subjected to ultracentrifugation (100,000g, 2 h) to obtain a purified virus pellet.

## **Immunopurification**

Biotinylated anti- $\alpha 1\text{AT}$  IgG (1 mg) was immobilised on streptavidin-agarose resin (Thermo Sci., UK) following manufacturer's instructions. CHO-K1 cells were transiently transfected with pcDNA3- $\alpha 1\text{AT}$  as described above. 4 days post-transfection, media were harvested, clarified by centrifugation (600g, 10 min) and passed through the IgG column by gravity flow. Following 3 washes with 10 ml PBS,  $\alpha 1\text{AT}$  was eluted using 0.1 M glycine-HCl, pH 2.8, neutralised by adding 2.5M Tris-HCl buffer, pH 8.0 and concentrated using Amicon protein concentrators (10 kDa MWCO).

## **Glycan analysis of purified glycoproteins**

Purified CHPV or immunopurified  $\alpha 1\text{AT}$  were resolved by SDS-PAGE, under reducing conditions followed by transfer to PVDF membranes which were then stained with Coomassie Blue (0.1% w/v in 30% methanol (v/v), 7% glacial acetic acid (v/v) in water) for 15 min, and destained in 50% methanol (v/v), 10% glacial acetic acid (v/v) in water, followed by a wash in water. Membranes were then air-dried, and the bands corresponding to the proteins of interest cut out and subjected to a digest by peptide:N-glycanase F (NEB), at 5,000 units enzyme per ml following manufacturer's instructions, in a total volume of 100  $\mu\text{l}$  for 24 h. Supernatants containing released oligosaccharides were dried, resuspended in 30  $\mu\text{l}$  water and subjected to fluorescent labelling and HPLC analysis.

## **Digitonin extraction of cytosol**

MDBK cells were grown, treated and harvested as described above. Cell pellets were washed in PBS and resuspended in cold (4°C) PBS containing 10 µg/ml digitonin (optimal concentration determined empirically). Samples were then incubated at 4°C for 10 min under gentle agitation, followed by centrifugation at 1000g, 4°C, for 5 min. Supernatants were then removed. Pellets were resuspended in water and lysed by sonication (Branson sonifier, set. 3, 50% duty cycle, 20 bursts, on ice). Equivalent volumes of post-digitonin treatment supernatants and lysed pellets were then subjected to FOS analysis or immunoblotting.

## **Western blotting**

Following SDS-PAGE of crude cell lysates, proteins were transferred to PVDF membranes, which were then blocked in 5% (w/v) non-fat dry milk in PBS supplemented with 0.2% (v/v) Tween 20 at 4°C overnight. Primary and secondary antibodies were diluted in the blocking buffer and incubated with the membrane for 1–2 h, followed by 3 brief washes and 3×5 min washes in PBS supplemented with 0.05% (v/v) Tween 20. ECL substrate (GE Healthcare) was used for detection in all cases.

# Results

## ER lumenal FOS accumulate in CHO-K1 and MDBK cells following glucosidase inhibition

Following treatment of CHO-K1, MDBK and HL60 cells with NB-DNJ (1 mM) for 24 h, FOS were extracted, fluorescently labelled, purified and analysed by HPLC. Peaks were identified by calculating glucose units (GU) deduced by interpolation from a standard curve generated using a fluorescently labelled partial hydrolysate of dextran, and comparing the obtained GU to known values.

Aside from the dominant peaks corresponding to the cytosolic  $\text{Glc}_{1-3}\text{Man}_{4-5}\text{GlcNAc}_1$  FOS, an additional set of oligosaccharides was present in the cells (Fig. 2.1, A). The latter were identified as mainly  $\text{Glc}_{1-3}\text{Man}_7\text{GlcNAc}_2$ , with the triglycosylated species representing the major free oligosaccharide in MDBK and even more so in CHO-K1 cells. This was in agreement with the previously obtained data (Alonzi et al., unpublished). The presence of the  $\text{GlcNAc}_2$  residue at the reducing terminus of the glycan has been previously confirmed by MALDI-TOF. The accumulation of the additional set of FOS in MDBK and CHO-K1 cells was in contrast to typical FOS profiles, such as that obtained for HL60 cells. In the latter case, the FOS composition was very similar to that observed for CHO-K1 and MDBK, with the exception of  $\text{GlcNAc}_2$ -terminating peaks, predominantly represented by  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ , which was virtually absent in HL60 cells. To establish the subcellular localisation of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ , digitonin-mediated subcellular fractionation of NB-DNJ-treated MDBK cells was carried out. The procedure was optimised to ensure optimal separation of free glycans in the cytosol and within the endomembrane system. BiP/GRP78 and NAM were used as markers of ER lumen and cytosol, respectively. However, although the separation of protein markers was essentially complete at 25  $\mu\text{g/ml}$  digitonin, lysosomal FOS ( $\text{Man}_{2-4}\text{GlcNAc}_1$ )

were extracted into the cytosolic fraction at this concentration of the detergent (data not shown).

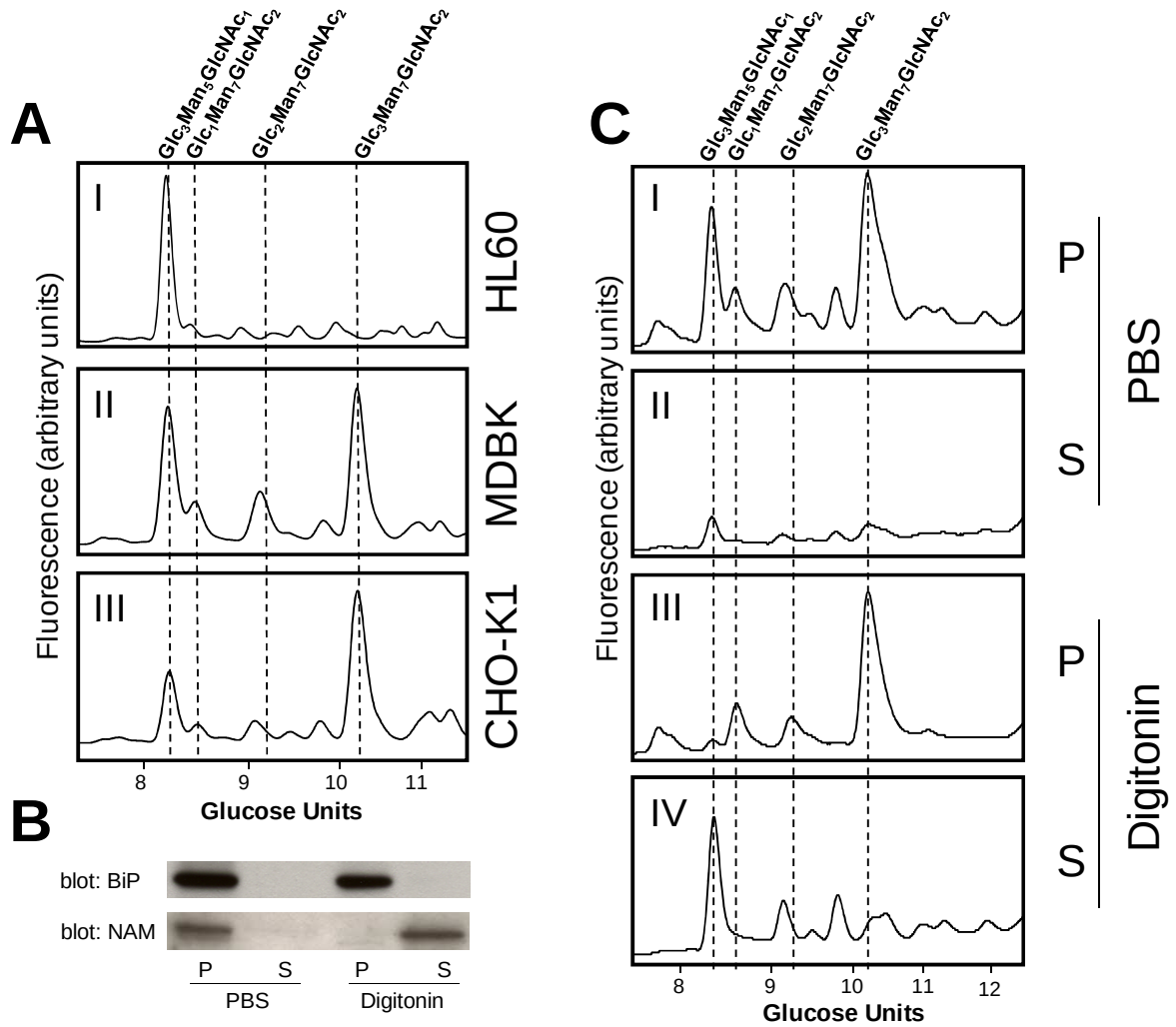


**Fig. 2.1. Glucosylated FOS accumulate in the lumen of endomannosidase-compromised cells.** A, HPLC analysis of 2-AA labelled FOS, isolated from HL60 (I), MDBK (II) or CHO-K1 (III) cells and purified by lectin affinity chromatography. Dashed lines indicate positions of  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  or  $\text{Glc}_{1-3}\text{Man}_7\text{GlcNAc}_2$ . HPLC profiles are representative of experiments carried out and are not drawn to the same scale of fluorescent intensity. B, immunoblotting of BiP and NAM in equivalent fractions of either pellet (P) or supernatant (S) following treatment of MDBK cells with either PBS or digitonin solution. C, HPLC analysis of 2-AA labelled FOS extracted from cells fractionated as in A.

It was therefore concluded that lower concentrations of digitonin were required to separate free oligosaccharides than those optimal for protein fractionation. The latter observation could be rationalised by the difference in size between proteins and FOS, the latter requiring lower levels of permeabilisation to penetrate membranes. Lysosomal and cytosolic ( $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$ ) FOS were used as a reference instead of luminal/cytosolic proteins. Op-

timal separation of the two populations of FOS was achieved at 10  $\mu$ g/ml digitonin (Fig. 2.1, B). In these conditions, Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> extraction from the cell was almost complete. Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>, however, remained in the cell pellet following treatment with digitonin, together with several smaller peaks, mainly Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> (Fig. 2.1, C). Similar results were obtained for CHO-K1 cells. The procedure was also carried out on human HEK-293T cells. The FOS profile of the latter was distinct from the other cell lines tested, showing high concentrations of Man<sub>7–9</sub>-containing glucosylated FOS rather than Man<sub>4–5</sub>-bearing species in CHO-K1, MDBK and HL60, presumably due to lower rates of cytosolic FOS processing by NAM. The identification of GlcNAc<sub>1</sub>/GlcNAc<sub>2</sub>-terminating FOS on the basis of their GU values was therefore problematic in HEK-293T cells since at higher molecular weights both types of free glycans coeluted by HPLC. However, digitonin-mediated cell fractionation demonstrated that the vast majority of glucosylated FOS were cytosolically localised (Fig. 2.2).

HEK-293T cells were therefore more similar to HL60 cells in their glucosylated FOS distribution. Interestingly, although the cytosolic FOS in HEK-293T cells failed to undergo extensive demannosylation resulting, in other cell lines, in Glc<sub>1–3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> and Glc<sub>1–3</sub>Man<sub>4</sub>GlcNAc<sub>1</sub>, the small amounts of glucosylated luminal FOS were represented almost exclusively by Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> (Fig. 2.2, peak 2), which is also the major luminal species in CHO-K1 and MDBK. This observation provides additional evidence for the exposure of the cytosolic and luminal FOS/N-linked glycans to different subsets of cellular glycosidases, with the cytosolic (but not luminal) mannosidase processing being most likely defective in HEK-293T cells.

Previous experiments have addressed the recovery of MDBK cells from treatment with NB-DNJ (Alonzi et al., unpublished). In these experiments, Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> was demonstrated to be accessible to ER glucosidases I and II since, following removal of NB-DNJ from the

medium after a 24 h treatment, the levels of the glycan were gradually reduced with a concomitant increase in  $\text{Man}_7\text{GlcNAc}_{1/2}$ . Together with the results of cell fractionation, these data suggest the localisation of  $\text{GlcNAc}_2$ -terminating FOS to the ER lumen, although the possibility that alternative ER glucosidase-positive structures (autophagic vesicles, EDEMosomes (Reggiori et al. 2010)) may contain these glycans, cannot be discarded.

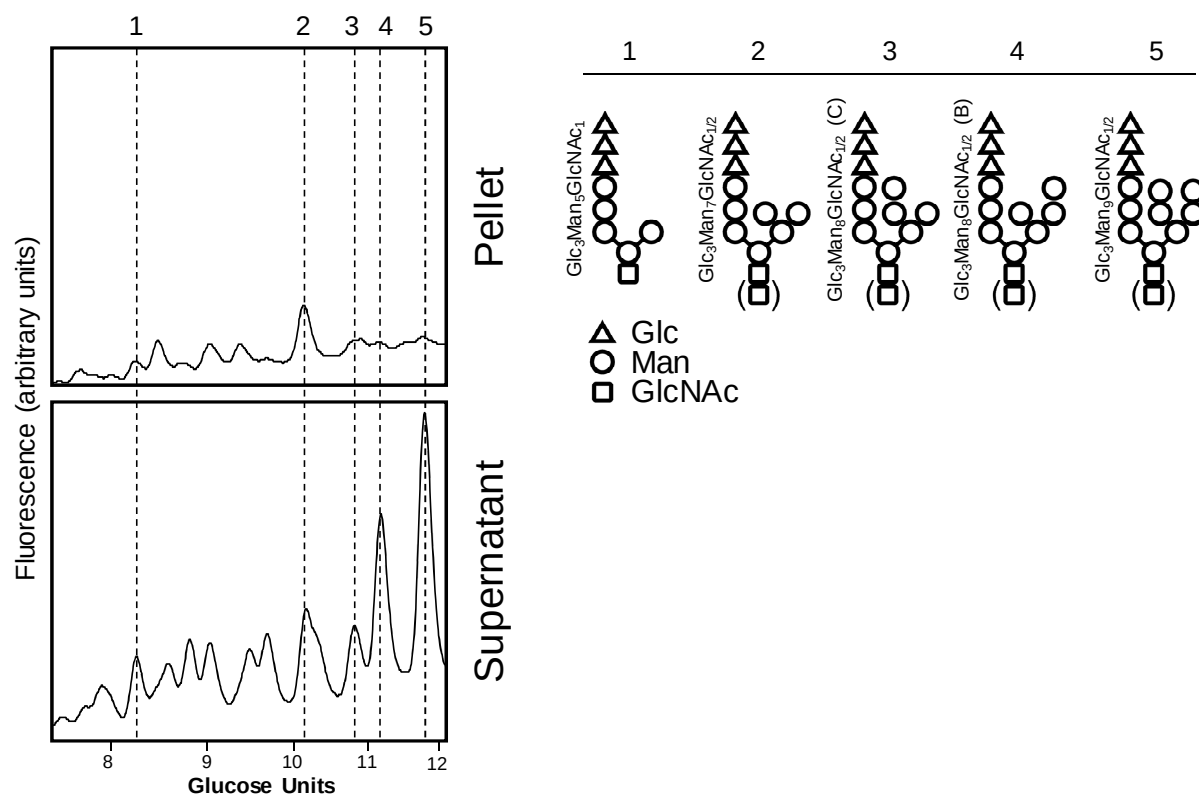


Fig. 2.2. **Digitonin fractionation of HEK-293T cells.** Cells were treated with NB-DNJ for 24 h and fractionated as described above. FOS present in the pellet (top panel) or supernatant (bottom panel) were extracted, fluorescently labelled, purified by lectin affinity chromatography and analysed by HPLC. Chromatograms are drawn to the same scale of fluorescence intensity. Peak numbers refer to the following FOS (schematic structures shown on the right): (1),  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$ , (2),  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_{1/2}$ , (3),  $\text{Glc}_3\text{Man}_8\text{GlcNAc}_{1/2}$  (isomer C), (4),  $\text{Glc}_3\text{Man}_8\text{GlcNAc}_{1/2}$  (isomer B), (5),  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_{1/2}$ .

Experiments carried out prior to this work (Alonzi et al., unpublished) have also demonstrated that the production of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  was sensitive to Z-VAD-fmk, a caspase inhibitor also shown to block peptide:N-glycanase, but not Q-VD-OPh, a related caspase inhibitor that does not have an effect on deglycosylation (Misaghi et al. 2004). This decrease was not due to

a decrease in protein synthesis as shown by a [ $^3\text{H}$ ]-leucine incorporation assay (Alonzi et al., unpublished). The effect of Z-VAD-fmk therefore indicated glycoprotein origin of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ .

Taken together, current and previous evidence has revealed a major steady-state population of  $\text{GlcNAc}_2$ -terminating FOS (N2-FOS) in CHO-K1 and MDBK cells but not in human cell lines following glucosidase inhibition. Some evidence was obtained supporting the hypothesis that this population was localised to the ER lumen and was glycoprotein-derived.

### **Thapsigargin treatment inhibits the production of free $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$**

To further investigate the distribution of N2-FOS, thapsigargin (TG), an inhibitor of retrograde ERGIC-to-ER but not anterograde COPII-mediated vesicular transport (Ying et al. 2002), was employed. Thapsigargin was used at concentrations up to 0.5  $\mu\text{M}$ . At these concentrations, cell growth was unaltered over a period of six days as verified by a growth curve assay (Fig. 2.3, A, B). Cotreatment of CHO-K1 and MDBK cells with NB-DNJ (1 mM) and TG showed a concentration-dependent decrease in the levels of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ . At the same time, glucosylated cytosolic FOS ( $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$ ) remained unaltered (Fig. 2.3, C, D).

To verify the effect of TG on glycoprotein processing, a similar experiment was carried out using BHK-21, a cell line with a characteristically high activity of Golgi endomannosidase. Cells were pretreated with cycloheximide to allow complete processing of FOS already present in the cell. Media were then replaced and supplemented with YB148, an inhibitor of the cytosolic mannosidase (Butters et al. 2009) employed to minimise cytosolic processing of

FOS, and NB-DNJ, either alone or in combination with TG (0.5  $\mu$ M). After a 24 h treatment, both ConA-bound and non-bound FOS were analysed (Fig. 2.4)

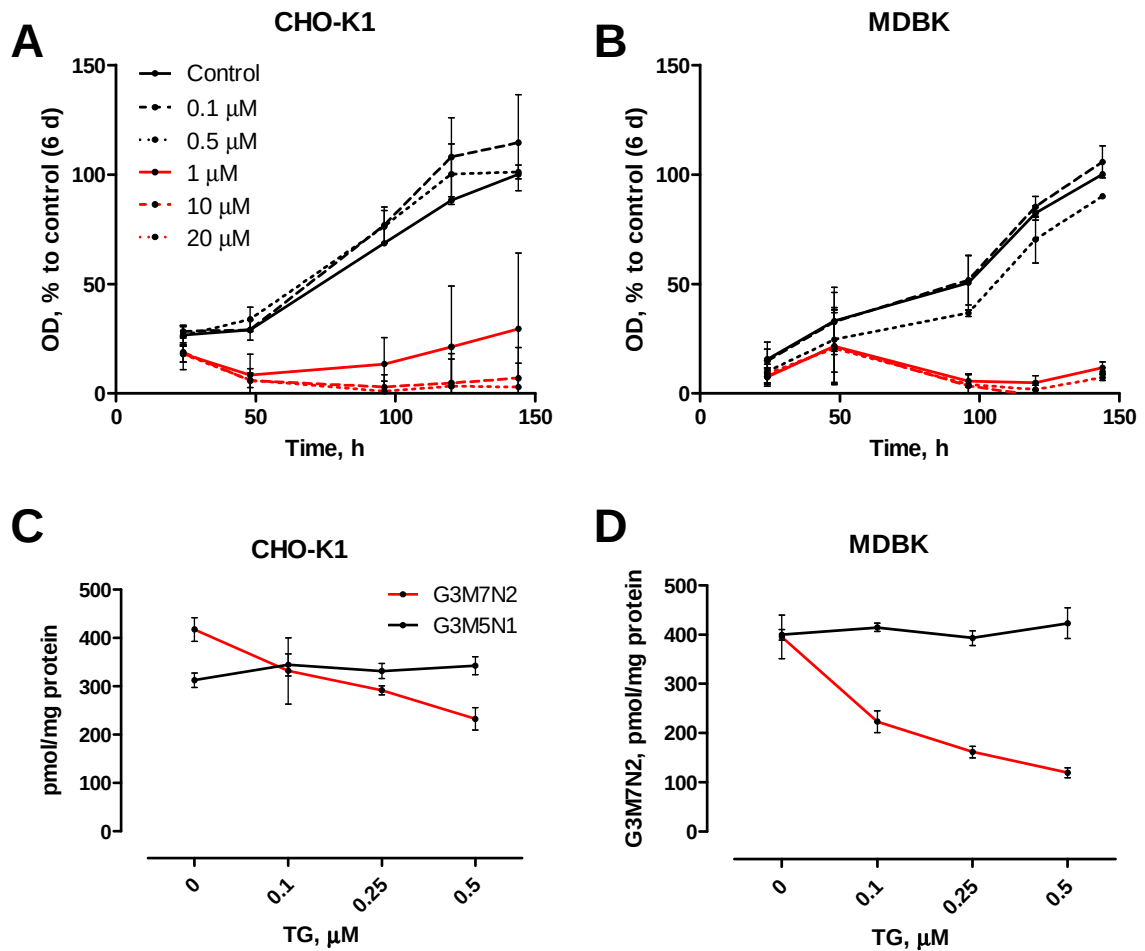


Fig. 2.3. **Thapsigargin induces a reduction in  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  but not  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$ .** A, growth curve of CHO-K1 cells, untreated (control) or treated with various concentrations of TG, and of MDBK cells (B). C, levels of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  and  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  in CHO-K1 cells, untreated or treated with increasing concentrations of TG, and in MDBK cells (D).

In ConA flow-through, the levels of  $\text{Glc}_3\text{Man}_1$ , a characteristic product of endomannosidase-mediated processing of triglycosylated oligosaccharides, were quantified. Since under the conditions of NB-DNJ treatment most protein-linked glycans were expected to remain in the triglycosylated form in the ER,  $\text{Glc}_3\text{Man}_1$  production was used as a control for the exposure of glycoproteins to the enzymes of the Golgi apparatus. Following treatment with TG and NB-

DNJ, the levels of the tetrasaccharide were increased dramatically compared to the cells treated with NB-DNJ alone (Fig. 2.4, B). At the same time, a decrease in the levels of glucosylated ConA-bound FOS, which contributed to a decrease in total FOS, was observed. However, the levels of deglucosylated FOS were not altered significantly. It was therefore speculated that since the levels of Glc<sub>3</sub>Man<sub>1</sub> increase in TG/NB-DNJ-treated BHK-21 cells, glycoproteins in the secretory pathway are subjected to more extensive processing by the enzymes in the Golgi apparatus. This was in agreement with the known ability of TG to inhibit retrograde transport to the endoplasmic reticulum (Ying et al. 2002). The levels of glucosylated FOS as well as the total free oligosaccharides produced decreased, concomitant with the increase in Glc<sub>3</sub>Man<sub>1</sub>. Since the levels of deglucosylated FOS did not compensate for the decreased production of glucose-bearing species, it was concluded that TG has a “bottleneck” effect on the retrograde trafficking of ERAD substrates from the post-ER compartments, limiting the rate of glycoprotein retrieval, which additionally contributes to their increased residence time in the ER-GIC/Golgi and therefore processing by the corresponding enzymes.

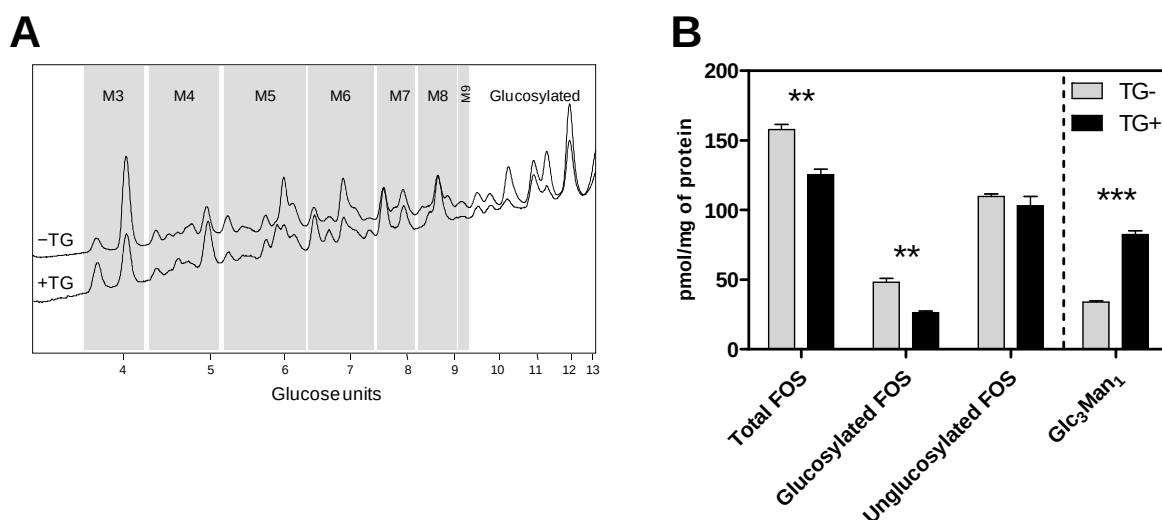


**Fig. 2.4. Thapsigargin effect on FOS in BHK-21 cells.** A, HPLC analysis of 2-AA-labelled ConA-binding FOS in BHK-21 cells incubated with or without TG as well as with NB-DNJ and YB148. Groups of peaks are structurally annotated on the diagram. B, quantitation of total, glucosylated and unglucosylated ConA-binding FOS and Glc<sub>3</sub>Man<sub>1</sub> (Non ConA-binding) oligosaccharide in cells incubated with (TG+) or without (TG-) thapsigargin as described above. Asterisks represent p-values in a two-tailed unpaired Student's t-test.

Taken together, the above data provided biochemical evidence that a selective decrease in the levels of Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> caused by TG treatment correlated with an increased exposure of ERAD substrates to Golgi-resident enzymes. This was consistent with the effects of TG previously demonstrated by means of microscopy (Ying et al. 2002).

### **Thapsigargin does not affect the glycosylation state of secreted glycoproteins**

To verify that the effect of TG was restricted to the production of free oligosaccharides and did not qualitatively affect N-glycosylation of proteins (e.g., by inhibiting ER or Golgi mannosidases), glycan analysis of two model secretory proteins was carried out,  $\alpha$ 1-antitrypsin and Chandipura virus (CHPV) glycoprotein (Fig. 2.5).

$\alpha$ 1-antitrypsin (A1AT) was chosen because of its recently demonstrated cycling between the ER and Golgi in the secretory pathway (Reiterer et al. 2010). The glycoprotein was transiently expressed in CHO-K1 cells in serum-free medium, either without supplements or containing NB-DNJ and/or TG. Recombinant  $\alpha$ 1-antitrypsin was immunopurified, and the protein-linked glycans were analysed as described in Materials and Methods (Fig. 2.5, A). Following treatment with NB-DNJ, the majority of A1AT-derived glycans collapsed to Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>, additionally verifying that the latter is the final product of Golgi processing of triglycosylated oligosaccharides. TG treatment did not qualitatively alter the glycosylation state of A1AT, with or without NB-DNJ.

CHPV glycoprotein is a close homologue of the vesicular stomatitis virus glycoprotein, a well-studied model substrate for the secretory pathway. CHPV is characterised by broad host specificity, allowing for easy expression of its glycoprotein even in MDBK cells which are

extremely difficult to transfect with plasmid DNA. The protocol for virus purification was optimised to ensure maximum purity of viral particles from cell and serum glycoproteins.

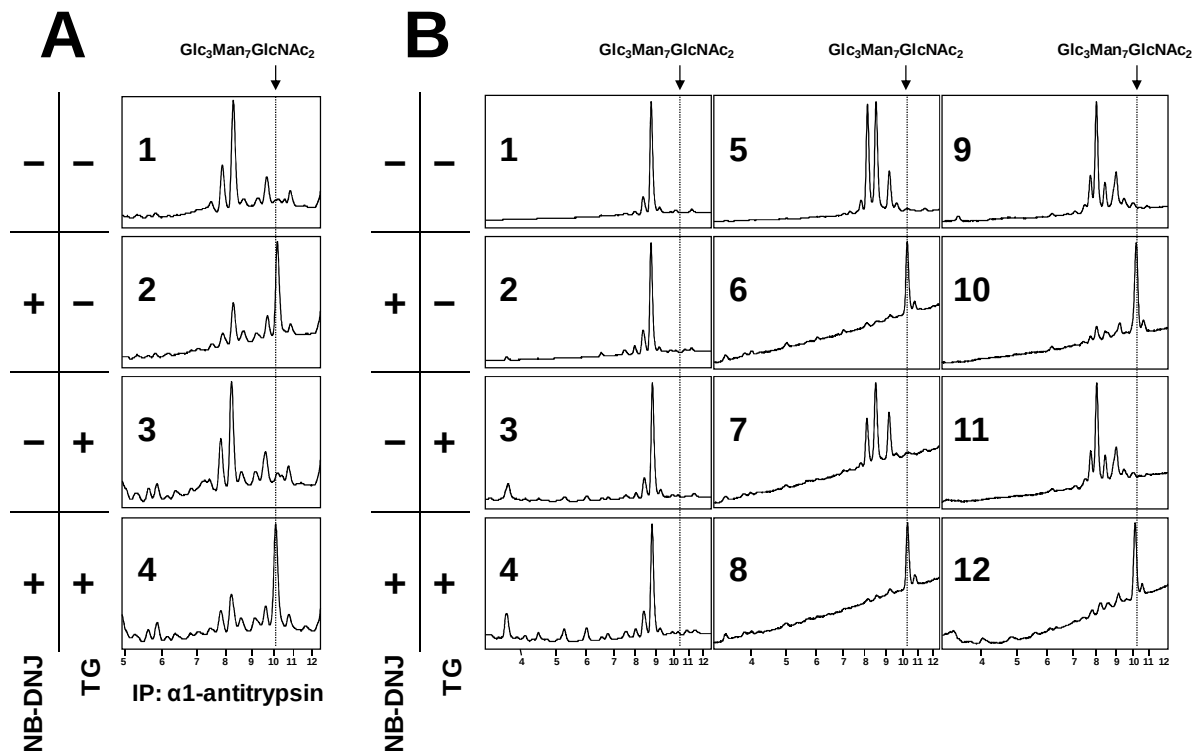


**Fig. 2.5. Thapsigargin does not affect the glycosylation state of secreted glycoproteins.** A, HPLC analysis of α1-antitrypsin glycosylation. CHO-K1 cells were transiently transfected with an α1-antitrypsin-encoding plasmid and incubated for 96 h in control conditions (1) or in the presence of NB-DNJ (2, 4) and/or TG (3, 4). Secreted α1-antitrypsin was immunopurified, its glycans released using PNGase and fluorescently labelled. Arrow and dotted line indicate the position of Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> on the chromatograms. B, HPLC analysis of fluorescently labelled PNGase-released CHPV glycoprotein oligosaccharides. Virus was produced in BHK-21 (1—4), MDBK (5—8) or CHO-K1 (9—12) host cells, untreated (1, 5, 9) or treated with NB-DNJ (2, 4, 6, 8, 10, 12) and/or TG (3, 4, 7, 8, 11, 12). Arrows and dotted lines indicate the position of Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> on the chromatograms.

Several approaches of purification prior to PNGase digestion were attempted, including direct ultracentrifugation of viral medium, in-gel digestion of bands in SDS-PAGE-separated viral pellets, ConA affinity purification, TX114 extraction of envelope proteins, ultrafiltration of viral medium and continuous sucrose gradient purification. The final protocol involved PEG6000/NaCl-mediated virus precipitation, followed by ultrasonic disruption of aggregates, sucrose cushion sedimentation (Fig. 2.6, A) and filtration to remove non-specific clumps. The

resulting filtrates were then subjected to ultracentrifugation, and viral pellets were separated using SDS-PAGE. CHPV glycoprotein was identified by size on a Coomassie Blue-stained gel (Fig. 2.6, B), and its glycosylation analysed as described above (Fig. 2.5, B)

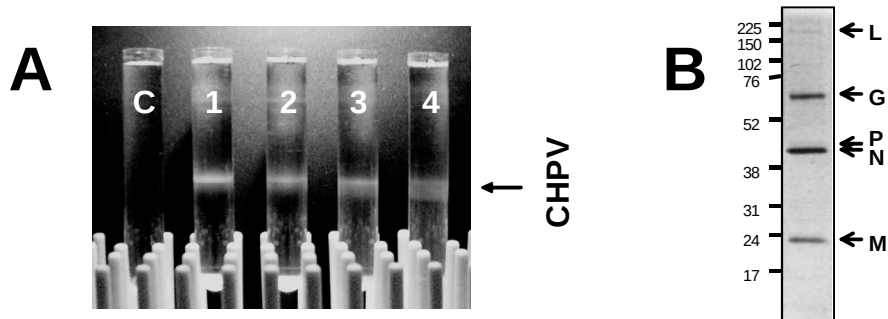


**Fig. 2.6. Purification of CHPV.** A, Sucrose gradient purification of CHPV virions from growth medium of cells treated with or without NB-DNJ and/or TG: c, uninfected control, 1, CHPV, 2, CHPV+NB-DNJ, 3, CHPV+TG, 4, CHPV+NB-DNJ / TG. Arrow indicates the CHPV-containing band. B, SDS-PAGE of the purified virus. Staining carried out using Coomassie blue, bands identified based on predicted molecular weight: L, large protein, G, glycoprotein, P, phosphoprotein, N, nucleoprotein, M, matrix protein.

In a manner similar to A1AT, CHPV-derived glycans collapsed to a single  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  peak when NB-DNJ was added to the medium post-infection (Fig. 2.5). The inclusion of TG did not alter CHPV glycoprotein glycosylation in the presence or absence of the glucosidase inhibitor.

It was therefore concluded that TG does not directly affect protein-linked glycan composition. This finding is likely valid for recycling proteins since A1AT has been shown to traffic between Golgi and ER prior to secretion. However, glycan analysis of continuously recycling proteins (i.e., ER-resident factors bearing an ER retention motif such as GRP94 or BiP) has not been carried out. Such experiments would further confirm the absence of a direct effect of thapsigargin on protein glycosylation. Overall, evidence has been obtained to suggest that the effect of TG on  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  is restricted to the pool of free glycans and is correlated to an increased exposure of recycling secretory cargo to Golgi enzymes.

## Differential effect of ERAD-enhancing factors on luminal and cytosolic FOS

The current view on ERAD attributes a major role in marking glycoproteins for degradation to EDEM1—3 (see Chapter 1). Overexpression of EDEM1 has been previously shown to enhance ERAD (Hosokawa et al., 2006; Oda et al., 2006; Ron et al., 2011). We therefore asked whether this enhancement would be reflected in the production of either type of FOS generated in our model. EDEM1 of murine origin was overexpressed in CHO-K1 cells treated with NB-DNJ, and the levels of glucosylated FOS were compared to those in mock-transfected cells. A significant increase in the production of FOS was observed in EDEM1-transfected cells compared to mock-transfected cells (Fig. 2.7 and Table 2.1).

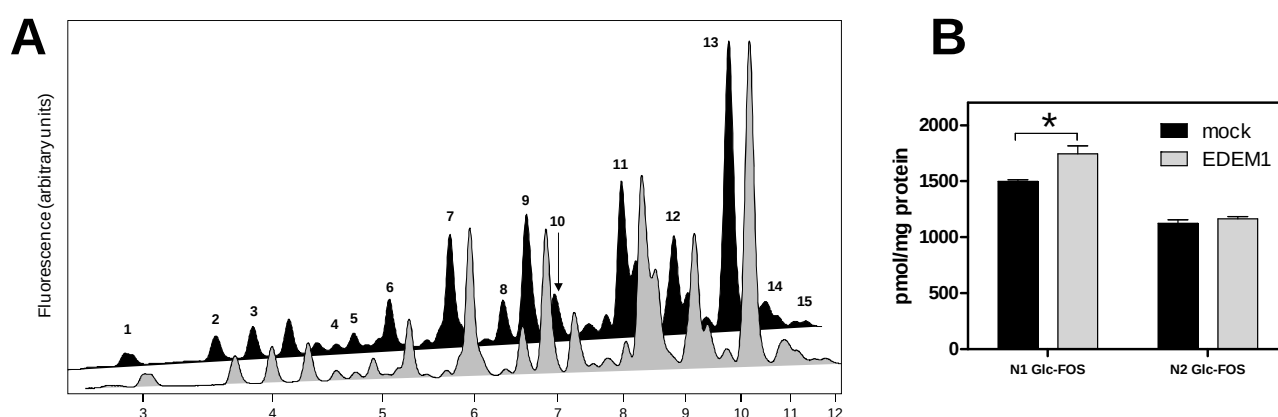


**Fig. 2.7. EDEM1 effect on FOS in CHO-K1 cells.** A, HPLC analysis of fluorescently labelled FOS extracted from mock- or EDEM-transfected CHO-K1 cells, treated with NB-DNJ (1 mM). Peaks were identified and structurally annotated as shown in Table 2.1. B, relative quantities of GlcNAc<sub>1</sub> and GlcNAc<sub>2</sub>-terminating glucosylated FOS, plotted in percent to total FOS. Asterisks represent p-values obtained for a two-tailed unpaired Student's t-test.

The increase primarily involved GlcNAc<sub>1</sub>-terminating FOS, whereas the levels of GlcNAc<sub>2</sub>-terminating species did not change significantly. EDEM1 overexpression therefore increased the relative amount of GlcNAc<sub>1</sub>-terminating FOS.

Table 2.1. **FOS in transfected CHO-K1 cells.** FOS were extracted from transfected CHO-K1 cells incubated for 24 h with NB-DNJ, fluorescently labelled with 2-AA and analysed by HPLC (Fig. 2.7). Glucose units were calculated by interpolation from a standard curve obtained by analysing a 2-AA labeled glucose oligomer standard (partial hydrolysate of dextran).

| Peak number | Glucose units (GU) | Structure                                                                                                    |
|-------------|--------------------|--------------------------------------------------------------------------------------------------------------|
| 1           | 3.08               | Man <sub>2</sub> GlcNAc <sub>1</sub>                                                                         |
| 2           | 3.79               | Man <sub>3</sub> GlcNAc <sub>1</sub> (linear)                                                                |
| 3           | 3.99               | Man <sub>3</sub> GlcNAc <sub>1</sub> (core)                                                                  |
| 4           | 4.61               | Man <sub>4</sub> GlcNAc <sub>1</sub> (linear)                                                                |
| 5           | 4.94               | Man <sub>4</sub> GlcNAc <sub>1</sub>                                                                         |
| 6           | 5.35               | Glc <sub>1</sub> Man <sub>4</sub> GlcNAc <sub>1</sub>                                                        |
| 7           | 6.01               | Glc <sub>2</sub> Man <sub>4</sub> GlcNAc <sub>1</sub>                                                        |
| 8           | 6.61               | Glc <sub>1</sub> Man <sub>5</sub> GlcNAc <sub>1</sub>                                                        |
| 9           | 6.91               | Glc <sub>3</sub> Man <sub>4</sub> GlcNAc <sub>1</sub>                                                        |
| 10          | 7.26               | Glc <sub>2</sub> Man <sub>5</sub> GlcNAc <sub>1</sub>                                                        |
| 11          | 8.21               | Glc <sub>3</sub> Man <sub>5</sub> GlcNAc <sub>1</sub>                                                        |
| 12          | 9.06               | Glc <sub>3</sub> Man <sub>6</sub> GlcNAc <sub>1</sub> /Glc <sub>2</sub> Man <sub>7</sub> GlcNAc <sub>1</sub> |
| 13          | 10.12              | Glc <sub>3</sub> Man <sub>7</sub> GlcNAc <sub>2</sub>                                                        |
| 14          | 11.39              | Glc <sub>3</sub> Man <sub>8</sub> GlcNAc <sub>1/2</sub>                                                      |
| 15          | 11.74              | Glc <sub>3</sub> Man <sub>9</sub> GlcNAc <sub>1/2</sub>                                                      |

The effect of other ERAD factors proposed to act upstream or downstream of EDEM1 was then investigated: ER mannosidase I (MAN1B1), which is a well known modulator of ERAD (reviewed in Spiro 2004), and Hrd1, a major ERAD-specific E3 ubiquitin-ligase, respectively. Both of these factors have been demonstrated to upregulate glycoprotein degradation following overexpression in mammalian cells (see Chapter 1). However, no significant changes in the production of luminal or cytosolic free oligosaccharides were detected in either case (Fig. 2.8, A). This was not due to the failure of expression of ER mannosidase I or Hrd1. In the former case an increase in specific activity was confirmed by a specific and significant upregulation of Glc<sub>3</sub>Man<sub>8</sub>GlcNAc<sub>2</sub> isomer B (lacking the D2 mannose residue) but not isomer C

(lacking the D3 mannose residue) (Fig. 2.8, B). In the case of Hrd1, expression was verified by Western blotting for the S-peptide used as an epitope tag (Fig. 2.8, C).

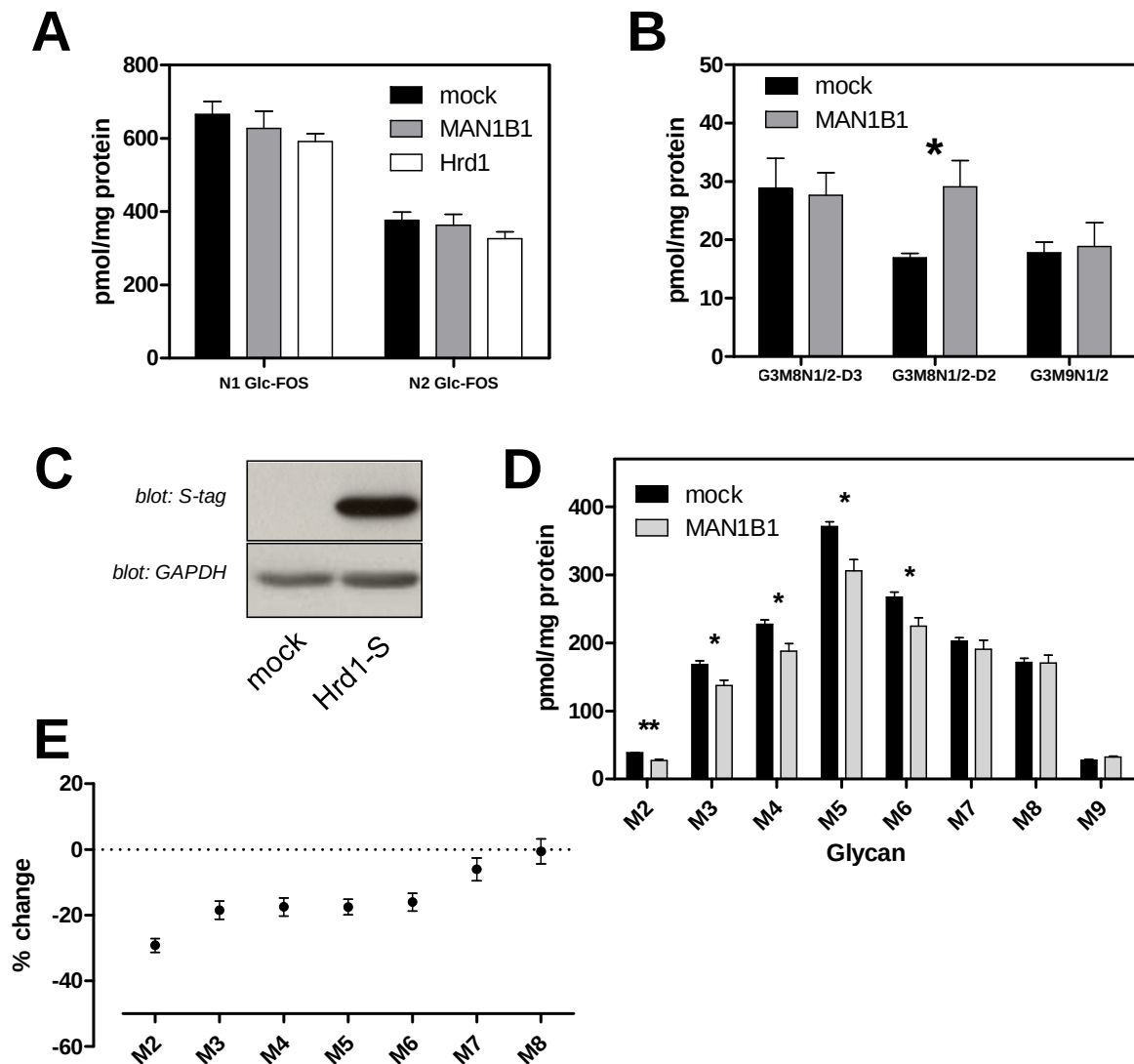


**Fig. 2.8. Effect of ER mannosidase I and Hrd1 on FOS in CHO-K1 cells.** A, quantitation of GlcNAc<sub>1</sub> and GlcNAc<sub>2</sub>-terminating FOS in mock-, ER mannosidase I- (MAN1B1) or Hrd1-transfected CHO-K1 cells treated with NB-DNJ for 24 h. B, confirmation of increased ER mannosidase I activity in transfected CHO-K1 cells. MAN1B1-transfected cells show increased concentration of Glc<sub>3</sub>Man<sub>8</sub>GlcNAc<sub>1/2</sub> isomer B (lacking D2) but not isomer C (lacking D3) or Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>1/2</sub>. C, immunostaining of S-tagged Hrd1 in transfected CHO-K1 lysates. D, levels of Man<sub>2</sub>—Man<sub>9</sub> FOS in YB148-treated mock- or MAN1B1-transfected CHO-K1 cells. E, relative levels of Man<sub>2</sub>—Man<sub>8</sub> FOS in ER mannosidase I-overexpressing CHO-K1 cells treated with YB148, plotted in per cent to the levels of corresponding FOS in mock-transfected cells.

It was speculated that failure of ER mannosidase I to upregulate ERAD was due to glucosidase inhibition employed to stabilise FOS and prevent their transport across the ER or lysosomal membranes, since it has been suggested that the ERAD-enhancing effect of ER mannosidase I stems primarily from its ability to remove the acceptor D1 mannose (Avezov et al. 2008), a residue which is inaccessible in the conditions of glucosidase inhibition. To test this hypothesis, the enzyme was overexpressed in CHO-K1 cells treated with YB148 to inhibit the cytosolic mannosidase. The effect of ER mannosidase I overexpression on FOS in this case was more pronounced (Fig. 2.8, D). However, a decrease in the levels of free glycans was observed rather than an expected increase. The change mainly affected glycans between Man<sub>2</sub>—Man<sub>6</sub> (Fig. 2.8, E). In fact, a consistent inverse correlation between the number of mannose residues and the percentage of decrease was observed.

To further explore this observation, ER mannosidase I was overexpressed in HEK-293T cells. The latter were chosen for two reasons. Firstly, the cell line allows for very high levels of transfection efficiency (>90% as verified by transfection of a GFP-encoding plasmid (data not shown)) using Fugene 6. Secondly, HEK-293T cells were noted to produce characteristically high percentages of high-mannose FOS (Man<sub>7</sub>–Man<sub>8</sub>), which was expected to result in more pronounced changes in the steady-state FOS profiles of untreated cells following ER mannosidase I overexpression. Since HEK-293T cells did not produce large amounts of luminal FOS, digitonin-mediated cell fractionation was employed to separate them from cytosolic FOS present in much higher concentrations. As expected, untreated HEK-293T cells did not accumulate luminal FOS, with virtually all free glycans detected in the cytosolic fraction. The effect of ER mannosidase I was therefore analysed in three groups of samples: untreated/cytosolic, NB-DNJ-treated/cytosolic and NB-DNJ-treated/luminal. Interestingly, a highly pronounced decrease in total FOS levels was observed for all three groups compared to mock-transfected cells (Fig. 2.9, A). The percentage of decrease was not significantly altered be-

tween either luminal or cytosolic free glycans in NB-DNJ-treated cells, as well as those in untreated cells, and measured on average approx. 25% in all three cases (Fig. 2.9, B). Although the general downregulation of FOS production was similar to that observed in YB148-treated CHO-K1 cells, the correlation between depletion and the degree of demannosylation of free glycans was not observed. In fact, the percentage of Man<sub>2</sub>-Man<sub>4</sub> to total FOS was elevated in untreated ER mannosidase I-transfected HEK-293T cells (Fig. 2.9, C).

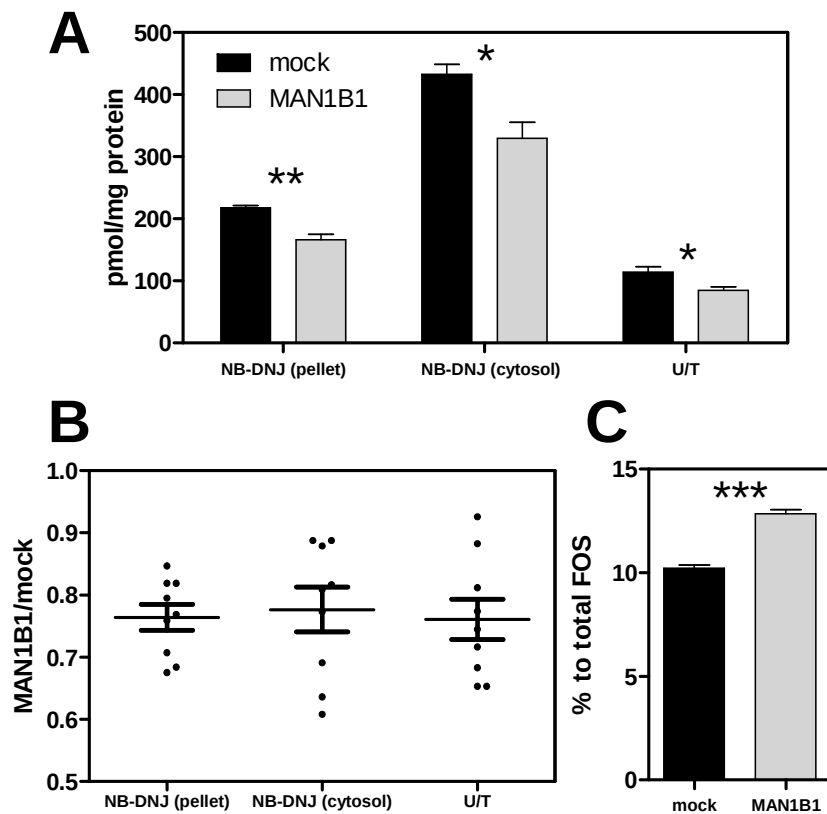


**Fig. 2.9. Effect of ER mannosidase I on FOS in HEK-293T cells.** A, quantitation of FOS in mock- or MAN1B1-transfected HEK-293T cells. Transfected cells were incubated with or without NB-DNJ for 24 h, followed by digitonin fractionation. FOS in the pellet and supernatant (cytosol) were extracted, 2-AA labelled, affinity purified and analysed by HPLC. B, ratio of FOS levels following mock- or MAN1B1 transfection in the samples from A. C, relative levels of small (Man<sub>2</sub>—Man<sub>4</sub>) FOS in untreated HEK-293T cells, plotted in per cent to total FOS. Asterisks represent p-values obtained for two-tailed unpaired t-tests.

Experiments to elucidate the nature of ER mannosidase I-mediated decrease in FOS production, as well as differences in the observed effects in HEK-293T and CHO-K1 cells, are currently under way. At present, there is no sufficient explanation for the described results.

The above data have demonstrated a differential effect of EDEM1 (but not ER mannosidase I or Hrd1) on the production of cytosolic and luminal FOS following glucosidase inhibition. It was next asked if the proposed localisation of the two pathways of FOS production could also influence differential upregulation. Recently, overexpression of cytosolic mannosidase (NAM) was shown to enhance glycoprotein degradation in HeLa cells (Bernon et al. 2010). Although the authors proposed a non-specific mechanism involving perturbation of N-glycosylation via an increase in the cellular levels of free mannose leading to underglycosylation of nascent proteins, the possibility of a more specific influence of NAM on ERAD and hence on FOS levels was explored. Given the exclusive cytosolic localisation of NAM, the enzyme was an attractive candidate for selectively modulating the production of cytosolic FOS.

Before assessing the effect of NAM overexpression or inhibition following glucosidase inhibition, the modulation of enzyme activity was verified by treating mock- or NAM-transfected CHO-K1 cells (Fig. 2.10, A) with a range of YB148 concentrations. Titration with the inhibitor increased the amount of total FOS in the cells in a concentration-dependent manner. As expected, the effect was significant for Man<sub>6</sub>-Man<sub>8</sub> glycans representing the major substrates of NAM (Fig. 2.10, B). Conversely, overexpression of NAM (over 14-fold on average, as assessed by immunoblotting of total cell lysates) shifted the YB148 dose-effect curve towards lower FOS levels (Fig. 2.10, C). CHO-K1 cells were then cotreated with NB-DNJ and YB148. In these conditions, the relative levels of glucosylated GlcNAc<sub>1</sub>-FOS were significantly decreased, whereas those of GlcNAc<sub>2</sub>-FOS (primarily Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>) were unaffected (Fig. 2.10, D, E).

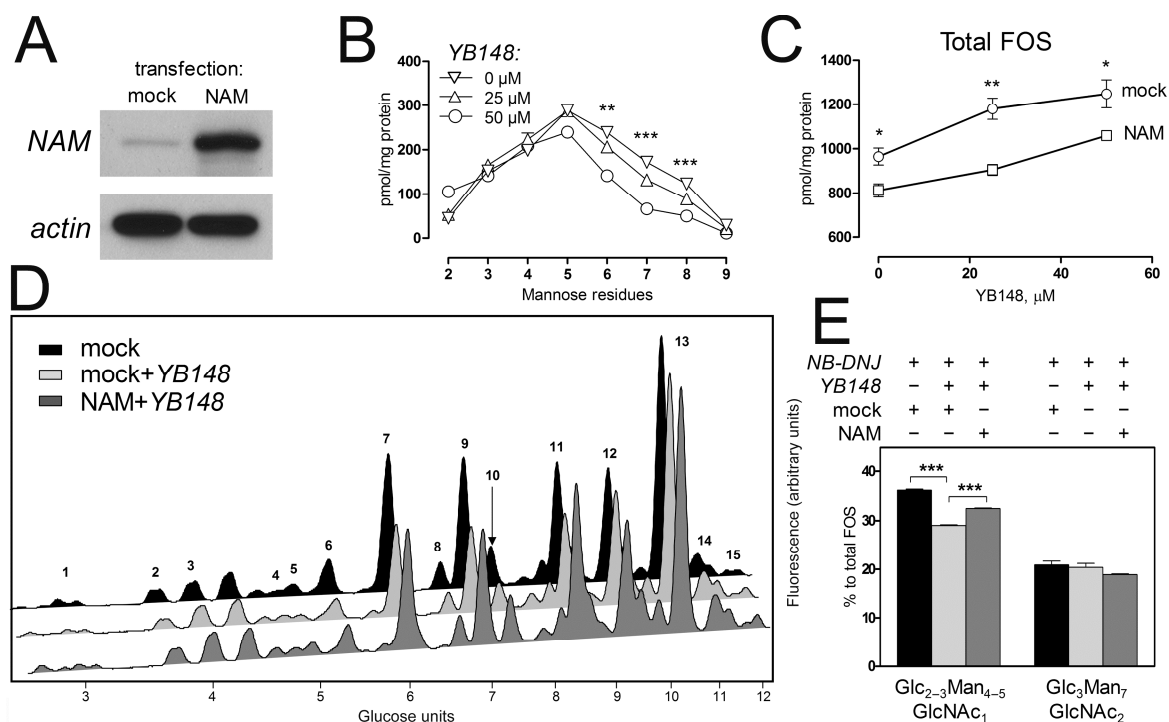


**Fig. 2.10. Cytosolic mannosidase activity affects conventional ERAD but not the alternative degradation pathway.** A, immunoblot demonstrating overexpression of NAM in transfected CHO-K1 cells. B, analysis of fluorescently labelled FOS extracted from mock-transfected CHO-K1 cells incubated for 48 h with 0, 25 or 50  $\mu$ M YB148. FOS were separated using HPLC, peak areas quantified, and the corresponding amounts plotted in pmol per 1 mg total protein against the number of mannose residues in each species. C, total amounts of FOS in mock- or NAM-transfected CHO-K1 cells treated with increasing concentrations of YB148, plotted in pmol per mg total protein. D, HPLC analysis of fluorescently labelled FOS extracted from mock- or NAM-transfected CHO-K1 cells, treated with NB-DNJ (1 mM) and with or without YB148 (50  $\mu$ M). Peaks were identified and structurally annotated as shown in Table 2.1. E, relative quantities of Glc<sub>2-3</sub>Man<sub>4-5</sub>GlcNAc<sub>1</sub> and Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>, per cent to total FOS. Results in all plots and histograms are means  $\pm$  S.E.M ( $n = 3$ ). For panels B, C and E: \*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$  (panel A: one-way ANOVA, panels C and E: two-tailed unpaired Student's  $t$  test).

The decrease in the former was not compensated for by an accumulation of higher-mannose glycans. Overexpression of NAM in NB-DNJ/YB148-treated cells partially rescued the FOS phenotype, confirming the specificity of the inhibitory effect of YB148. Since the levels of deglycosylated FOS were not affected, and the effect on the luminal and cytosolic populations of glucosylated FOS was markedly different, it was concluded that NAM affects ERAD in a specific manner rather than by disrupting the formation of complete glucosylated lipid-linked glycan precursors as suggested by Bernon et al. On the other hand, the selective effect

of NAM activity modulation on cytosolic but not luminal FOS further underlines the topological differences between the two pathways of free glycan formation.

## Discussion

Using FOS analysis coupled with glucosidase inhibition, a new population of glycoprotein-derived oligosaccharides was identified. As opposed to the canonical route of glycoprotein ERAD/FOS production, in which free glycans are released by the cytosolic PNGase following dislocation of proteins across the ER membrane, the novel FOS production pathway resulted in the luminal localisation of released oligosaccharides. In the steady state, the latter primarily comprised  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ , which appears to be the final product of glycan processing within the secretory pathway of deglycosylation-compromised cells, as evidenced by glycan analysis of CHPV and A1AT in NB-DNJ-treated CHO-K1 and MDBK cells. Glycoprotein origin of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  was previously confirmed in PNGase inhibition experiments (Alonzi et al., unpublished).

Two major questions arise regarding the production of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ . Firstly, how are luminal FOS connected to glycoprotein degradation? “Classic” cytosolic FOS have been identified as biomarkers of ERAD since their release from glycoproteins is coupled with retrotranslocation (upstream) and ubiquitin-mediated proteasomal degradation (downstream). Whether luminal FOS could be considered byproducts of an alternative ERAD pathway, and if so, what defines this route as opposed to the other disposal mechanisms, are the main questions arising from the existence of large steady-state populations of luminal FOS. Here, evidence is presented that the production of luminal FOS may indeed be coupled with glycoprotein degradation and constitutes a distinct disposal pathway for recycled proteins. The second major question arises from the cell line-specific differences in the accumulation of glucosylat-

ed luminal FOS, specifically Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>. This glycan was present in large amounts in CHO-K1 and MDBK but not HEK-293T, HL60, BHK-21 or other cell lines tested in this work or in previously carried out glucosidase inhibitor screenings. It is therefore important to establish whether the processes underlying luminal FOS production are restricted to a limited number of cell lines such as CHO-K1 and MDBK, or whether they are universal but somehow masked in other cell lines. The obvious hypothesis stems from the common feature shared by CHO-K1 and MDBK cells but not generally observed in most mammalian cell lines or tissues. This feature is the compromised activity of Golgi endomannosidase, an enzyme capable of removing the glucosyl cap from protein-linked and free oligosaccharides. Hypothetically, NB-DNJ-resistant deglucosylation of oligosaccharides prior to their release from degrading glycoproteins would prevent their accumulation in the ER lumen, obscuring the detection of the pathway. The data on the correlation between the exposure to Golgi enzymes and the production of Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>, discussed below, further support the hypothesis on endomannosidase involvement. While CHO-K1 endomannosidase is known to contain a mutation causing its mislocalisation and complete loss of enzymatic activity (Torossi et al. 2007), MDBK endomannosidase has been shown to be active *in vitro* but not in glucosidase-inhibited cells (Karaivanova et al. 1998). The involvement of endomannosidase in ER-associated degradation and the trafficking of free oligosaccharides are addressed in Chapter 3. The nature of MDBK endomannosidase activity *in vitro* but not in glucosidase-inhibited cells is addressed in Chapter 4.

Establishing a direct connection between luminal FOS production and glycoprotein degradation is not a straightforward task. Studies of protein degradation essentially involve tracking the fate of a single protein substrate. However, FOS analysis presents a generalised view of ERAD in the cell. Glycans can be released from specific cellular glycoproteins *in vitro*, but no connection between a free glycan and its glycoprotein of origin can be made once the cleav-

age occurs in the cell. It is therefore problematic to unequivocally determine the fate of proteins post-lumenal deglycosylation, especially if the pathways resulting in the generation of both cytosolic and lumenal FOS converge at the level of the ubiquitin/proteasome system. However, some preliminary data have been obtained pointing to the connection between lumenal FOS and ERAD. Most importantly, in pulse-chase experiments the production of Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> appears to be blocked by proteasome inhibitors to the same extent as that of Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> (Kukushkin et al., unpublished). This finding suggests physical interactions between the factors of lumenal deglycosylation and the components of the “glycoprotein degradation complex” including retrotranslocation, ubiquitination and proteasomal degradation machinery. It could be speculated that proteasomal blockage results in the “clogging” of the glycoprotein disposal route and therefore inhibits deglycosylation.

Although the ultimate outcome of the two pathways is likely to be the same, the differences between the degradation routes producing lumenal or cytosolic FOS do not appear to be limited to the site of deglycosylation. Firstly, disrupting retrograde transport to the ER causes a decrease in free Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> but does not affect Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> or change the composition of protein-linked glycans, including those on recycling substrates. This correlated with an increased exposure to the Golgi enzymes, including endomannosidase in cell lines other than CHO-K1/MDBK, further supporting the hypothesis on the involvement of the latter in masking the production of lumenal FOS in most cell lines following glucosidase inhibition. Taken together, these data strongly suggest that the production of lumenal FOS is linked to trafficking between the ER and post-ER compartments. Whether or not the extent of such trafficking is substrate-specific, is not clear. It is tempting to speculate that an alternative degradation pathway is utilised by misfolded glycoproteins dependent on ER-Golgi recycling for efficient quality control (Haynes et al. 2002).

Further confirming the intrinsic differences between the two pathways of glycoprotein degradation, it was shown that at least two reported ERAD enhancers (EDEM1 and NAM) differentially affect luminal/cytosolic FOS production following glucosidase inhibition, i.e. influence the levels of  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  but not  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ . If both types of free oligosaccharides were produced in a single degradation event and only differed in the orientation of the deglycosylation reaction relative to the ER membrane, any enhancers/inhibitors of ERAD would be expected to affect the levels of luminal and cytosolic FOS to the same extent. On the other hand, the observed differential effect of EDEM1 and NAM on the two types of FOS suggests that the production of each type of glycans is part of an independent glycoprotein degradation process. However, the factors involved are yet to be identified.

Unexpected results have been obtained in ER mannosidase I overexpression experiments. The effect of the enzyme has been analysed in CHO-K1 and HEK-293T cell lines. In untreated CHO-K1 cells, the cytosolic processing of FOS occurs extremely fast, making detection of significant differences challenging. This is opposed to HEK-293T cells, where relatively high levels of  $\text{Man}_6$ — $\text{Man}_8$  are detectable in the cytosol without in the absence of inhibitors. YB148 was therefore employed to artificially decrease the rate of FOS catabolism in non-glucosidase-inhibited CHO-K1 cells, whereas in HEK-293T cells the effect of ER mannosidase I was analysed in untreated cultures.

Despite the expectation for an increase in global FOS production, no such trend was observed in any of the experiments. In fact, a decrease in the steady-state FOS levels was consistent in both NB-DNJ-treated and untreated HEK-293T cells. In CHO-K1, no effect was observed following glucosidase inhibition, but a subtle decrease was seen in YB148-treated cells. However, the latter was different from that demonstrated by HEK-293T cells, where the levels of larger FOS species were decreased more significantly than those of smaller glycans, whereas

the opposite was true for CHO-K1. It is not unlikely that the difference may stem from off-target effects of YB148, for example, on Golgi mannosidases.

Although ER mannosidase I overexpression experiments failed to establish the effect of the enzyme on either luminal or cytosolic FOS, the results obtained may prove important for elucidating the role of the enzyme in ERAD regulation. Much attention has been devoted to the action of ER mannosidase I in the glycoprotein degradation pathway; however, the data presented here appear to be the first indication that an increase in its activity may in fact down-regulate ERAD on the global scale. Further control experiments are required to confirm the obtained results.

Overall, in the present chapter biochemical evidence is provided for the existence of an alternative glycoprotein degradation pathway, which involves the production of free oligosaccharides in the ER lumen. The pathway appears to be linked to post-ER trafficking, and regulated in a manner distinct from that of the conventional ERAD. However, both routes ultimately lead to proteasomal destruction of misfolded substrates.

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# Chapter 3. Endoplasmic reticulum-associated degradation downstream of processing by endomannosidase

## Introduction

Endo- $\alpha$ -mannosidase is a Golgi/ERGIC enzyme involved in N-linked glycan processing in the glycoprotein secretory pathway. It is the only known endoglycosidase, acting on the  $\alpha(1,2)$  linkage within the A branch of an N-linked oligosaccharide to yield Glc<sub>1–3</sub>Man (depending on the glucosylation state of the substrate) (Lubas et al. 1987; Lubas et al. 1988; Roth et al. 2002). In contrast to glucosidase II, endomannosidase preferentially acts on oligosaccharides with truncated mannose chains (Lubas et al. 1988). Since deglucosylation is a prerequisite for the formation of complex carbohydrate units essential for glycoprotein function (Lodish et al. 1984), ER glucosidase inhibition was originally expected to block oligosaccharide processing. However, early studies indicated that glucosidase inhibitors failed to halt N-linked glycan processing completely (Lodish et al. 1984; Romero et al. 1985; Gross et al. 1986). Endomannosidase, first discovered in rat Golgi membranes, was shown to provide an alternative deglucosylation route in oligosaccharide processing. Further evidence confirmed that the pathway functions *in vivo* in the absence of glucosidase inhibitors (Weng et al. 1996).

Endomannosidase has been proposed to act in the post-ER quality control system based on (i) the implication that the removal of the terminal mannose residue by endomannosidase would not allow for re-entry of the glycoprotein into the calnexin/calreticulin cycle, mediated by UDP-Glc:glycoprotein glucosyltransferase (Sousa et al. 1992); (ii) co-localisation of endomannosidase with calreticulin and co-purification of the two proteins on an affinity matrix (Spiro et al. 1996); and (iii) the mutually exclusive subcellular distribution of endomannosidase and glucosidase II, an enzyme participating in the quality control of proteins in the ER involving the calnexin/calreticulin cycle (Zuber et al. 2000, reviewed in Roth et al. 2003). However, a more recent finding that under the conditions of glucosidase inhibition, endomannosidase non-selectively processes mutated variants of  $\alpha$ 1-antitrypsin, causing the secretion of the misfolded null-Hong Kong  $\alpha$ 1-antitrypsin, established that endomannosidase does not discriminate between misfolded and properly folded glycoproteins, therefore denying endomannosidase a role in quality control (Torossi et al. 2006).

Studies on free oligosaccharides accumulating in the presence of glucosidase inhibitors (see Chapter 2) have suggested a possible link between the absence of endomannosidase activity and the production of glucosylated luminal FOS (predominantly  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ ), which are thought to represent an alternative glycoprotein degradation pathway. Additional data supported the involvement of Golgi-resident enzymes in the biogenesis of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  (see Chapter 2). The aim of this part of the project was therefore to establish the role of endomannosidase in glycoprotein degradation in general, and specifically in the alternative  $\text{GlcNAc}_2$ -FOS-generating route discussed in Chapter 2. To analyse the involvement of the enzyme in these processes on a global level rather than within the disposal pathways of a particular protein, FOS analysis in cells treated with glucosidase inhibitors was again employed.

# Materials and methods

## Materials

For sources of materials, see Chapter 2. Additionally, N-(6'-(4"-azido-2"-nitrophenylamino)hexyl)-1-deoxynojirimycin (NAP-DNJ) was provided by M. Lee (Oxford Glycobiology Institute).

## Cell culture and transfection of plasmids

For cell culture conditions and transfection of plasmids, see Chapter 2.

## Free oligosaccharide analysis

FOS isolation, fluorescent labeling, ConA affinity purification and HPLC analysis were carried out as described in Chapter 2

## Antibodies

The following primary antibodies were used: mouse monoclonal to protein disulphide isomerase (PDI), 2 µg/ml; mouse monoclonal to giantin, 1 µg/ml (both – Enzo Life Sci); mouse monoclonal to GM130, 1.25 µg/ml, mouse monoclonal to BiP/GRP78, 0.5 µg/ml (both – BD Biosciences); mouse monoclonal to ERGIC53, 10 µg/ml (Santa Cruz Biotechnology); rabbit polyclonal to hemagglutinin (HA tag), 1 µg/ml for immunofluorescence, 0.5 µg/ml for immunoblotting (Sigma); mouse monoclonal to  $\beta$ -actin, 2 µg/ml (Abcam). The following secondary antibodies were used: goat polyclonal to mouse IgG, horseradish peroxidase (HRP)

conjugate, 2 µg/ml; goat polyclonal to rabbit IgG, HRP conjugate, 1 µg/ml (both – Abcam), goat polyclonal to mouse IgG, Alexa Fluor 633 conjugate, 4 µg/ml; goat polyclonal to rabbit IgG, Alexa Fluor 488 conjugate, 2 µg/ml (both – Invitrogen).

## Plasmid Construction

Full-length human endomannosidase open reading frame (MANEA) was amplified from an IMAGE cDNA clone (Geneservice, UK) using the following primers:

(P1) 5'-CATTGAATTCTGTGGAATTGTGAGCGGATA-3';

(P2) 5'-TAGCTCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTAAGAAACAGGCAGCTGGCGATCT-3'.

The resulting hemagglutinin-tagged product was PCR purified, digested with EcoRI and XhoI, and the obtained fragment was ligated into the pLEXm vector following gel purification of both digested vector and insert. Empty plasmid was used as the mock vector.

## Compound treatments

In glucosidase inhibition experiments, cells were treated with either N-butyl-deoxynojirimycin (NB-DNJ) at a high concentration (1 mM), or NAP-DNJ at a low concentration (5 µM). The latter compound was previously reported to be a more potent inhibitor of both glucosidases I and II than NB-DNJ (Rawlings et al. 2009), however in cultured cells at low concentrations it has been noted to cause the accumulation of primarily monoglucosylated GlcNAc<sub>1</sub>-terminating FOS (Lee et al., unpublished). Additionally, cells were treated with swainsonine (SW, 100 µM) to inhibit lysosomal FOS processing, YB148 (Compound 1 (Butters et al. 2009) at 100 µM), an inhibitor of cytosolic mannosidase, to stabilise the cytosolic pool of FOS, and dithiothreitol (DTT, 2 mM), to induce unfolded protein response (UPR).

## **Indirect immunofluorescence**

Transfected CHO-K1 cells were grown overnight on coverslips pre-treated with poly-L-lysine bromide (50 µg/ml). Cells were washed with PBS, fixed with paraformaldehyde (2%, v/v) for 30 min, blocked in 5% (w/v) bovine serum albumin in PBS for 30 min, permeabilised with Triton X-100 (1%, v/v) for 20 min, followed by an incubation with primary antibodies diluted in the blocking buffer, for 1 h. Cells were then washed in PBS supplemented with Tween 20 (1%, v/v), 3×15 min, followed by incubation with secondary antibodies diluted in the blocking buffer, for 1 h. After washing as previously, coverslips were mounted on microscopy slides using Vectashield mounting medium with DAPI (Vector Laboratories). Fluorescent images were obtained using the DeltaVision deconvolution-enhanced wide field fluorescent microscope (Olympus APO 100×/1.4) coupled to a CoolSNAP HQ camera (Photometrics). Deconvolution was carried out using the Applied Precision Resolve 3D algorithm. Images were analysed using ImageJ.

## **Endomannosidase assay**

Endomannosidase assay was carried out as described and discussed in Chapter 4.

## **GlcNAc<sub>1</sub>/GlcNAc<sub>2</sub> Analysis of Glucosylated FOS**

GlcNAc<sub>1</sub>- and GlcNAc<sub>2</sub>-reducing terminating forms of FOS with longer retention times can co-elute on HPLC. Therefore, to accurately determine the ratio between the two groups of glucosylated FOS, a series of enzyme digests was carried out. To isolate glucosylated FOS from deglycosylated species, total 2-AA labelled FOS were first digested with jack bean  $\alpha$  mannosidase. Briefly, purified, dried FOS were resuspended in citrate buffer (10 mM, pH 4.5) containing zinc acetate (0.2 mM) and 100 µU jack bean  $\alpha$ -mannosidase (Oxford Glyco-

biology Institute), and incubated at 37°C for 24 h. Samples were deproteinated by ultrafiltration as above, and glucosylated FOS were isolated using preparative HPLC. Eluates were dried, and the resulting FOS were digested 37°C for 24 h using recombinant human endomannosidase expressed and purified as described in Chapter 4. Approx. 0.1 µg of purified protein was used per reaction. Following deproteination, reaction products were analysed by HPLC.

## **Western blotting**

Immunoblotting was carried out as described in Chapter 2. Densitometry was carried out using ImageJ.

# Results

## **Recombinant endomannosidase is functional and localised to the Golgi apparatus**

CHO-K1 cells were transiently transfected with hemagglutinin-tagged human endomannosidase (MANEA). Expression was verified by indirect immunofluorescence and western blotting (Fig 1). Recombinant endomannosidase showed some co-localisation with ERGIC53 (ERGIC marker) and to a greater extent GM130 (cis-Golgi marker), but most significantly with giantin, a more distal (cis- to medial) Golgi marker. No co-localisation with ER marker PDI was detected (Fig 1, A). Overall, the observed distribution of recombinant endomannosidase in CHO-K1 cells agreed with the previously reported localisation of the endogenous enzyme *in vivo*. No changes in endomannosidase localisation were detected with any of the treatments used (data not shown).

It has been previously reported that endomannosidase is post-translationally modified by phosphorylation, which occurs in the Golgi apparatus (Torossi et al. 2010). Western blotting of cell lysates following expression of endomannosidase resulted in a double band similar to the one observed previously, consistent with the reported post-translational processing (Fig. 3.1, A).

Enzymatic activity of recombinant endomannosidase was confirmed by an enzyme assay. Post-nuclear membrane extracts of CHO-K1 cells were incubated with fluorescently labeled oligosaccharide substrates, Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> and Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub>, in the presence of glucosidase and mannosidase inhibitors. Following an overnight reaction, HPLC analysis showed no peak shift caused by mock-transfected CHO-K1 extracts. A complete shift of the substrate

peaks to a single peak corresponding to  $\text{Man}_4\text{GlcNAc}_1$ , the predicted endomannosidase product, was observed in the case of MANEA-transfected cells (Fig. 3.1, C).

Finally, the action of recombinant endomannosidase in the glycoprotein-processing pathway was verified by analysing Concanavalin A (Con A) affinity chromatography flow-through following purification of free oligosaccharides extracted from cells treated with NB-DNJ (1 mM). ConA binds most FOS, but low-molecular weight sugars containing up to one alpha-mannosyl residue are generally detected in the flow-through. A peak with  $\text{GU}=3.47$  corresponding to  $\text{Glc}_3\text{Man}_1$  (Mellor et al. 2004), a characteristic endomannosidase product in the presence of glucosidase inhibitors, was shown to increase over 5-fold in Con A flow-through of extracts from MANEA-transfected cells (Fig. 3.1, D). This peak was further confirmed to correspond to a tetrasaccharide by MALDI-TOF (data not shown).

### **Endomannosidase causes a decrease in total FOS and a relative increase in small FOS concentrations following glucosidase inhibition**

Following a 24 h treatment with NB-DNJ (1 mM), the major peaks in CHO-K1 FOS HPLC profiles corresponded to triglycosylated oligosaccharides  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  and  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  (Fig. 3.2, A, and Table 3.1), consistent with data reported in Chapter 2. Endomannosidase expression was expected to alter the ratio between lumenal and cytosolic glucosylated FOS in accordance to the proposed mechanism of degradation of recycled proteins in the ER. However, the ratio between the dominant FOS peaks was not altered in MANEA-transfected cells. The HPLC profiles of FOS in mock-transfected or endomannosidase-overexpressing cells following NB-DNJ treatment were qualitatively identical at higher retention times ( $\text{GU}>5$ ). At the same time, the total levels of FOS were significantly decreased (Fig. 3.2, B). This decrease was composed of polymannose glycans with 5 and more mannose

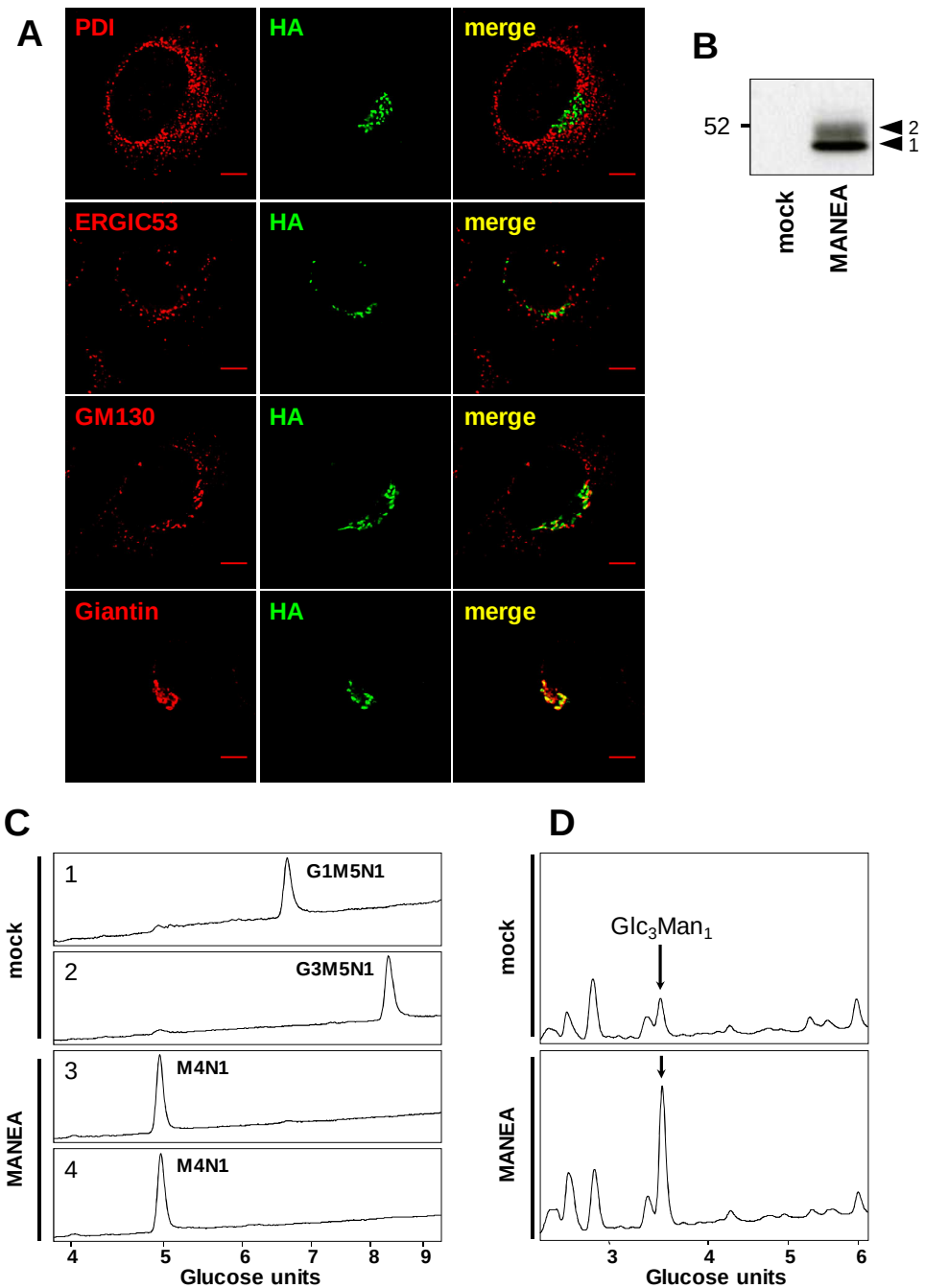


**Fig. 3.1. Recombinant endomannosidase is functional in CHO-K1 cells.** A, colocalisation of HA-tagged endomannosidase in transfected CHO-K1 cells with ER (PDI), ERGIC (ERGIC53), cis-Golgi (GM130) and cis- to medial Golgi (giantin) markers. Size bars, 5  $\mu$ m. B, immunoblot showing expression of endomannosidase in MANEA-transfected cells. Bands are likely to represent unmodified (1) and post-translationally modified (2) endomannosidase (not verified experimentally). C, endomannosidase activity in transfected cell extracts. 2-AA labeled Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> (G1M5N1) (1, 3) or Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> (G3M5N1) (2, 4) were used as substrates in an overnight reaction in the presence of NB-DNJ, DMJ and EDTA. Reaction mixtures were deproteinated and analysed by HPLC. Peak shifts were observed in MANEA-transfected (3, 4) but not in mock-transfected (1, 2) cell extracts. D, HPLC analysis of 2-AA labeled ConA flow-through of transfected cell lysates shows an average 5.4-fold increase in Glc<sub>3</sub>Man<sub>1</sub> (G3M1) levels (data derived from the results of three independent experiments; the result was significant with  $p=0.0034$ )

residues, but primarily all glucosylated oligosaccharides (“large FOS”, Fig. 3.2, A). However, the relative contribution of FOS with shorter retention times (“small FOS”) to the total FOS pool appeared to be increased in endomannosidase-transfected CHO-K1 cells (Fig. 3.2, C).

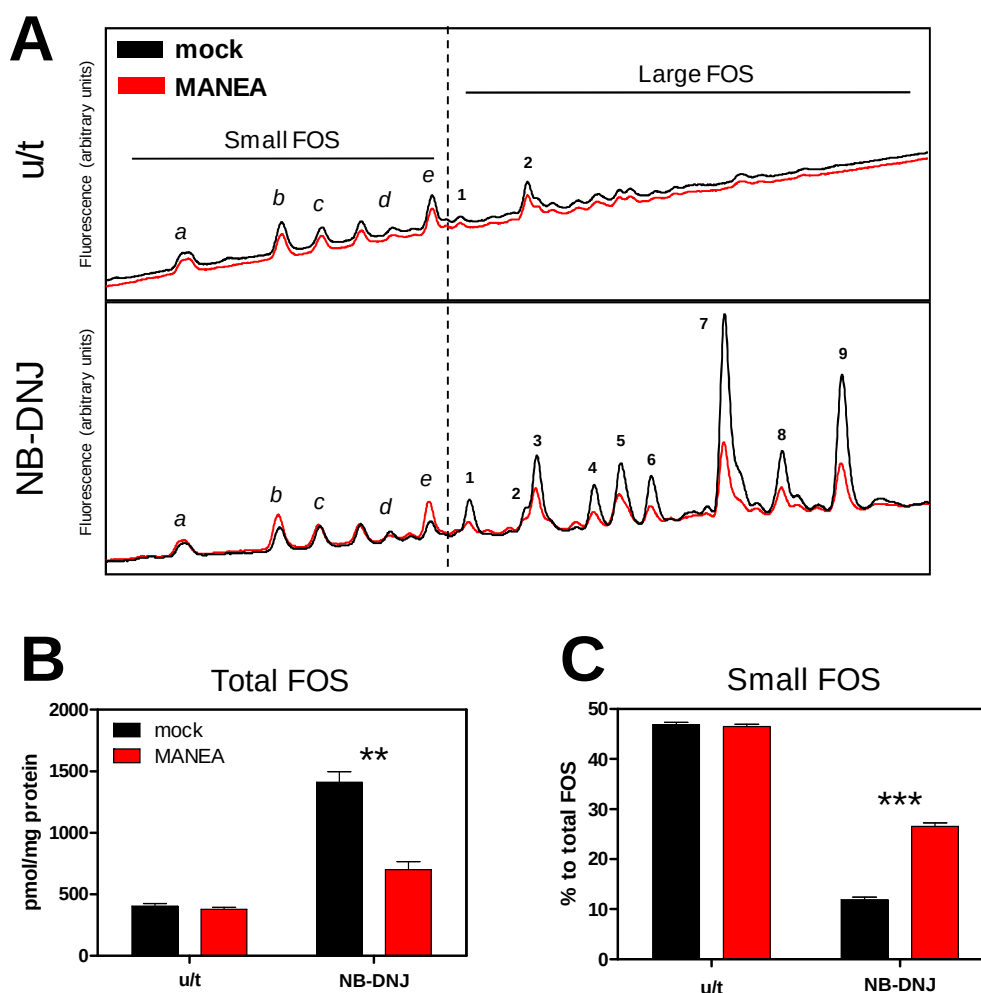


**Fig. 3.2. Endomannosidase effect on free oligosaccharides in NB-DNJ-treated cells.** A, HPLC analysis of FOS extracted from mock- or MANEA-transfected CHO-K1 cells incubated for 24 h without inhibitors (u/t) or with NB-DNJ. Identified large FOS peaks are numbered and structurally annotated as shown in Table 3.1 and Table 3.3. Small FOS were further analysed as discussed below. Peaks were labeled and structurally annotated as shown in Table 3.2. B, total amounts of FOS in the cells, plotted in pmol per 1 mg total protein. C, proportion of small FOS in the cells, plotted in percent to total FOS. Data are from three independent experiments, asterisks represent p-values in a two-tailed unpaired Student’s t-test (\*,  $p < 0.05$ , \*\*,  $p < 0.01$ , \*\*\*,  $p < 0.001$ ).

FOS status of untransfected cells, untreated or treated with NB-DNJ, was also assessed and in most cases was not different from that of mock-transfected cells. A decrease in the levels of

Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> and a concomitant increase in the levels of Glc<sub>3</sub>Man<sub>4</sub>GlcNAc<sub>1</sub> were significant in mock-transfected CHO-K1 cells, however the differences observed were negligible compared to those between mock- and MANEA-transfected cells (Table 3.2).

Table 3.1. **Large FOS in cells following various treatments.** FOS were extracted from mock- or MANEA-transfected CHO-K1 cells incubated for 24 h under various conditions, fluorescently labeled with 2-AA and analysed by HPLC (Figs. 2–5). Glucose units were calculated by interpolation from a standard curve obtained by analyzing a 2-AA labeled glucose oligomer standard (partial hydrolysate of dextran).

| Peak no. | Glucose units<br>(GU) | Structure                                                                                                    | Abbreviation  |
|----------|-----------------------|--------------------------------------------------------------------------------------------------------------|---------------|
| 1        | 5.35                  | Glc <sub>1</sub> Man <sub>4</sub> GlcNAc <sub>1</sub>                                                        | G1M4N1        |
| 2        | 5.82                  | Man <sub>5</sub> GlcNAc <sub>1</sub>                                                                         | M5N1          |
| 3        | 6.01                  | Glc <sub>2</sub> Man <sub>4</sub> GlcNAc <sub>1</sub>                                                        | G2M4N1        |
| 4        | 6.61                  | Glc <sub>1</sub> Man <sub>5</sub> GlcNAc <sub>1</sub>                                                        | G1M5N1        |
| 5        | 6.91                  | Glc <sub>3</sub> Man <sub>4</sub> GlcNAc <sub>1</sub>                                                        | G3M4N1        |
| 6        | 7.26                  | Glc <sub>2</sub> Man <sub>5</sub> GlcNAc <sub>1</sub>                                                        | G2M5N1        |
| 7        | 8.21                  | Glc <sub>3</sub> Man <sub>5</sub> GlcNAc <sub>1</sub>                                                        | G3M5N1        |
| 8        | 9.06                  | Glc <sub>3</sub> Man <sub>6</sub> GlcNAc <sub>1</sub> /Glc <sub>2</sub> Man <sub>7</sub> GlcNAc <sub>2</sub> | G3M6N1/G3M7N2 |
| 9        | 10.12                 | Glc <sub>3</sub> Man <sub>7</sub> GlcNAc <sub>2</sub>                                                        | G3M7N2        |
| 10       | 5.74                  | Man <sub>5</sub> GlcNAc <sub>1</sub>                                                                         | M5N1          |
| 11       | 5.99                  | Man <sub>5</sub> GlcNAc <sub>2</sub>                                                                         | M5N2          |
| 12       | 6.86                  | Man <sub>6</sub> GlcNAc <sub>1</sub>                                                                         | M7N1          |
| 13       | 7.28                  | Man <sub>7</sub> GlcNAc <sub>1</sub>                                                                         | M7N1          |
| 14       | 7.72                  | Man <sub>7</sub> GlcNAc <sub>1</sub>                                                                         | M7N1          |
| 15       | 8.36                  | Man <sub>8</sub> GlcNAc <sub>1</sub> /Man <sub>8</sub> GlcNAc <sub>2</sub>                                   | M8N1/M8N2     |
| 16       | 8.73                  | Man <sub>8</sub> GlcNAc <sub>1</sub> /Man <sub>8</sub> GlcNAc <sub>2</sub>                                   | M8N1/M8N2     |
| 17       | 9.39                  | Man <sub>9</sub> GlcNAc <sub>1</sub> /Man <sub>9</sub> GlcNAc <sub>2</sub>                                   | M9N1/M9N2     |

Table 3.2. **Quantitation of FOS in CHO-K1 and HEK-293 cells.** Values are presented for untransfected or mock-transfected CHO-K1 (A, B) or HEK-293T cells (C, D), untreated (A, C) or treated with NB-DNJ (B, D). P-values are given for a two-tailed unpaired Student's t-test and summarised as follows: ns, not significant ( $p>0.05$ ), \*,  $p<0.05$ . Data are average of three independent experiments.

#### A. CHO-K1, untreated

| FOS               | Untransfected      |       | mock               |       | Difference,<br>mock to<br>untransfected | p    | Summary |
|-------------------|--------------------|-------|--------------------|-------|-----------------------------------------|------|---------|
|                   | pmol/mg<br>protein | SEM   | pmol/mg<br>protein | SEM   |                                         |      |         |
| M2N (a)           | 62.28              | 3.32  | 50.41              | 2.86  | -11.87                                  | 0.05 | ns      |
| M3N (b)           | 32.13              | 0.32  | 30.78              | 0.97  | -1.36                                   | 0.96 | ns      |
| M3N (c)           | 49.23              | 0.09  | 45.53              | 2.87  | -3.70                                   | 0.71 | ns      |
| M4N (d)           | 5.56               | 0.50  | 6.97               | 0.15  | 1.41                                    | 0.07 | ns      |
| M4N (e)           | 65.12              | 0.63  | 63.81              | 4.81  | -1.32                                   | 0.78 | ns      |
| G1M4N1            | 8.35               | 0.50  | 7.48               | 0.45  | -0.88                                   | 0.58 | ns      |
| M5N (all isomers) | 99.31              | 2.04  | 96.59              | 4.04  | -2.72                                   | 0.76 | ns      |
| M6N (all isomers) | 58.49              | 1.37  | 54.79              | 1.63  | -3.70                                   | 0.76 | ns      |
| M7N (all isomers) | 14.97              | 0.15  | 16.50              | 0.45  | 1.53                                    | 0.07 | ns      |
| M8N (all isomers) | 17.82              | 0.05  | 17.81              | 0.92  | -0.01                                   | 0.56 | ns      |
| M9N               | 5.46               | 0.21  | 6.78               | 0.22  | 1.32                                    | 0.25 | ns      |
| Total FOS         | 437.15             | 10.71 | 426.96             | 17.48 | -10.19                                  | 0.69 | ns      |

#### B. CHO-K1, NB-DNJ 1 mM

| FOS                   | Untransfected      |       | mock               |       | Difference,<br>mock to<br>untransfected | p    | Summary<br>pmol/mg<br>protein |
|-----------------------|--------------------|-------|--------------------|-------|-----------------------------------------|------|-------------------------------|
|                       | pmol/mg<br>protein | SEM   | pmol/mg<br>protein | SEM   |                                         |      |                               |
| M2N (a)               | 20.93              | 0.31  | 21.67              | 0.27  | 0.74                                    | 0.15 | ns                            |
| M3N (b)               | 24.97              | 1.39  | 27.71              | 0.44  | 2.74                                    | 0.13 | ns                            |
| M3N (c)               | 36.95              | 2.71  | 37.17              | 0.78  | 0.22                                    | 0.94 | ns                            |
| M4N (d)               | 4.61               | 0.36  | 6.34               | 0.63  | 1.73                                    | 0.08 | ns                            |
| M4N (e)               | 24.01              | 0.84  | 23.10              | 1.01  | -0.91                                   | 0.53 | ns                            |
| G1M4N1                | 52.95              | 3.46  | 59.97              | 1.14  | 7.02                                    | 0.13 | ns                            |
| G2M4N1                | 107.63             | 7.06  | 121.40             | 2.09  | 13.77                                   | 0.13 | ns                            |
| G1M5N1                | 52.41              | 2.64  | 45.17              | 1.42  | -7.24                                   | 0.07 | ns                            |
| G3M4N1                | 97.29              | 6.66  | 121.33             | 1.40  | 24.03                                   | 0.02 | *                             |
| G2M5N1                | 45.78              | 2.43  | 43.30              | 0.85  | -2.48                                   | 0.39 | ns                            |
| G3M5N1                | 325.69             | 16.66 | 232.10             | 2.24  | -93.59                                  | 0.01 | *                             |
| G3M6N1—2/<br>G2M7N1—2 | 149.00             | 8.35  | 133.31             | 1.91  | -15.68                                  | 0.14 | ns                            |
| G3M7N2                | 387.97             | 23.54 | 352.22             | 6.46  | -35.75                                  | 0.22 | ns                            |
| Total FOS             | 1604.17            | 87.78 | 1483.17            | 26.71 | -121.00                                 | 0.26 | ns                            |

#### C. HEK-293T, untreated

| FOS               | Untransfected      |      | mock               |      | Difference,<br>mock to<br>untransfected | p    | Summary |
|-------------------|--------------------|------|--------------------|------|-----------------------------------------|------|---------|
|                   | pmol/mg<br>protein | SEM  | pmol/mg<br>protein | SEM  |                                         |      |         |
| M2N (a)           | 4.40               | 0.50 | 3.26               | 0.12 | -1.15                                   | 0.09 | ns      |
| M3N (b)           | 1.23               | 0.18 | 0.72               | 0.06 | -0.51                                   | 0.05 | ns      |
| M3N (c)           | 4.51               | 0.06 | 3.60               | 0.41 | -0.91                                   | 0.09 | ns      |
| M4N (d)           | 1.63               | 0.46 | 0.60               | 0.14 | -1.04                                   | 0.10 | ns      |
| M4N (e)           | 7.61               | 0.64 | 5.69               | 0.68 | -1.92                                   | 0.11 | ns      |
| M5N (all isomers) | 30.70              | 1.38 | 28.26              | 2.24 | -2.44                                   | 0.41 | ns      |
| M6N (all isomers) | 20.13              | 0.32 | 19.50              | 0.97 | -0.62                                   | 0.57 | ns      |
| M7N (all isomers) | 15.91              | 0.19 | 15.16              | 1.00 | -0.75                                   | 0.50 | ns      |

|                   |        |      |        |      |        |      |    |
|-------------------|--------|------|--------|------|--------|------|----|
| M8N (all isomers) | 19.51  | 0.85 | 16.74  | 0.99 | -2.77  | 0.10 | ns |
| M9N               | 26.43  | 1.05 | 24.25  | 1.13 | -2.18  | 0.23 | ns |
| Total FOS         | 134.53 | 3.36 | 119.45 | 7.63 | -15.07 | 0.15 | ns |

#### D. HEK-293T, NB-DNJ 1 mM

| FOS                   | Untransfected      |       | mock               |       | Difference,<br>mock to<br>untransfected | p    | Summary |
|-----------------------|--------------------|-------|--------------------|-------|-----------------------------------------|------|---------|
|                       | pmol/mg<br>protein | SEM   | pmol/mg<br>protein | SEM   |                                         |      |         |
| M2N (a)               | 17.64              | 0.98  | 20.91              | 3.67  | 3.27                                    | 0.44 | ns      |
| M3N (b)               | 5.78               | 0.67  | 5.50               | 0.06  | -0.28                                   | 0.70 | ns      |
| M3N (c)               | 8.94               | 0.32  | 9.34               | 0.65  | 0.40                                    | 0.61 | ns      |
| M4N (d)               | 4.96               | 0.07  | 5.45               | 0.19  | 0.49                                    | 0.07 | ns      |
| M4N (e)               | 13.02              | 1.50  | 13.49              | 0.86  | 0.47                                    | 0.80 | ns      |
| G1M4N1                | 22.32              | 0.66  | 23.91              | 0.58  | 1.59                                    | 0.15 | ns      |
| M5N                   | 15.26              | 1.01  | 15.54              | 1.07  | 0.28                                    | 0.86 | ns      |
| G2M4N1                | 26.74              | 0.81  | 26.77              | 1.44  | 0.03                                    | 0.98 | ns      |
| G1M5N1                | 18.05              | 0.67  | 18.29              | 0.72  | 0.24                                    | 0.82 | ns      |
| M6N1—2                | 34.69              | 0.53  | 33.80              | 0.46  | -0.89                                   | 0.28 | ns      |
| G2M5N1                | 3.40               | 0.32  | 4.05               | 0.60  | 0.65                                    | 0.39 | ns      |
| G3M5N1                | 33.11              | 0.95  | 34.41              | 1.67  | 1.31                                    | 0.53 | ns      |
| G3M6N1—2/<br>G2M7N1—2 | 35.78              | 0.83  | 37.48              | 0.77  | 1.70                                    | 0.21 | ns      |
| G3M7N1—2              | 72.65              | 1.23  | 72.26              | 1.34  | -0.39                                   | 0.84 | ns      |
| G3M8N1—2              | 107.08             | 1.47  | 105.02             | 3.37  | -2.06                                   | 0.61 | ns      |
| G3M9N1—2              | 112.27             | 1.44  | 106.69             | 2.20  | -5.58                                   | 0.10 | ns      |
| Total FOS             | 759.01             | 11.92 | 762.87             | 15.63 | 3.87                                    | 0.85 | ns      |

## Small FOS correspond to putative endomannosidase products

To determine the origin of the “small FOS” that were relatively increased following endomannosidase transfection in CHO-K1 cells, the corresponding GU values were determined and used to compare with those previously obtained for a range of cellular FOS. To further confirm the structure of the glycans, the eluted “small FOS” were digested with *A. saitoi*  $\alpha$ 1,2-mannosidase, and the migration of the peaks was analysed by HPLC (Fig. 3.3).

Five peaks (Fig. 3.3, *a—e*) were identified as different isomers of GlcNAc<sub>1</sub>-reducing terminus Man<sub>2</sub>–Man<sub>4</sub> oligosaccharides (Table 3.3). Of the identified FOS, only a single species bore the D1 mannose residue (Man<sub>4</sub>GlcNAc<sub>1</sub>, linear isomer – see Table 3.3, D1 mannose shown in bold). In sharp contrast to the other identified Man<sub>2</sub>–Man<sub>4</sub> oligosaccharides, this linear isomer of Man<sub>4</sub>GlcNAc<sub>1</sub> was not increased in endomannosidase-expressing cells (Table 3.2).

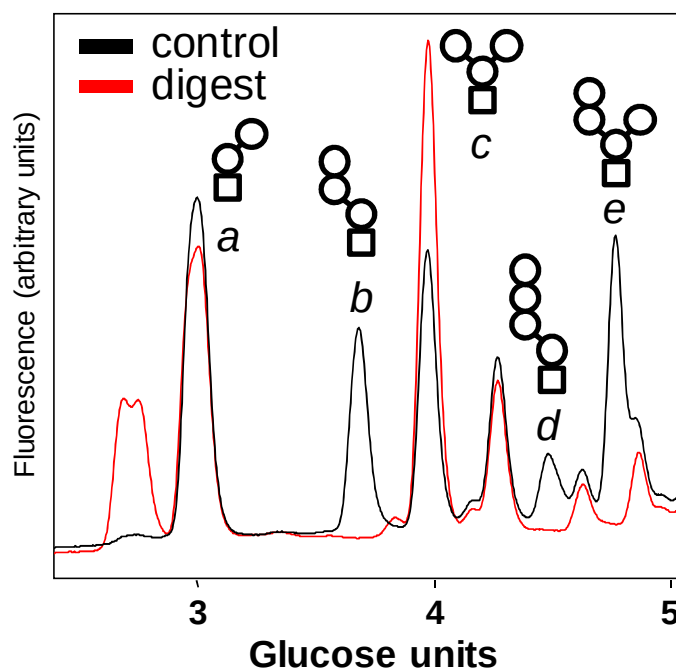
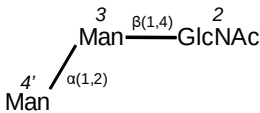
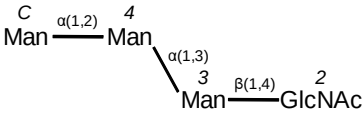
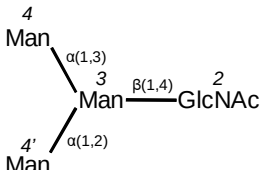
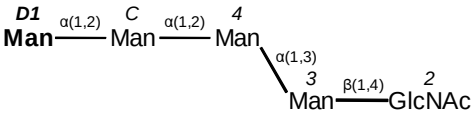
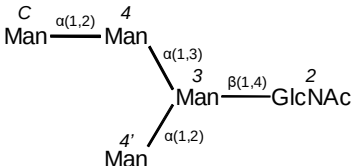


Fig. 3.3. **Identification of small FOS.** 2-AA labeled small FOS were purified by preparative HPLC, digested with *A. saitoi*  $\alpha$ 1,2-mannosidase, and the reaction products were analysed by HPLC. Peak labels and schematic structures refer to Table 3.3.

Since the D1 mannose residue is removed by endomannosidase, the selective increase in the production of FOS lacking this residue suggests that they have originated from endomannosidase-processed glycoproteins. It was previously suggested that endomannosidase-mediated deglycosylation acts as a “bypass mechanism” allowing glycoproteins to evade quality control without compromising their end glycosylation (Torossi et al. 2006). However, the described data hereby suggest the existence of a pathway in which glycoproteins are exposed to post-ER compartments and specifically endomannosidase prior to their deglycosylation in the ER.

In contrast to FOS produced in the conventional ER-confined pathway in the conditions of glucosidase blockage, the glycans released from ERAD substrates processed by endomannosidase are deglycosylated and therefore do not accumulate in the cell. They get rapidly processed by cytosolic and then lysosomal enzymes. The detected “small FOS” are therefore transient and their levels could not be quantitatively compared with those of glucosylated FOS generated as a result of the conventional glycoprotein ERAD. Hence, the decrease in to-

**Table 3.3. Characterisation of small FOS in transfected CHO-K1 cells treated with NB-DNJ.** Transfected CHO-K1 cells were treated with NB-DNJ (1 mM) for 24 h, followed by FOS extraction, fluorescent labeling and analysis by HPLC (Fig. 3.2). Peak areas were analysed and compared for mock- and MANEA-transfected cells using two-tailed Student's t-tests. The nomenclature used refers to the structure below.

| Peak ID  | Nominal formula                                | Structure                                                                           | Per cent in mock (SEM) | Per cent in MANEA (SEM) | <i>p</i> |
|----------|------------------------------------------------|-------------------------------------------------------------------------------------|------------------------|-------------------------|----------|
| <i>a</i> | M2N<br>(Man <sub>2</sub> GlcNAc <sub>1</sub> ) |    | 1.70<br>(0.15)         | 2.84<br>(0.06)          | 0.0019   |
| <i>b</i> | M3N<br>(Man <sub>3</sub> GlcNAc <sub>1</sub> ) |    | 1.40<br>(0.11)         | 3.15<br>(0.10)          | 0.0008   |
| <i>c</i> | M3N<br>(Man <sub>3</sub> GlcNAc <sub>1</sub> ) |  | 1.47<br>(0.09)         | 2.54<br>(0.08)          | 0.0020   |
| <i>d</i> | M4N<br>(Man <sub>4</sub> GlcNAc <sub>1</sub> ) |  | 0.69<br>(0.09)         | 0.63<br>(0.02)          | 0.5678   |
| <i>e</i> | M4N<br>(Man <sub>4</sub> GlcNAc <sub>1</sub> ) |  | 0.89<br>(0.07)         | 3.09<br>(0.22)          | 0.0002   |

tal FOS observed in endomannosidase-expressing CHO-K1 cells could be explained by a greater deglycosylated-to-glucosylated FOS ratio and consequently higher susceptibility of the total FOS pool to cytosolic and lysosomal glycosidases. However, endomannosidase-mediated deglycosylation could also prevent some glycoproteins from recycling back into the ER and entering the degradation pathway, resulting in decreased overall FOS release. Such

stabilisation of glycoproteins by endomannosidase in the conditions of glucosidase inhibition has been previously demonstrated for mutated variants of  $\alpha$ 1-antitrypsin (Torossi et al. 2006).

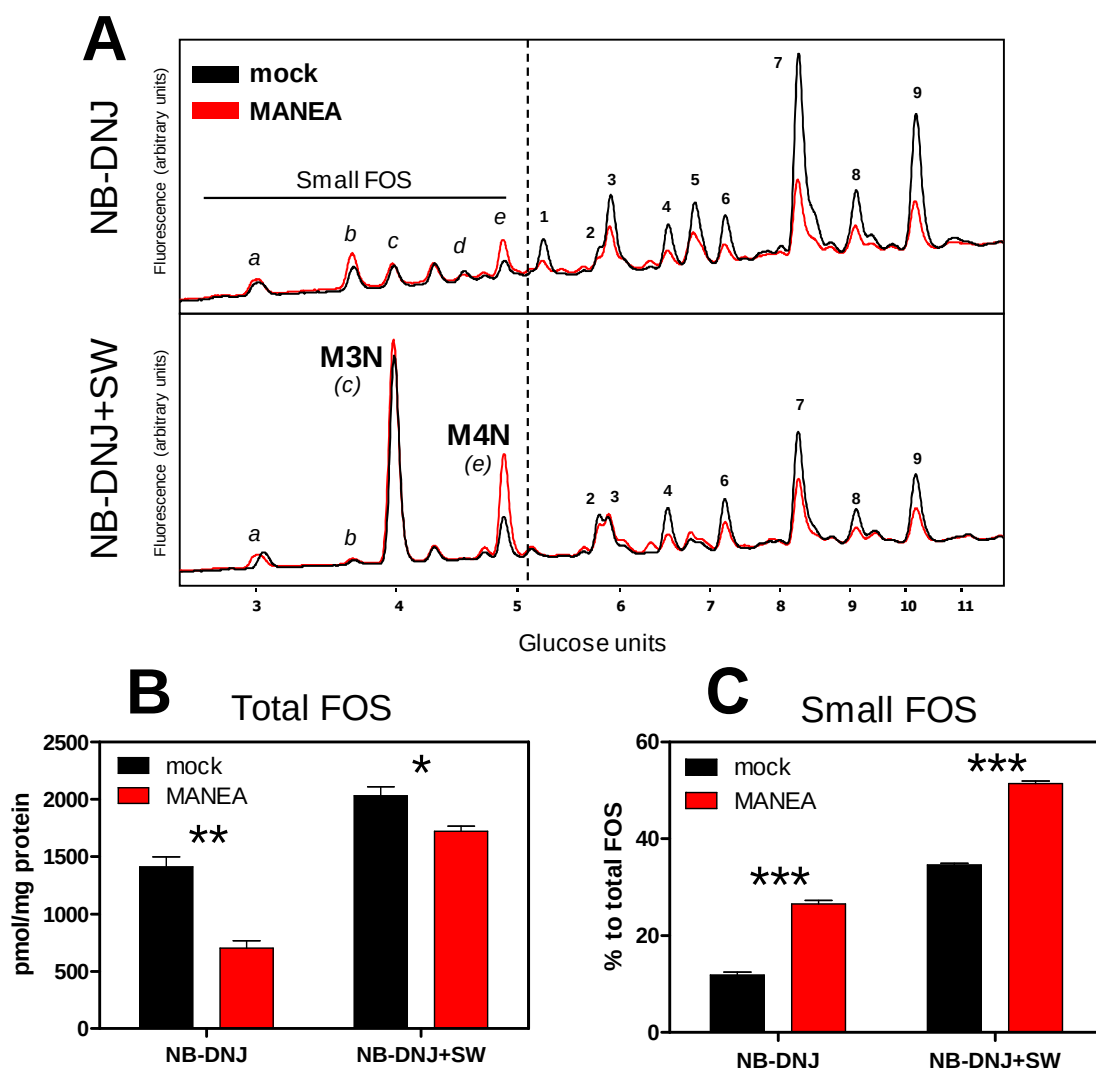


**Fig. 3.4. Swainsonine attenuates the effect of endomannosidase on total FOS levels.** Transfected cells were incubated in the presence of NB-DNJ (1 mM) with or without SW (100  $\mu$ M) for 24 hours. A, HPLC analysis of 2-AA labeled FOS extracted from transfected cells, showing a build-up in small FOS (a–e) following SW/NB-DNJ treatment, more pronounced in endomannosidase-transfected cells. B, quantitation of total FOS (pmol per 1 mg total protein). C, quantitation of small FOS (per cent to total FOS).

To investigate whether both the “FOS-destabilising” and the “glycoprotein-stabilising” mechanisms take place in the cell at the same time, swainsonine treatment was employed. The latter compound is a mannosidase inhibitor primarily used for late-Golgi glycoprotein processing perturbation, but also known to be a potent lysosomal mannosidase inhibitor (Butters

et al. 2009). Swainsonine therefore arrests the catabolism of FOS at the late stages, predominantly causing the accumulation of the core-type  $\text{Man}_3\text{GlcNAc}_1$  (Fig. 3.4, A).

Following cotreatment of transfected CHO-K1 cells with swainsonine and NB-DNJ, the levels of FOS were decreased in endomannosidase-expressing cells, but to a significantly lesser extent than in cells treated with NB-DNJ alone (approx. 44% of the decrease seen in the latter case; Fig. 3.4, B). As expected, the proportion of small FOS was increased in MANEA-expressing cells in both case (Fig. 3.4, C). Since the catabolism of FOS is blocked in the presence of swainsonine, the extent of free oligosaccharide depletion still observed upon swainsonine/NB-DNJ cotreatment was indicative of decreased production rather than increased degradation of FOS, supporting the previous finding that the secretion of glycoproteins is enhanced by endomannosidase in the conditions of glucosidase inhibition. It was therefore concluded that both stabilisation of glycoproteins and destabilisation of FOS contribute to the decrease in FOS levels observed in endomannosidase-transfected CHO-K1 cells.

## **Glucosylated luminal FOS are not the primary source of small FOS**

Glucosidase inhibition by NB-DNJ in a high concentration results in the accumulation of two types of glucosylated FOS, namely cytosolic and luminal. The former are generated by the cytosolic PNGase at the stage of the ER-cytosol retrotranslocation of the glycoprotein targeted for degradation. Rapid action by ENGase results in characteristic  $\text{GlcNAc}_1$ -reducing terminating configuration of the cytosolic FOS.

The production of  $\text{GlcNAc}_2$ -reducing terminating luminal FOS occurs in the ER lumen and is addressed in Chapter 2. In the glucosylated form luminal FOS fail to get transported across the ER membrane and could potentially become subject to ER/Golgi vesicular trafficking and

endomannosidase-mediated deglycosylation. The resulting oligomannose glycans would then escape the ER/Golgi network and enter the cytosolic FOS degradation pathway. Although theoretically possible, this pathway seemed unlikely to provide the primary source for the up-regulation of “small FOS” correlated with endomannosidase expression in the conditions of NB-DNJ treatment (see Fig. 3.2), since the incubation of CHO-K1 cells with NAP-DNJ at 5  $\mu$ M resulted in a similar endomannosidase dependence of total FOS levels and the relative amounts of Man2–Man4 FOS (Fig. 3.5, A, B and C).

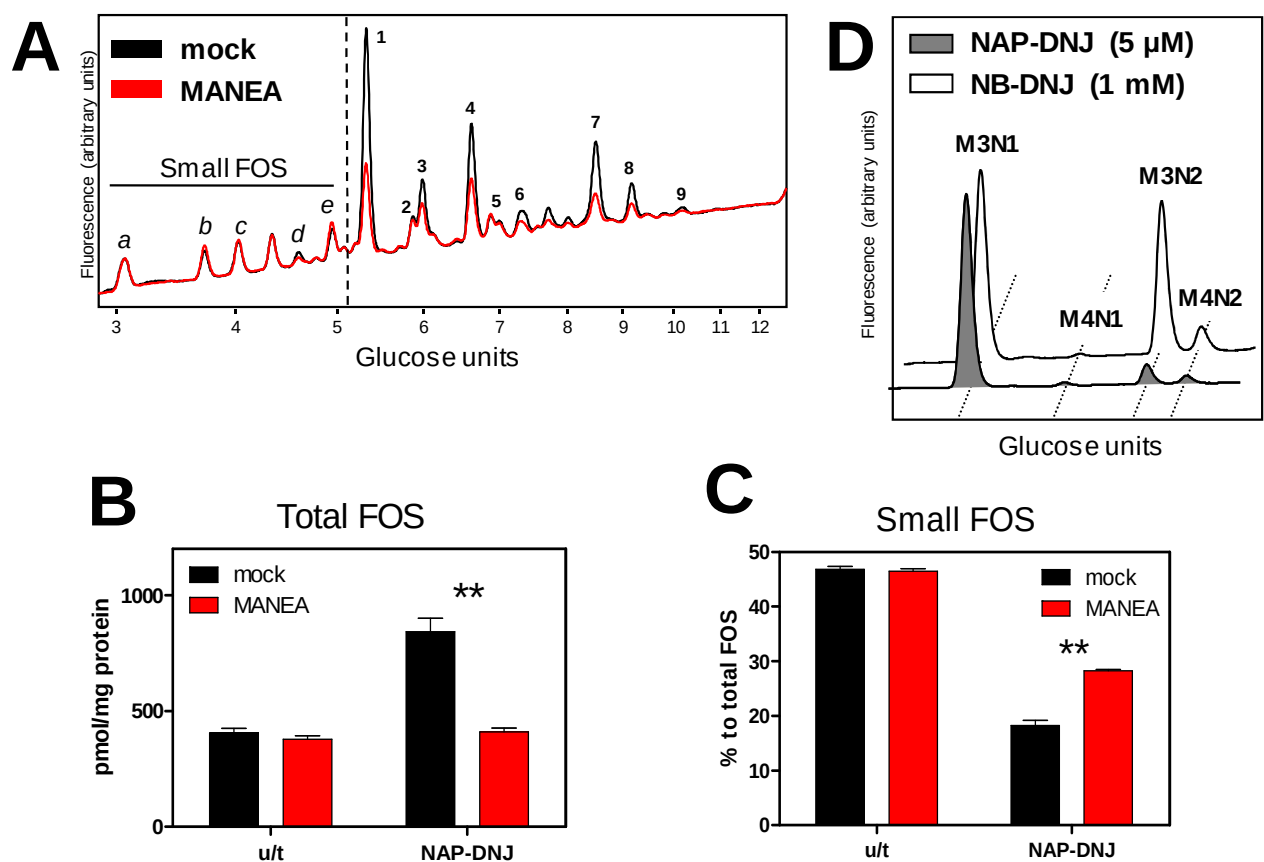


**Fig. 3.5. Independence of endomannosidase effect from glucosylated luminal FOS.** A, HPLC analysis of FOS extracted from mock- or MANEA-transfected CHO-K1 cells incubated for 24 h with NAP-DNJ (5  $\mu$ M). B, quantitation of total FOS (pmol per 1 mg total protein). C, quantitation of small FOS (per cent to total FOS). D, HPLC analysis of 2-AA labeled oligosaccharides obtained by digesting the FOS extracted from NAP-DNJ- or NB-DNJ- treated cells with jack bean  $\alpha$ -mannosidase, followed by isolation of glucosylated FOS and further digestion with recombinant endomannosidase catalytic fragment. Resulting peaks are structurally annotated on the diagram (M3N1=Man<sub>3</sub>GlcNAc<sub>1</sub>, M4N1=Man<sub>4</sub>GlcNAc<sub>1</sub>, M3N2=Man<sub>3</sub>GlcNAc<sub>2</sub>, M4N2=Man<sub>4</sub>GlcNAc<sub>2</sub>).

The treatment with low-concentration NAP-DNJ results in the accumulation of primarily monoglucosylated GlcNAc<sub>1</sub>-reducing terminating oligosaccharides, with hardly any GlcNAc<sub>2</sub>-reducing terminating FOS produced (see Materials and Methods and Fig. 3.6).

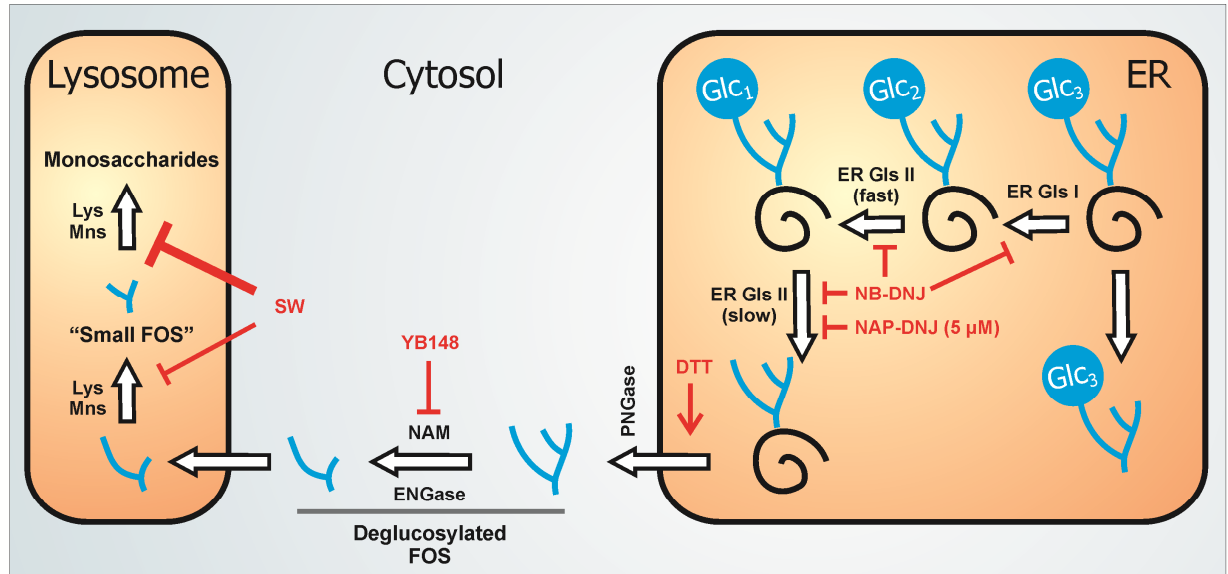


**Fig. 3.6. Treatments used to manipulate cellular FOS.** For detailed overview of ERAD and FOS trafficking, see Chapter 1. Triglycosylated N-linked oligosaccharides are sequentially processed by ER glucosidases (ER Glcs) I and II. NB-DNJ inhibits both enzymes, whereas NAP-DNJ at a low concentration (5 μM) predominantly inhibits the rate-limiting second trimming by ER glucosidase II, resulting in mainly monoglucosylated glycans. Misfolded proteins are degraded via ERAD which involves retrotranslocation and glycan removal by PNGase, resulting in FOS. DTT induces UPR and therefore upregulates ERAD and the amount of FOS produced. GlcNAc<sub>2</sub>-reducing terminating FOS can also be produced lumenally and in the triglycosylated form do not escape into the cytosol. Deglucosylated FOS are processed in the cytosol by ENGase and NAM, which is inhibited by YB148. Man<sub>5-6</sub>GlcNAc<sub>1</sub> FOS are transported into the lysosome and further processed by a lysosomal mannosidase (Lys Mns) which is inhibited by swainsonine. "Small FOS" are intermediates in the lysosomal FOS degradation.

Therefore, to confirm that the primary source of the upregulated "small FOS" is the deglucosylation of glycoproteins rather than deglucosylation of luminal FOS, the ratio between GlcNAc<sub>1</sub>- and GlcNAc<sub>2</sub>-reducing terminating glucosylated oligosaccharides was evaluated in NB-DNJ- (1 mM) and NAP-DNJ- (5 μM) treated cells. For this purpose, total FOS isolated from CHO-K1 cells were first digested with jack bean α-mannosidase. This treatment results in all non-glucosylated FOS being processed up to Man<sub>1</sub>GlcNAc<sub>1-2</sub>.

Glucosylated species are protected from extensive trimming by the glucose residues and as a result are processed up to Man<sub>4-5</sub> oligosaccharides. The resulting difference in molecular weight allows for separation of glucosylated and deglycosylated FOS using preparative HPLC. Following isolation of the glucosylated species, the glycans were digested with the recombinant human endomannosidase catalytic fragment, resulting in Man<sub>3-4</sub>GlcNAc<sub>1-2</sub> oligosaccharides. These final products are clearly distinguishable by HPLC, facilitating discrimination between GlcNAc<sub>1</sub> and GlcNAc<sub>2</sub>-reducing terminating FOS (Fig. 3.5, D). In cells treated with 5  $\mu$ M NAP-DNJ GlcNAc<sub>1</sub>-reducing terminating glycans comprised approx. 90% of total glucosylated FOS, compared to approx. 55% in cells treated with 1 mM NB-DNJ.

These data confirm the observation that treatment of CHO-K1 cells with NAP-DNJ in a low concentration results in the accumulation of glucosylated FOS that are almost exclusively cytosolic. At the same time, endomannosidase expression together with the treatment causes an approx. 50% upregulation of non-D1-terminating Man<sub>2</sub>–Man<sub>4</sub> oligosaccharides (Fig. 3.5, C). It was therefore concluded that the primary source of “small FOS” is not glucosylated luminal FOS but glycoproteins undergoing ERAD through an ER-Golgi-ER shuttling route.

### **The effect of endomannosidase on FOS is dependent on substrate glucosylation**

To verify that the processing of glucosylated substrates by endomannosidase is the cause of the changes in the FOS profiles of CHO-K1 cells expressing the enzyme, glucosidase inhibitors were substituted for either dithiothreitol, a commonly used UPR-inducing agent, or YB148, a cytosolic mannosidase inhibitor arresting the catabolism of FOS following their release in the cytosol (Butters et al. 2009). The effects of these compounds on free oligosaccharides paralleled those of glucosidase inhibitors, namely induction of unfolded protein response

and increasing the rates of ERAD (Pahl et al. 1995), or stabilising the cytosolic pool of FOS, respectively. However, glucosidase-mediated deglycosylation of N-linked glycans was unaffected (see Fig. 3.7).

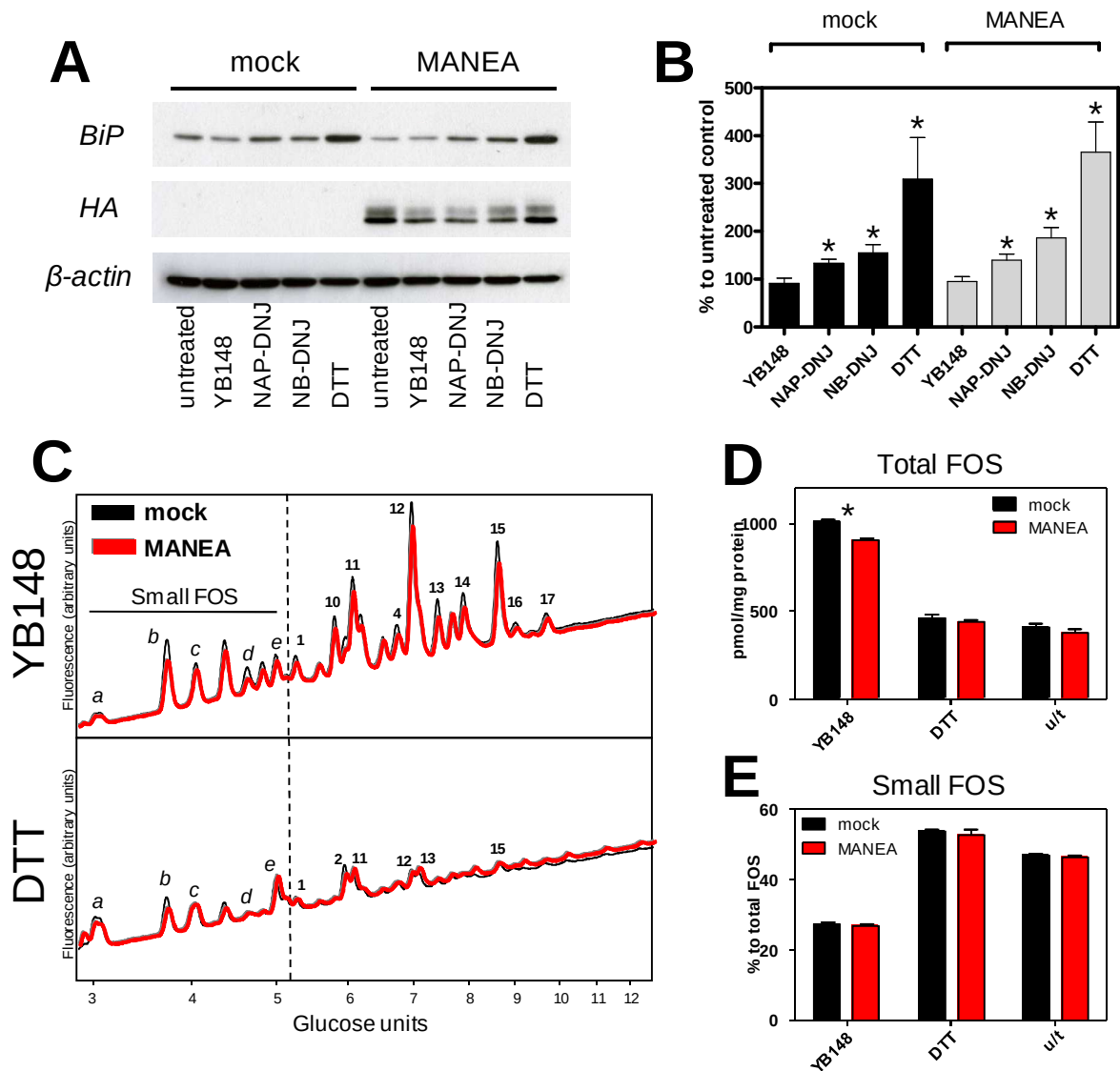


**Fig. 3.7. Endomannosidase effect on free oligosaccharides in the presence of YB148 or DTT.** A, western blotting analysis of crude protein extracts from mock- or MANEA-transfected CHO-K1 cells following treatment with YB148 (100  $\mu$ M), DTT (2 mM), NAP-DNJ (5  $\mu$ M) or NB-DNJ (1 mM). B, densitometric evaluation of BiP upregulation in CHO-K1 cells. BiP/GRP78 blots were analysed using ImageJ, standardised to  $\beta$ -actin loading controls, and the resulting values plotted in percent to untreated controls. Asterisks represent p-values in a single-sample Student's t-test. C, HPLC analysis of FOS extracted from mock- or MANEA-transfected CHO-K1 cells incubated for 24 h with YB148 or DTT. D, quantitation of total FOS (pmol per 1 mg total protein). E, quantitation of small FOS (per cent to total FOS).

To monitor the induction of UPR, the levels of BiP/GRP78 were compared densitometrically following western blotting of cell lysates. As expected, DTT caused the strongest upregulation of BiP, whereas treatment with glucosidase inhibitors resulted in a less pronounced response (Fig. 3.7, A, B). No significant changes in BiP levels were detected upon treatment with YB148. Consistent with the expected effect of YB148, the compound caused a 2.5-fold increase in total FOS levels, whereas DTT did not significantly affect the total amount of FOS produced (Fig. 3.7, C, D). The increase in FOS levels by YB148 and the stimulation of BiP/GRP78 expression by DTT confirm the expected effects of the compounds on ERAD. However, the effect of endomannosidase on FOS in cells treated with YB148 or DTT was much less pronounced than in the case of glucosidase inhibitors. Endomannosidase expression in YB148-treated CHO-K1 cells caused a subtle but significant decrease in the amounts of total FOS (Fig. 3.7, C, D). No changes in the relative amounts of Man<sub>2</sub>–Man<sub>4</sub> oligosaccharides were detected, consistent with the YB148-mediated arrest of FOS catabolism on the early stages (Fig. 3.7, E).

The effect of endomannosidase in DTT-treated cells was not significant. This could be partly due to the fast turnover of FOS not stabilised by glycosidase inhibitors, and also due to selective retention of some of the newly synthesised proteins in the ER following treatment by DTT (Braakman et al. 1992; Kaji et al. 1993; Valetti et al. 1994), resulting in decreased overall exposure of cellular glycoproteins to endomannosidase. Taken together, these findings confirm that the observed effect of endomannosidase expression in CHO-K1 cells is directly related to the processing of glucose-bearing substrates, the amounts of which are increased due to glucosidase inhibition.

## Endomannosidase knockdown in HEK-293T cells leads to increased FOS production

To verify the findings of the effect of endomannosidase on FOS production, shRNA-mediated knockdown of the enzyme in HEK-293T cells was performed. Four shRNA-encoding vectors (pGFP-V-RS, #1–#4, OriGene) were tested for their ability to knock down endomannosidase. Prior to the knockdown experiments, two commercially available antibodies (Sigma Cat. No. HPA011046 and SAB2101423) were tested in western blotting experiments. However, detection of specific bands at the predicted molecular weight did not succeed for any of the cell lines tested, suggesting that endogenous endomannosidase in the cells used was below the level of immunological detection. The latter forced efficient knockdown to be verified by co-transfecting HEK-293T cells with the shRNA-encoding vectors and the MANEA-HA plasmid, similar to the strategy recently employed for ER mannosidase I (Termine et al. 2009). HA-tagged endomannosidase expression was then assayed using western blotting (Fig. 3.8, A).

Out of the four shRNA constructs tested, #4 appeared to be most efficient (>90% attenuation of MANEA-HA overexpression). Knockdown was verified by an activity assay using a triglucosylated substrate. Breakdown of 2-AA labelled  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_2$  oligosaccharide (1 pmol in 10  $\mu\text{l}$ ) in cell extracts was slowed down dramatically following transfection with #4. At the linear phase of the reaction, the slope of the kinetic curve was decreased 9.7-fold (Fig. 3.8, B).

The construct was employed in subsequent experiments alongside with the scrambled control vector. HEK-293T cells transfected with the shRNA-encoding plasmid were treated with NB-DNJ (1 mM), and FOS were analysed as described for CHO-K1 cells. Endomannosidase knockdown resulted in both an absolute increase in total FOS produced, and a relative in-

crease (per cent to total FOS) in glucosylated oligosaccharides (Fig. 3.8, C, D). The latter included  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$ , representing the major product of glucosylated FOS cytosolic processing (see Chapter 2), and a group of incompletely demannosylated lumenal and cytosolic FOS composed of  $\text{Glc}_3\text{Man}_{7-9}\text{GlcNAc}_{1/2}$  oligosaccharides. At the same time, a relative increase was observed in some of the deglycosylated FOS, most strikingly the  $\text{Man}_6\text{GlcNAc}_{1/2}$  isomer A, representing a characteristic product of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_{1/2}$  deglycosylation by endomannosidase or demannosylation of the equivalent  $\text{Man}_7$  and  $\text{Man}_8$  species (generated from  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_{1/2}$  or  $\text{Glc}_3\text{Man}_8\text{GlcNAc}_{1/2}$  species, respectively). Peak identity was based on the reported GU value for the corresponding oligosaccharide (Alonzi et al. 2008), and the structure was confirmed by digesting total 2-AA labeled FOS with recombinant endomannosidase catalytic fragment, resulting in the shift of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_{1/2}$  GU value to that of the peak downregulated upon endomannosidase knockdown (Fig. 3.8, E).

Although no statistically significant changes in small FOS corresponding to  $\text{Man}_{2-4}\text{GlcNAc}_1$  were observed, the decreased level of  $\text{Man}_6\text{GlcNAc}_{1/2}$  isomer A reflects the reduction in endomannosidase activity in a similar fashion to the increase in the relative  $\text{Man}_{2-4}$  FOS levels following recombinant endomannosidase expression in CHO-K1 cells. The less pronounced changes in the levels of free oligosaccharides in HEK-293T cells may be due to cell-specific kinetics of cytosolic and lysosomal FOS degradation, which is supported by the fact that  $\text{Man}_{7-9}$  FOS are primarily associated with the cytosol and become subject to active cytosol-to-lysosome transport in the  $<\text{Man}_7$  state (Chantret et al. 2010). It is therefore likely that  $\text{Man}_6\text{GlcNAc}_{1/2}$  exists in both cytosol and lysosome, resulting in a higher overall half-life in the cell, which could lead to higher levels of the oligosaccharide in the steady state and greater susceptibility to endomannosidase knockdown.

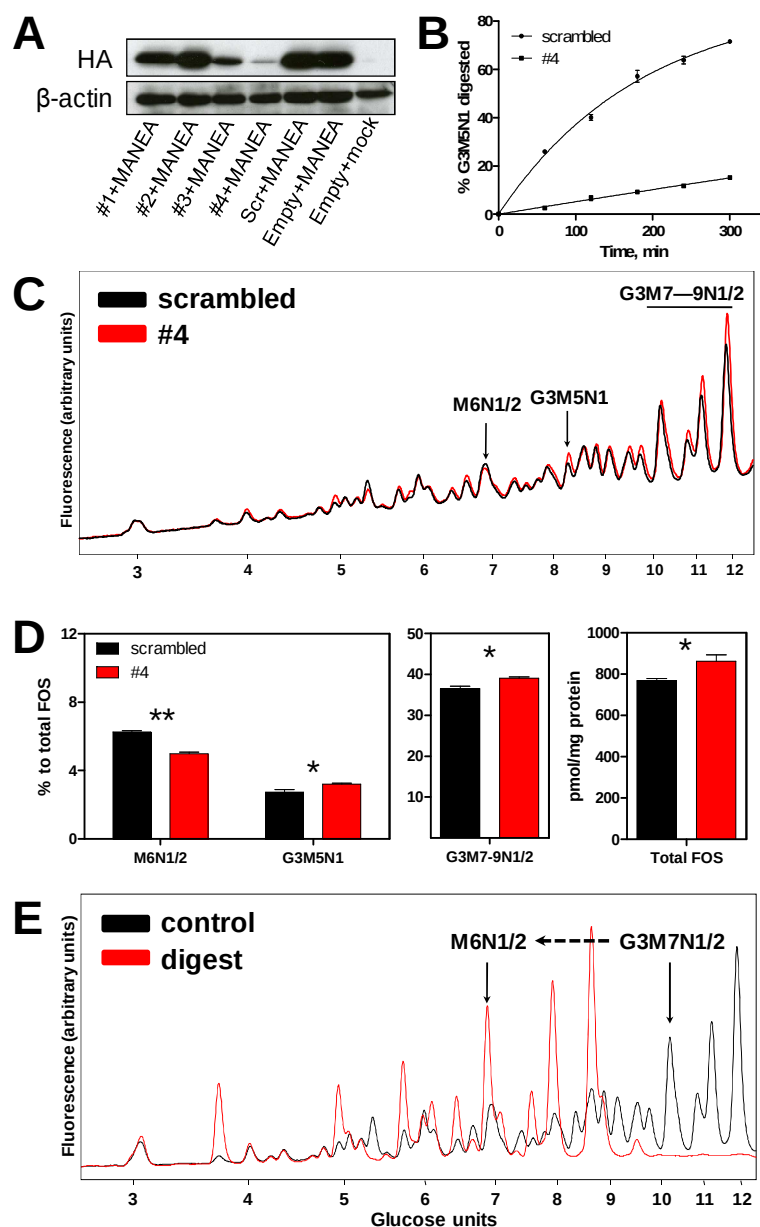


**Fig. 3.7. Effect of endomannosidase knockdown on free oligosaccharide production.** A, western blotting analysis of MANEA-HA overexpression in HEK-293T cells upon co-transfection with MANEA/mock plasmids and pGFP-V-RS vectors containing shRNA-encoding inserts (#1—#4), a scrambled insert (scr) or no insert (empty). B, endomannosidase activity in transfected cell membrane extracts. 2-AA labeled  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  (1 pmol in 10  $\mu\text{l}$ ) was used as a substrate in the presence of NB-DNJ, DMJ and EDTA. Reactions were carried out for various time periods, deproteinated and analysed by HPLC. C, HPLC analysis of 2-AA labeled FOS extracted from HEK-293T cells transfected with vectors containing the shRNA insert #4 or the scrambled insert and treated with NB-DNJ (1 mM) for 24 h. D, relative amounts of  $\text{Man}_6\text{GlcNAc}_{1/2}$  (M6N1/2),  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  (G3M5N1),  $\text{Glc}_3\text{Man}_7-9\text{GlcNAc}_{1/2}$  (G3M7—9N1/2) in NB-DNJ-treated HEK-293T cells transfected with #4/scrambled, plotted in percent to total FOS, and total levels of FOS extracted from the cells, plotted in pmol per 1 mg total protein. Data shown are representative of three independent experiments. E, HPLC analysis of 2-AA labeled FOS extracted from HEK-293T cells treated with NB-DNJ (1 mM) and incubated with either PBS (control) or human endomannosidase catalytic fragment (digest) for 16 h.

# Discussion

Glucosylated FOS generated in the presence of glucosidase inhibitors are a valuable analysis tool primarily due to their arrested catabolism. Cells deficient in endomannosidase activity (CHO-K1 and MDBK) treated with glucosidase inhibitors are unable to remove glucose residues from protein-linked glycans using either of the two available mechanisms. Using this model, two separate populations of glycoprotein-derived FOS were identified, of which one (cytosolic) was accumulating in all cell lines tested, whereas the other (lumenal) was only present in large amounts in MDBK and CHO-K1 cells (see Chapter 2). Together with additional evidence pointing to the involvement of the Golgi apparatus in the pathway leading to the production of lumenal FOS, these data have allowed proposing that the absence of endomannosidase activity is a prerequisite for the accumulation of glucosylated lumenal FOS. Two possibilities for the role of endomannosidase were considered:

1. Endomannosidase deficiency leads to a glycoprotein degradation pathway that does not exist when the enzyme is active. Post-ER deglucosylation therefore rescues potential ERAD substrates, allowing them to proceed in the secretory pathway. This would suggest that the fate of a glycoprotein molecule in the Golgi apparatus does not entirely depend on its folding status, since it is unlikely that removal of glucose residues itself could physically affect folding.
2. By deglucosylating glycoproteins which are subsequently degraded, endomannosidase activity masks a “recycling” ERAD pathway that exists in all cells. Post-ER deglucosylation therefore simply prevents lumenal FOS from accumulating. In this case, the degradation pathways are dependent on ERAD determinants which are present in the substrate molecule irrespective of its deglucosylation.

In either case, a selective decrease in Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> FOS following endomannosidase overexpression in CHO-K1 cells was anticipated. Unexpectedly though, the ratio between luminal and cytosolic FOS was not altered in MANEA-transfected cells. Instead, the observed effect involved a global decrease in both luminal and cytosolic FOS. It was further shown that the decrease in the levels of glycoprotein-derived oligosaccharides stems from both the downregulation of glycoprotein degradation and the production of deglycosylated FOS that do not stably accumulate in the cell. The latter is of particular importance since it is the first direct indication that endomannosidase-processed glycoproteins can undergo ERAD rather than progressing in the secretory pathway.

Contrary to the initial hypothesis, endomannosidase overexpression affected not only luminal, but also cytosolic FOS. Since the ratio between the two populations remained unaltered, it is likely that the mechanism underlying FOS reduction is the same in both cases. It had to be therefore concluded that recycling between ER and Golgi does not necessarily lead to the production of luminal FOS, but is characteristic of proteins destined for both of the ERAD pathways discussed in Chapter 2. The seeming contradiction of this conclusion to those of Chapter 2 is resolved by assuming that the commitment of glycoproteins to either the “cytosolic FOS” or the “luminal FOS” pathway is determined not by a single ER-Golgi-ER translocation, but rather by the level of exposure to the Golgi apparatus enzymes. In the simplest explanation, differential exposure could stem from a different number of recycling rounds, i.e. the more times a glycoprotein recycles between ER and Golgi, the more likely is it to become substrate to the alternative luminal FOS-generating ERAD. However, highly overexpressed endomannosidase would saturate the post-ER deglycosylation pathway, leading to efficient deglycosylation of all recycling proteins independently of their Golgi residence time. The influence of the latter on the ERAD route is therefore masked in an endomannosidase-overexpressing cell. The reverse experimental approach, i.e. reduction of endomannosidase

activity in MANEA-positive cells, should provide a better model for studying the connection between luminal FOS, endomannosidase activity and Golgi residence time. However, shRNA-mediated knockdown employed in this work does not completely abolish enzyme activity or result in an effect strong enough to evaluate the cytosolic/luminal FOS ratio. It would therefore be of great interest to assess the effect of endomannosidase pharmacological inhibition on FOS in NB-DNJ-treated cells, specifically in the HL60 cell line previously shown to accumulate a pattern of FOS highly similar to that of CHO-K1 and MDBK cells, with the exception of the luminal Glc<sub>3</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> free oligosaccharide.

To sum up, it was sought to determine whether luminal FOS-generating ERAD is specific to endomannosidase-deficient cells, or simply unmasked when post-ER deglycosylation is impaired. Evidence was obtained that endomannosidase processing does not necessarily prevent glycoprotein degradation, pointing to the latter option. However, endomannosidase expression affected the accumulation of cytosolic FOS to the same extent as that of the luminal species, indicating that recycling of ERAD substrates could at least to some extent take place in both of the degradation sites.

The extent to which endomannosidase-mediated deglycosylation occurs upstream of ER-associated degradation *in vivo* in the absence of glucosidase inhibitors is currently unknown. However, the existence of this route demonstrated in the present study raises several intriguing questions concerning glycoprotein quality control and ER-associated degradation. Since misfolded mutant glycoproteins are, at least in some cases, able to proceed in the secretory pathway following processing by endomannosidase (Torossi et al. 2006), it is considered unlikely that an additional quality control checkpoint operates at a post-ER location. On the other hand, the fact that anterograde (secretion) and retrograde (degradation) pathways may co-exist at this stage challenges this view and raises the possibility that, should the post-ER sorting mechanism be present, the abnormal secretion could be a consequence of its oversatura-

tion. This speculation appears even more reasonable in view of the fact that following glucosidase inhibition the traffic through the endomannosidase pathway would be expected to greatly exceed that in normal physiological conditions. One possibility for the quality mechanism underlying retrieval of misfolded substrates from the Golgi to the ER is binding to ER-resident chaperones bearing ER retention signals, e.g. BiP or GRP94.

Overall, by employing the method of free oligosaccharide analysis to gain a global view of ER-associated degradation, (i) it was established that endomannosidase activity does not directly affect the production of either luminal or cytosolic FOS, and (ii) a novel glycoprotein processing route which coexists with the canonical pathways for secretion and ER-associated degradation (Fig. 3.9) was identified. This route involves anterograde transport of glycoproteins from the endoplasmic reticulum, followed by their deglucosylation by endomannosidase and subsequent retrograde transport resulting in ERAD and the production of deglucosylated free oligosaccharides. The latter could be generated in both the “luminal” and the “cytosolic” pathways discussed in Chapter 2.

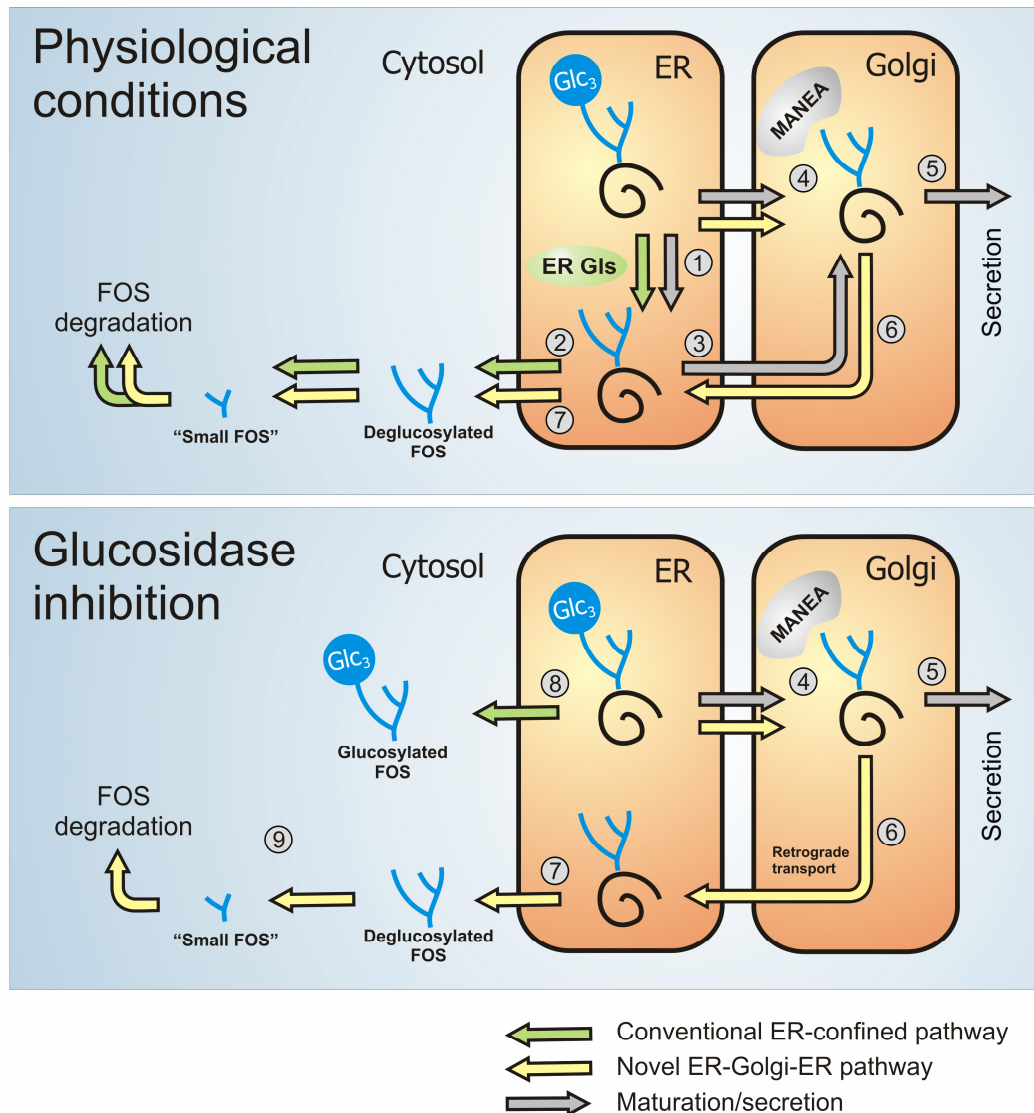


Fig. 3.9. **Proposed model for the novel ERAD pathway.** In physiological conditions (top panel), nascent glycoproteins are deglucosylated predominantly by ER glucosidases I and II (ER Glcs) as part of ER quality control (1). Aberrant glycoproteins are subjected to the conventional ER-confined degradation pathway, resulting in the production of deglucosylated FOS (2). Normal glycoproteins proceed further to the Golgi apparatus for maturation and secretion (3). A fraction of glucosylated glycoproteins escapes the quality control in the ER and gets transported into the Golgi, allowing for deglucosylation by endomannosidase (MANEA) (4). The resulting glycoproteins then enter normal maturation pathways (5). Alternatively, endomannosidase-processed glycoproteins can be targeted back to ER (6) and become subject to ERAD targeting, producing FOS (7). However, in physiological conditions the FOS generated in the latter pathway are indistinguishable from those produced in the conventional ER-confined ERAD. This is opposed to the conditions of glucosidase inhibition (lower panel). In this case, conventional ERAD results in the production of glucosylated FOS that accumulate in the cytosol (8). The ER-Golgi-ER pathway retains a functional deglucosylation step, therefore resulting in deglucosylated FOS (7), eventually subjected to complete degradation via  $\text{Man}_2\text{-Man}_4$  "small FOS" (9).

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# Chapter 4. Species specificity of endomannosidase activity

## Introduction

Studying glycoprotein degradation by employing free oligosaccharide analysis has allowed us to gain a global view of the enzymatic and trafficking processes underlying ERAD targeting. The data presented in Chapter 3 have emphasised the involvement of endomannosidase, a post-ER enzyme, in processing misfolded proteins that are subsequently subjected to one of the two degradation pathways characterised in Chapter 2.

Endomannosidase activity is cell-specific and varies dramatically between tissues and organisms. Previous studies have assessed the degree of endomannosidase involvement in the processing of viral envelope glycoproteins in the presence of glucosidase inhibitors (Karaivanova et al. 1998). This was shown to range between complete absence of processing to essentially unimpaired deglycosylation. The same study has analysed endomannosidase activity by measuring the processing of oligosaccharide substrates by various cell extracts, and produced somewhat conflicting results. In some cases, the measured endomannosidase activity was consistent between *in vitro* tests and cellular experiments, e.g. relatively high levels were demonstrated in both cases for BHK-21, BW5147, HepG2 cells, and no activity was detected in CHO-K1 cells in either of the experiments (Karaivanova et al. 1998). However, MDBK as

well as MDCK cells were unable to process glucosylated viral envelope glycoproteins, but demonstrated average levels of endomannosidase activity in cell extracts.

Experiments on free as well as protein-linked glycans generated in the presence of glucosidase inhibitors have additionally indicated that MDBK cells appear to be similar to CHO-K1 in the processing of recycling glycoprotein substrates (see Chapter 2). These data therefore supported the absence of endomannosidase activity in MDBK cells. However, the striking contrast between the reported MDBK and CHO-K1 *in vitro* endomannosidase activity raised questions about the importance of the pathway *in vivo*. Since the enzyme that is present and active in the cell does not appear to be necessarily involved in glycoprotein deglucosylation, two explanations seemed likely: either endomannosidase conservation is dependent on some additional functions rather than its deglucosylating capacity, or the processing of glycoproteins by endomannosidase requires specific conditions/substrates different from those employed in the experimental system used.

Understanding glycoprotein processing in MDBK cells is of particular importance because of the use of the cell line in studying viral infection. Bovine cells serve as a host cell line for the bovine viral diarrhea virus (BVDV), a surrogate model for the hepatitis C virus (HCV) (Xue et al. 1993). Several inhibitors targeting viral glycosylation have been developed as a potential HCV treatment, some of which have been tested in the MDBK-BVDV model prior to *in vivo*/clinical trials (Branza-Nichita et al. 2001; Jordan et al. 2002).

The aim of this part of the project was to investigate the cell specificity of endomannosidase activity in more detail in attempt to understand the role of the enzyme in glycan processing and/or other cellular processes better. In particular, experiments were focused on MDBK endomannosidase and the apparent discrepancy between its activity *in vitro* and in cell culture. Additionally, BHK-21 and CHO-K1 cells were used as a positive and negative control, re-

spectively. While BHK-21 demonstrated unambiguously high endomannosidase activity, CHO-K1 cells have been shown to express a mutant form of the enzyme which is mislocalised and devoid of enzymatic activity due to a formation of an internal disulphide bond (Torossi et al. 2007).

## Materials and methods

### Post-nuclear membranes concentration

*In vitro* experiments were carried out on postnuclear membranes of the various cell lines. For this purpose, a previously described method for purifying and concentrating membrane proteins (Lubas et al. 1987) was used. Harvested, washed cells were resuspended in 4 volumes 0.1 M NaMES buffer (pH 6.5), containing 0.5 mM dithiothreitol, and disrupted using a Branson sonifier (3×10 sec bursts, set. 3). The lysates were then subjected to a low-speed centrifugation (600g, 10 min), followed by a high-speed centrifugation (135,200×g, 15 min at 4°C) of the obtained supernatant. The resulting pellet was washed in the lysis buffer, and the high-speed centrifugation repeated. After discarding the supernatant, the washed pellet was resuspended in 100 µl 0.1 M NaMES buffer (pH 6.5), containing 0.02% NaN<sub>3</sub>. An aliquot was taken for protein assay.

### *In vitro* endomannosidase assay

Glucosylated free oligosaccharide substrates for endomannosidase assays were isolated from NB-DNJ-treated MDBK cells as described in Chapter 2. Individual substrates were then purified by preparative HPLC and concentrated by drying and resuspending in the appropriate amount of water. Endomannosidase activity was determined in a manner similar to methods

previously described (Lubas et al. 1987). 20 µl Aliquots of the membrane preparations diluted up to a total protein concentration of 10 or 20 mg/ml were pre-incubated for 1 hour in 0.1 M NaMES buffer (pH 6.5), containing 10 mM EDTA, 2 mM NB-DNJ, 2 mM deoxymannojirimycin and 0.2% Triton X-100, followed by addition of the 2-AA labeled substrate. Reaction was stopped by the addition of 2 µl glacial acetic acid and the reaction mix was passed through a 10,000 molecular weight cut-off filter. The resulting solution was then either analysed by HPLC, or relabeled with 2-AA as described previously and purified using PGC and ethyl acetate phase separation. PGC column purification was carried out as described in Chapter 2. The resulting solution in 50% acetonitrile was then subjected to ethyl acetate extraction of hydrophobic non-carbohydrate contaminants (i.e., free 2-AA). Ethyl acetate was added to the samples at a ratio of 3:2 (v/v), vortexed for one minute and centrifuged briefly. The upper organic phase was discarded, and the procedure was repeated three times. Samples were dried, resuspended in 50% acetonitrile and analysed by HPLC.

## Construction of plasmids for bacterial expression

Truncated human endomannosidase ORF (hMANEA) was amplified from an IMAGE cDNA clone in a pCR4 plasmid (IMAGE:9021009, GenBank® BC137016.1; Geneservice) using the primers:

5'-TATATAAGAATCCCATATGGGTTTAAAAATGCTGAGACCAAATACAGC-3'

5'-ATAATTGTAGCTCGAGTCCAGAACTCAAAACGAAAGC-3'

For the construct with the C-terminal hemagglutinin tag (hMANEA-HA), the reverse primer was replaced with the following:

5'-TAGCTCTCGAGTTAAGCGTAATCTGGAACATCGTATGGGTAAGAAACAGGCAGCTGGCGATCT-3'

The resulting product was gel purified and digested with NdeI and XhoI, and the fragment obtained was ligated into the pET15b expression vector following gel purification of both digested vector and insert.

## **Amplification of MDBK MANEA ORF**

Total RNA was isolated from trypsinised, washed with PBS, pelleted MDBK cells using TRI-reagent (Sigma, UK) following manufacturer's instructions. RNA was dissolved in DEPC-treated water, aliquoted and stored at  $-70^{\circ}\text{C}$ . For 3'-end amplification, cDNA was synthesised using a dT<sub>12</sub>-AP primer (5'-GACTCGAGTCGACATCGTTTTTTTTTTTTTT-3'). 3'RACE procedure (Frohman et al. 1988) was carried out with a gene-specific forward primer (P1: 5'-CCCAAAGGGAAGCACAACCCCC-3') and an adaptor primer (AP: 5'-GACTCGAGTCGACATCG-3') using a gradient of annealing temperatures (50–60°C) and extended first cycle DNA synthesis time. Nested PCR was carried out on the obtained product using P1 as a forward primer and an extra gene-specific reverse primer designed to anneal to the 3'-UTR of the cDNA (P2: 5'CCCATAACCCAAAACAAAAGG3').

For 5'-end amplification, cDNA was synthesised using the P2 primer. The desired fragment was amplified using direct PCR, using a forward primer annealing to the predicted 5'-UTR (P3: 5'CCATTTTGGAGTTTAAAGGATGA3') and a reverse primer other than P2 (P4: 5'TCCTGGGCCCACACTTGGA3'). The complete ORF was obtained by ligating the two fragments using a unique BamHI restriction site located within the overlap. The resulting sequence, termed bMANEA, was then cloned into the pET15b vector as described for hMANEA.

## Endomannosidase expression in *E. Coli*

The following protocol was optimised extensively primarily for highest levels of purity of the end products, but also for higher yields. BL21/DE3 *E. Coli* competent cells (Stratagene, UK) were transformed with pET15b-hMANEA, pET15b-bMANEA or pET15b-hMANEA-HA according to the manufacturer's instructions. A culture volume of 10 ml was inoculated with a single clone and incubated at 37°C at 200—250 RPM overnight. If higher amounts were required, the culture was further split and incubated as appropriate. For large-scale expression, 10 ml culture per 1L standard LB medium inoculation ratios were used. Cultures were grown to OD<sub>600</sub>=0.7, followed by addition of IPTG up to a concentration of 1 mM. Induction was carried out for 30 min, and cultures were rapidly cooled to 4°C by vigorous stirring in a glass beaker immersed in a dry ice/methanol bath. Cultures were then centrifuged at 6000g, media discarded, and pellets stored at –70°C. Expression was verified by subjecting aliquots of induced/uninduced cultures to reducing SDS-PAGE followed by either Coomassie Blue staining or immunoblotting against 6×His tag or HA tag.

## Inclusion body isolation

The following procedure is described for a culture volume of 1 L. In most cases, the protocol was scaled up 3× per extraction. Bacterial pellets were thawed at room temperature and re-suspended in 13 ml buffer 1 (Tris-HCl buffer 50 mM, sucrose 25% (w/v), NaEDTA 1 mM, β-mercaptoethanol 10 mM, sodium azide, 0.1% w/v, pH 8.0). The resulting suspension was sonicated (Branson sonifier set. 5, 30×1 s pulses, on ice), supplemented with lysozyme (100 μl, 50 mg/ml), deoxyribonuclease (250 μl, 1 mg/ml in 50% (v/v) glycerol and 75 mM NaCl) and MgCl<sub>2</sub> (50 μl, 0.5 M) and vortexed. Following addition of 12.5 ml buffer 2 (Tris-HCl buffer 50 mM, Triton X-100 1% (v/v), sodium deoxycholate 1% (w/v), NaCl 100 mM, β-mercaptoethanol 10 mM, sodium azide 0.1% (w/v), pH 8.0), the suspension was vortexed and

incubated for 1 h at room temperature. NaEDTA was added (350  $\mu$ l, 0.5 M in 50 mM Tris-HCl buffer, pH 8.0), after which the suspension was vortexed and frozen in liquid nitrogen, followed by thawing at 37°C for 30 min or until complete defrosting. MgCl<sub>2</sub> was added again (200  $\mu$ l, 0.5 M) and incubated for 30 min at room temperature, followed by addition of NaEDTA (350  $\mu$ l, 0.5 M in 50 mM Tris-HCl buffer, pH 8.0) and centrifugation (30 min, 11,000g, 4°C). The supernatant was discarded, and the obtained pellet was resuspended in 10 ml cold (4°C) buffer 3 (Tris-HCl buffer 50 mM, Triton X-100 0.5% (v/v), NaCl 100 mM,  $\beta$ -mercaptoethanol 10 mM, sodium azide 0.1% w/v, pH 8.0), and the after sonication (Branson sonifier set. 5, 30 $\times$ 1 s pulses, on ice) the centrifugation step was repeated. The wash was then repeated, substituting buffer 3 for the same buffer without Triton X-100. Following centrifugation, the pellet was used for affinity purification.

## **Ni<sup>2+</sup>-affinity purification**

The inclusion bodies pellet was weighed, and the denaturation buffer was prepared so that addition of a 10 ml volume (here and below, volumes independent of the initial culture volume) would result in the following composition: GdHCl, 6M,  $\beta$ -mercaptoethanol 100 mM. The pellet was dissolved by rotating with the denaturation buffer at room temperature overnight, then placed at 37°C for 1 h, followed by a 10-fold dilution to the following final buffer composition: GdHCl, 6M, phosphate buffer, 0.1 M, NaCl 0.5 M, imidazole 10 mM,  $\beta$ -mercaptoethanol 10 mM, pH 8.0). The resulting solution was passed through a 0.22  $\mu$ m PVDF filter. A HisTrap HF column (5 ml cartridge, GE Healthcare) was preequilibrated with the sample buffer without  $\beta$ -mercaptoethanol. Soluble protein was loaded on the column at 1 ml/min, followed by a wash with the sample buffer at pH 5.3 (50 ml). Bound protein was eluted using sample buffer without  $\beta$ -mercaptoethanol at pH 4.5.

## **Refolding, concentration and dialysis**

Refolding was carried out by slowly diluting HisTrap-eluted GdHCl-solubilised protein at least 100-fold (to 30—50 µg/ml) in a cold (4°C) buffer containing 100 mM Tris-HCl buffer, 400 mM L-arginine, 2 mM NaEDTA, 5 mM reduced glutathione and 0.02% (w/v) sodium azide using a syringe, followed by stirring overnight at 4°C. The solution was then sterile filtered and concentrated using an Amicon nitrogen pressure concentrator (10 kDa cut-off filter) to approx. 10 ml. The resulting sample was passed through a 0.22 µm PVDF filter and subjected to extensive dialysis against 50 mM Tris-HCl buffer, pH 8.0 or 100 mM NaMES buffer, pH 6.5, at 4°C. Samples were then either concentrated in Amicon microconcentrators (10 kDa cut-off filters) to OD<sub>280</sub>=20 for crystallography trials, used immediately or lyophilised for later use in enzyme assays.

# **Results**

## **Development and verification of an in vitro endomannosidase assay**

In order to verify the previously reported data on the cell-specific variations of endomannosidase activity, a robust method of assaying it in cellular preparations was developed. The protocol was adapted from that employed by Spiro and colleagues (Lubas et al. 1987), but was modified for 2-AA labeled free oligosaccharides and separation using normal phase HPLC.

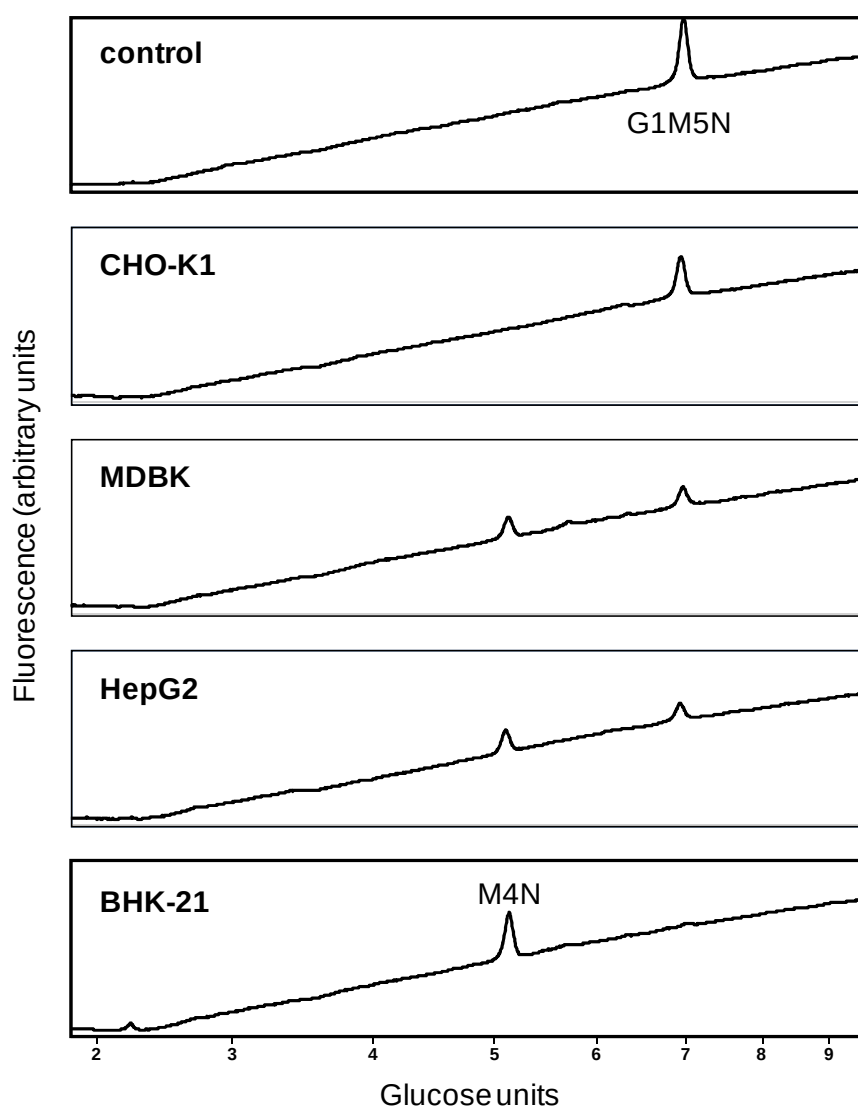


Fig. 4.1. ***In vitro* endomannosidase assay.** Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub>-2AA incubated for 1 h with membrane preparations from CHO-K1, MDBK, HepG2 and BHK-21 cell lines. 2-AA labeled substrate was added to the reaction mixture following a 1 h preincubation with NB-DNJ, DMJ, Triton X-100 and EDTA. Reaction products were filtered and analysed by NP-HPLC.

Endomannosidase extracts from BHK-21, MDBK, CHO-K1 and HepG2 cells were used to digest 2-AA labeled Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> oligosaccharide (0.3 pmol in 20 µl total vol.). In 1 h, 91.3% Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> was converted to a glycan coeluting with a Man<sub>4</sub>GlcNAc<sub>1</sub> standard by the BHK-21 extract (Fig. 4.1). Only 25% substrate was digested by the MDBK extract. Similar activity was seen for HepG2 cells. No digestion by CHO-K1 extracts was observed in 1 h or 24 h. The relative *in vitro* activity levels of endomannosidase derived from different cell lines were therefore consistent with those reported in the literature.

Several control experiments were carried out to verify that the measured activity was specific and representative of cellular endomannosidase. To detect the characteristic product of endomannosidase digestion of triglucosylated oligosaccharides, Glc<sub>3</sub>Man<sub>1</sub>, 2-AA-labeled Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> was incubated with a BHK-21 extract in the manner identical to the one for Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub>, and the reaction product subjected to relabeling with 2-AA. HPLC analysis of these samples (Fig. 4.2) showed a time-dependent increase in Man<sub>4</sub>GlcNAc<sub>1</sub> species, but also a corresponding increase of a peak with the glucose unit value of 3.47.

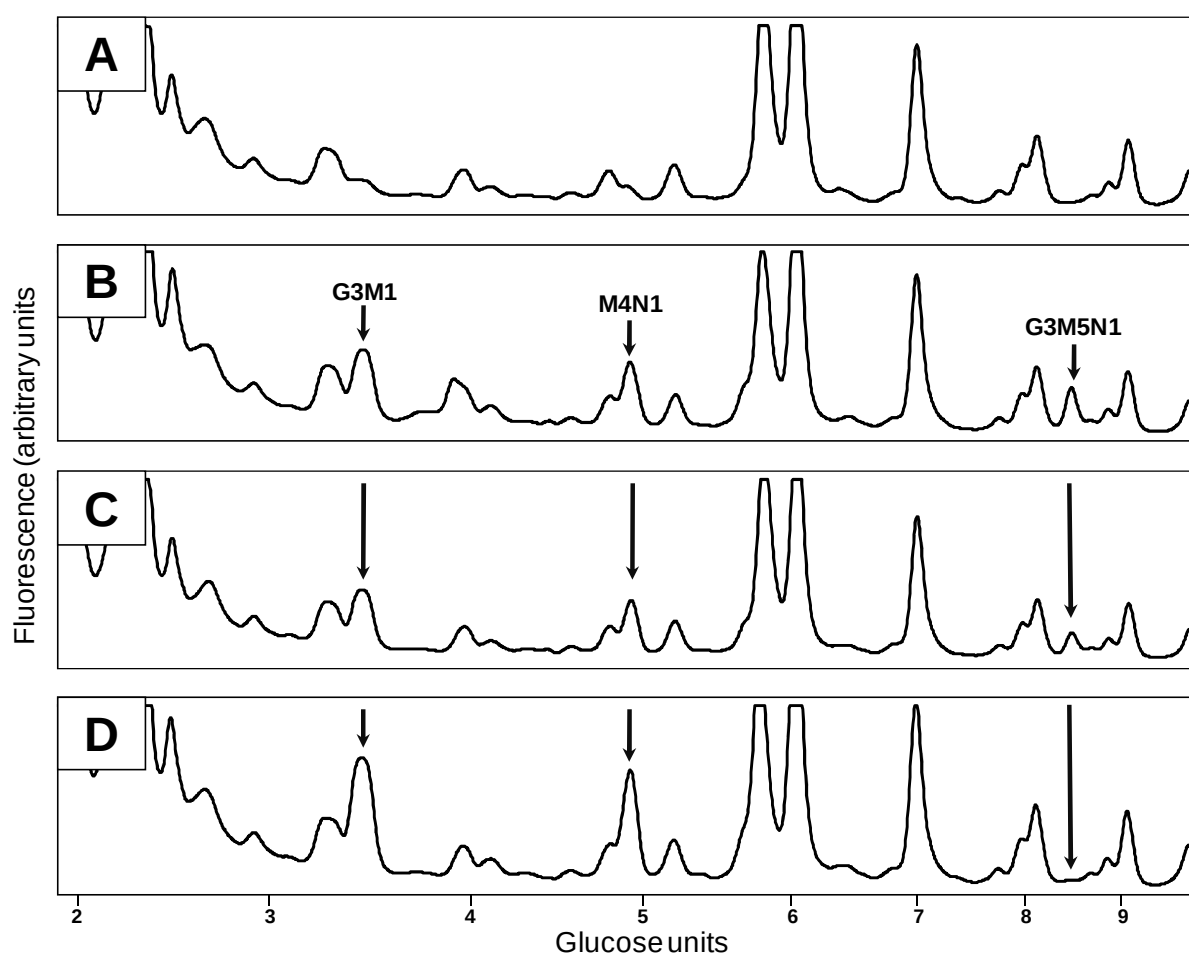


Fig. 4.2. ***In vitro* endomannosidase assay followed by relabeling of the reaction mixture.** Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub>-2AA was hydrolysed by BHK-21 endomannosidase. Membrane preparations were preincubated with NB-DNJ, DMJ, Triton X-100 and EDTA, followed by addition of substrate (B—D) and incubation for 30 min (B), 60 min (C) or 180 min (D) at 37°C. Control sample (A) was incubated for 180 min without substrate. Reaction products were filtered, relabeled with 2-AA, purified and analysed by NP-HPLC.

The ratio between the two peak areas was almost unchanged between samples incubated for different times, suggesting that the GU=3.47 peak corresponded to the expected second product of endomannosidase action,  $\text{Glc}_3\text{Man}_1$ . The GU value for the tetrasaccharide was in agreement with that previously reported in literature (Mellor et al. 2004) and observed in endomannosidase overexpression experiments (Chapter 3).

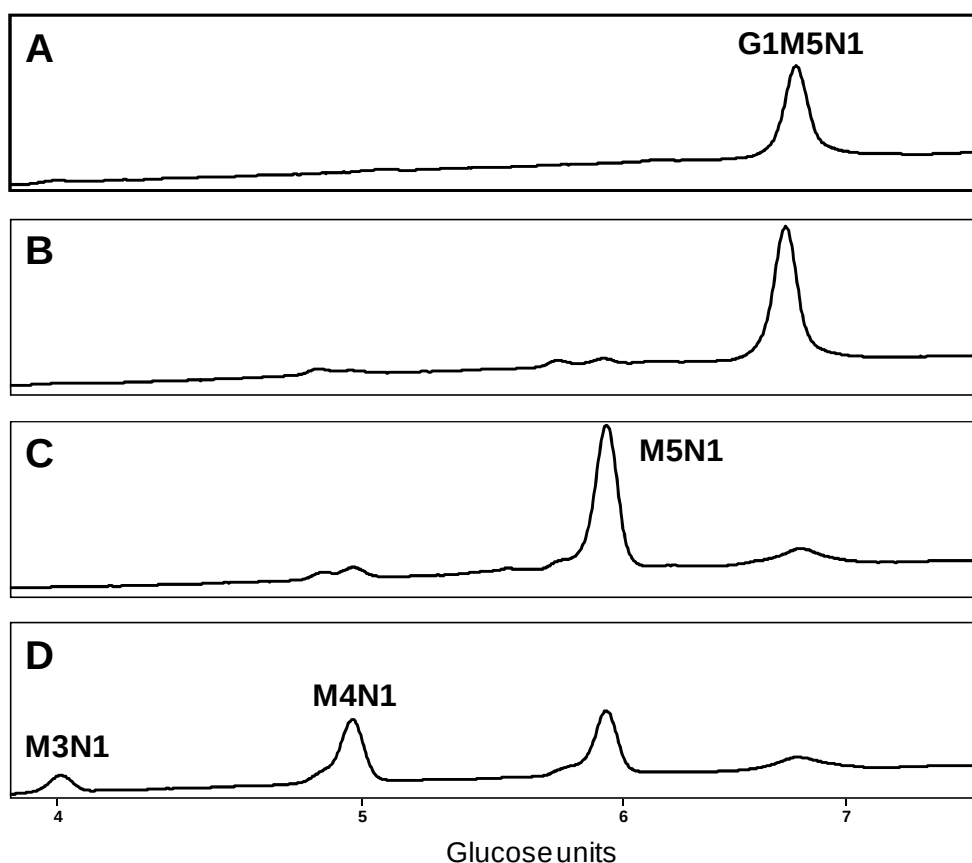


Fig. 4.3. **Endomannosidase activity is absent in CHO-K1 cells.**  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$ -2AA was incubated for 3 h with (B—D) or without (A) membrane preparations from CHO-K1 cells. 2-AA labeled substrate was added to the reaction mixture following a 1 h preincubation with (B) NB-DNJ, DMJ, TX100 and EDTA, (C), DMJ, TX100 and EDTA or (D) without enzyme inhibitors. Reaction products were filtered and analysed by HPLC.

To confirm that the absence of  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  digestion by CHO-K1 could be attributed to the lack of endomannosidase activity rather than general inactivation of glycosidases in the extracts (Fig. 4.3, B), the reaction was carried out without NB-DNJ (Fig. 4.3, C) and with no inhibitors (Fig. 4.3, D). In the absence of glucosidase inhibition, the  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  peak was shifted by approximately 1 glucose unit, corresponding to expected glucosidase-mediated

deglycosylation. In the absence of both glucosidase and mannosidase inhibitors a number of peaks with lower GU values were observed, pointing to normal *in vitro* glycan processing by glucosidases and mannosidases in CHO-K1 extracts.

## MDBK endomannosidase exhibits altered substrate specificity

A kinetic study was conducted to assess substrate specificity of BHK-21 and MDBK-derived endomannosidases. Reaction mixtures containing extracts from the two cell lines were incubated for different time periods with 2-AA-labeled  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  or  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  and analysed as described above.

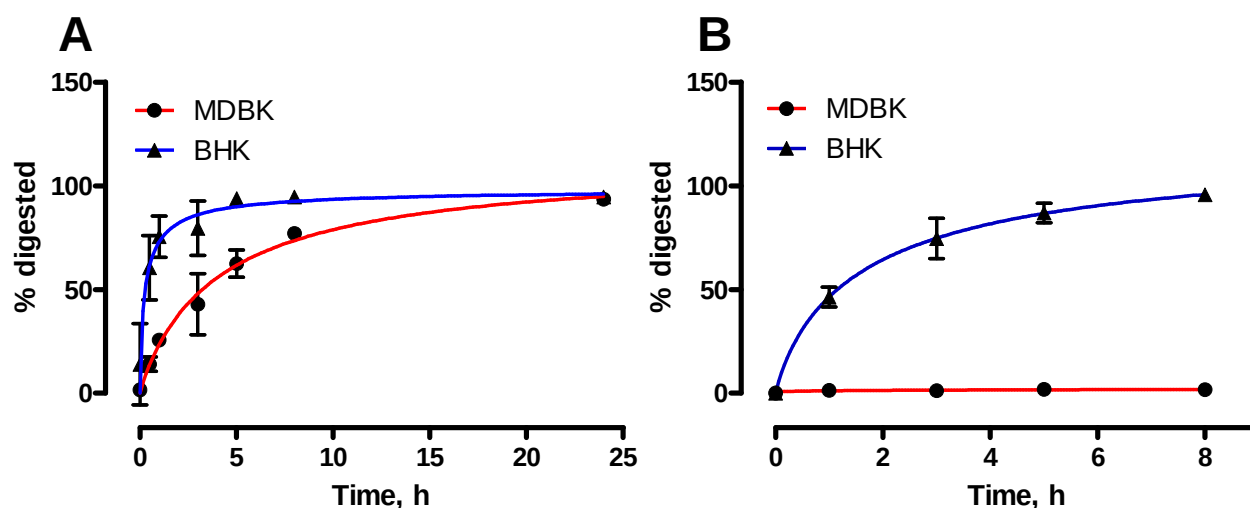


Fig. 4.4. **Kinetics of oligosaccharide processing by endomannosidase.** 2-AA labeled  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  (A) or  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  (B) were digested with endomannosidase. Substrates were incubated for various time periods with MDBK or BHK-21 membrane extracts (20 mg/ml total protein) following a 1 h preincubation with NB-DNJ, DMJ, TX100 and EDTA. Reaction products were filtered and analysed by NP-HPLC.

As expected, the BHK-21 extract hydrolysed both substrates faster than the MDBK extract (Fig. 4.4). However, a significant difference in substrate specificity was observed between the two cell lines. Both cell extracts processed  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  with only a moderate differ-

ence in kinetics. However,  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  was readily processed by the BHK-21 extract with only trace activity detected for MDBK. Importantly, no normalisation of endomannosidase levels in cell extracts was made. The observed differences in kinetics could therefore stem from both the differential enzymatic activity, and differential levels of endomannosidase expression in BHK-21 and MDBK.

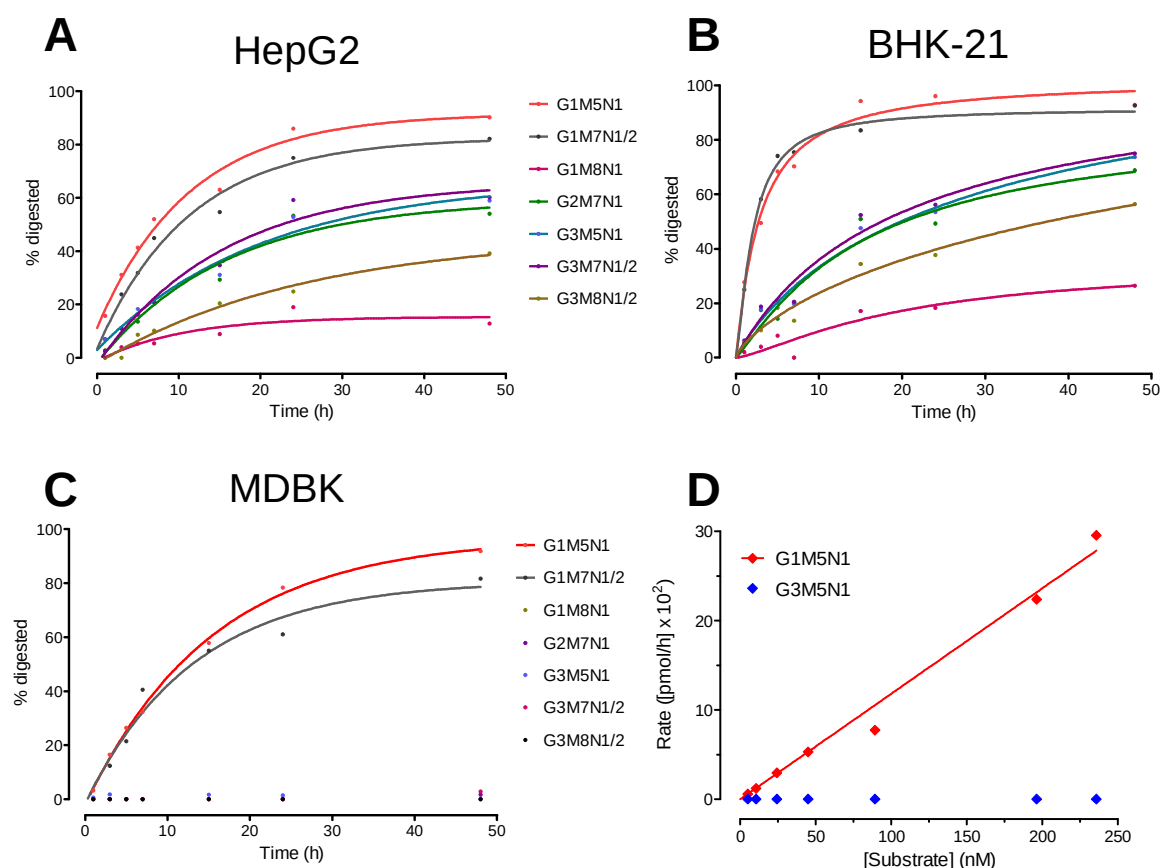


**Fig. 4.5. Comparison of endomannosidase specificity in different cell lines.** 2-AA labeled substrates were incubated for time periods indicated in the presence of membrane extracts from HepG2 (A), BHK-21 (B) or MDBK (C) cells (10 mg/ml total protein). Reaction products were filtered and analysed by HPLC. D, initial rate of substrate hydrolysis by MDBK endomannosidase, plotted as a function of substrate concentration. Reactions were carried out for 5 h and analysed as described above.

The observed specificity of the MDBK but not BHK-21 endomannosidase towards monoglucosylated glycans prompted an investigation of the substrate specificity in greater detail. To verify that the differences observed were not due to abnormal endomannosidase activity in BHK-21 cells, an additional assessment of enzyme specificity in HepG2 cell

extracts was made. Previous data have indicated that HepG2 cells were comparable with MDBK in the rates of hydrolysis of monoglucosylated glycans (Karaivanova et al. 1998). A range of mono-, di- and triglucosylated substrates with mannose branches of varying lengths was purified from MDBK cells treated with NB-DNJ for 24 h, and equal amounts of free oligosaccharides were incubated with cellular extracts, and the reaction mixtures after various incubation periods were then analysed as described above (Fig. 4.5, A—C).

As seen from the results, striking variation in the rates of hydrolysis was observed both between cell lines and between substrates processed by cell extracts of each type. In general, endomannosidase variants tend to preferentially process monoglucosylated oligosaccharides with truncated mannose chains, i.e. the highest levels of activity were observed for  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  and  $\text{Glc}_1\text{Man}_7\text{GlcNAc}_{1/2}$  in all cases. This was in accordance with previously reported data (Lubas et al. 1987; Lubas et al. 1988). However, the most striking observation was that MDBK endomannosidase demonstrated appreciable levels of activity exclusively with  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  and  $\text{Glc}_1\text{Man}_7\text{GlcNAc}_{1/2}$  (Fig. 4.5, C). As no digestion occurred with  $\text{Glc}_1\text{Man}_8\text{GlcNAc}_1$ , monoglucosylation is not the only factor in MDBK endomannosidase selectivity. For the two other cell lines tested, significant rates of reaction were observed for all oligosaccharides tested except  $\text{Glc}_3\text{Man}_8\text{GlcNAc}_{1/2}$  which was processed by endomannosidase from HepG2 cells but not BHK-21. Additionally, BHK-21 endomannosidase exhibited a more pronounced preference for  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  and  $\text{Glc}_1\text{Man}_7\text{GlcNAc}_{1/2}$  substrates than did endomannosidase from HepG2 cells.

To explore the possibility of substrate concentration being the limiting factor for MDBK endomannosidase activity, this enzyme was assayed with both  $\text{Glc}_1\text{Man}_5\text{GlcNAc}_1$  and  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  at a range of substrate concentrations for a 5-hour period, during which the rate of reaction was approximately constant as established from previous assays. No reaction with  $\text{Glc}_3\text{Man}_5\text{GlcNAc}_1$  was detected even at the highest substrate concentration tested

(Fig. 4.5, D). Consequently, it is highly unlikely to be a substrate for MDBK endomannosidase *in vivo*. However, the reaction rate with Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> increased with substrate concentration in a linear fashion. This indicates that the maximum substrate concentration tested, 0.2  $\mu$ M, was much lower than that required for enzyme saturation under the current conditions.

Since endomannosidase only processes glycosylated oligosaccharides (Lubas et al. 1987), it is clear from the data shown that the MDBK endomannosidase is distinctive in exhibiting extreme specificity for monoglucosylated glycans with fewer than eight mannose residues.

### **MDBK substrate specificity is determined genetically**

The possible reasons for the extreme substrate selectivity of MDBK endomannosidase were then investigated. The latter could be determined genetically, or, alternatively, could be conferred by additional cellular factors. To test which of the two hypotheses was true, human and MDBK endomannosidase variants were cloned and expressed in *E. Coli* in order to investigate their enzymatic properties *in vitro*.

Cloning of endomannosidase ORF from MDBK cell line posed a challenge due to the fact that two available bovine endomannosidase transcript sequences appeared to be incomplete, i.e. lacking the 5' (XM\_590447) or 3' (BC103138) ends of the ORF. The latter is likely to be a cloning artifact explained by the first strand synthesis poly-dT primer annealing to an A-rich region of the transcript.

Primer design was carried out using a draft template sequence predicted by comparing the two aforementioned incomplete bovine MANEA mRNA sequences and also by performing a

BLAST search across the bovine genome using a known human mRNA sequence (NM\_024641) in order to deduce regions flanking the ORF.

cDNA was synthesised from total RNA extracted from MDBK cells as described in Materials and Methods. MANEA cDNA presence was verified by PCR amplifying a short 217-bp fragment of the target gene (Primers: C\_F 5'-CTGTGAAAGCTTCCGTAGCC-3'; C\_R 5'AACCTGGAGCCAATGTCATC3'). Absence of genomic DNA contamination was controlled by amplifying a fragment of  $\beta$ -actin (Primers: bActin\_F 5'-GAGAAGCTGTGCTACGTCGC-3', bActin\_R 5'-CCAGACAGCACTGTGTTGGC-3'). The  $\beta$ -actin gene is located on two different exons, therefore the fragments amplified from cDNA and genomic DNA vary in size, being 261 bp in the former case and 350 bp in the latter case.

The 3'-end of the ORF was amplified using the 3'RACE procedure followed by nested PCR. For the former, a gene-specific forward primer (P1, see Materials and Methods) and an adaptor primer (AP) were used. The initial reaction failed to generate a specific product, presumably due to the length of the transcript and a high level of non-specific primer anneal. However, by carrying out nested PCR using the 3'RACE product as template, with P1 as a forward primer and an extra gene-specific reverse primer designed to anneal to the 3'-UTR of the cDNA (P2), the amplification of a specific 1056-bp fragment identical to the corresponding region of the predicted sequence was obtained. This fragment could not be amplified directly from total cDNA, suggesting that two rounds of "selection" by gene-specific amplification were required at this stage.

For amplification of the 5'-end of the ORF, cDNA was synthesised using the P2 primer. MANEA cDNA presence was verified the same way as for 3'-end amplification. The desired fragment was amplified using direct PCR, using a forward primer annealing to the predicted

5'-UTR (P3) and a reverse primer other than P2 (P4). The resulting 1101-bp fragment was identical to the corresponding region of the deduced sequence and contained a 660-bp overlap with the previously amplified 3'-end of the ORF. The complete ORF was obtained by ligating the two fragments using a unique BamHI restriction site located within the overlap. The resulting sequence (GenBank<sup>®</sup> accession JN385276) was identical to the predicted bovine MANEA ORF and was termed bMANEA (Fig. 4.6).

Human endomannosidase ORF was amplified from an IMAGE clone and termed hMANEA. Truncated versions of both hMANEA and bMANEA lacking the transmembrane fragment were then subcloned into the pET15b expression vector in frame with an N-terminal polyhistidine tag and carried out IPTG-induced expression in the BL21/DE3 strain of *E. Coli*. Both proteins were expressed in inclusion bodies. Western blotting of bacterial lysates using an anti-6×His antibody showed severe fragmentation of the products after 2 h of induction. However, after 30 min a single band was observed in both cases (Fig. 4.7, A). Although no fragments were detected in the latter case even at higher exposures, the bands appeared to deviate from the predicted molecular weight (52 kD). It was speculated that this was due to abnormal electrophoretic mobility of the endomannosidase variants. Similar deviation from the predicted molecular weight was observed following expression of full-length human endomannosidase in CHO-K1 cells. To verify this hypothesis, a C-terminal HA tag was cloned in frame with the hMANEA ORF and expressed the obtained product in the same way as hMANEA. Western blotting using antibodies to both the N-terminal and the C-terminal tags confirmed that the full-length product demonstrates abnormal electrophoretic mobility (Fig. 4.7, B) and therefore the single band stained with the anti-6×His antibody represented the uncleaved protein of interest.

|                    |                                                                                 |     |
|--------------------|---------------------------------------------------------------------------------|-----|
| Homo_sapiens       | MAKFRRRTC I I L A L F I L F I F S L M M G L K M L R P N T A T F G A P F         | 40  |
| Bos_taurus         | MAKFRRRTC F I L S L F I L F I F S L M M G L K M L R P N K A A F G D P F         | 40  |
| Cricetulus_griseus | MAKFRRKTC I I L S L F I L F I F S L M M G L K M L R P N T S A F G T P F         | 40  |
| Homo_sapiens       | G L D L L P E L H Q R T I H L G K N F D F Q K S D R I N S E T N T K N L K S V E | 80  |
| Bos_taurus         | G L D L L P E I R Q Q - T H L T K V F D S Q K S D K I S S E T N I K N L K S E E | 79  |
| Cricetulus_griseus | G L D L L P E L H P P N A P L E N K V D F Q K S D R I N M E T N T K D L K A A V | 80  |
| Homo_sapiens       | I T M K P S K A S E L N L D E L P P L N N Y L H V F Y Y S W Y G N P Q F D G K Y | 120 |
| Bos_taurus         | I T V K A S V A S E P H P E E P P L N Y Y L H V F Y Y S W Y G N P Q F D G K Y   | 119 |
| Cricetulus_griseus | E T V Q P P K A S E V N L E E L P P L N Y F L H V F Y Y S W Y G N P Q F D G K Y | 120 |
| Homo_sapiens       | I H W N H P V L E H W D P R I A K N Y P Q G R H N P P D D I G S S F Y P E L G S | 160 |
| Bos_taurus         | I H W N H P V L S H W D P Q I T K K Y P K G K H N P P D D I G S R F Y P E L G S | 159 |
| Cricetulus_griseus | I H W N H P V L E H W D P R I A K N Y P Q G I H T P P D D I G S S F Y P E L G S | 160 |
|                    | *                                                                               |     |
| Homo_sapiens       | Y S S R D P S V I E T H M R Q M R S A S I G V L A L S W Y P P D V N D E N G E P | 200 |
| Bos_taurus         | Y S S R D P S V I E T H M K Q M Y S A S I G V L A L S W Y P P D L N D E N G E P | 199 |
| Cricetulus_griseus | Y S S R D P S V I E T H M R Q M C S A S I G V L A L S C Y P P D S N D E N G E A | 200 |
| Homo_sapiens       | T D N L V P T I L D K A H K Y N L K V T F H I E P Y S N R D D Q N M Y K N V K Y | 240 |
| Bos_taurus         | T D N L V P T I L D K A H K Y N L K V T F H I E P Y K N R D D K T M Y Q N V K Y | 239 |
| Cricetulus_griseus | T D H L V P T I L D K A H K Y N L K V T F H V E P Y S N R N D Q N M Y T N V K Y | 240 |
| Homo_sapiens       | I I D K Y G N H P A F Y R Y K T K T G N A L P M F Y V Y D S Y I T K P E K W A N | 280 |
| Bos_taurus         | I I D K Y G N H P A F Y R Y K T K N G N A L P M F Y V Y D S Y I T A S Q K W A N | 279 |
| Cricetulus_griseus | I I D K Y G S H P A F Y R Y K T R T G H S L P M F Y I Y D S Y I T K P N V W A N | 280 |
| Homo_sapiens       | L L T T S G S R S I R N S P Y D G L F I A L L V E E K H K Y D I L Q S G F D G I | 320 |
| Bos_taurus         | L L T S S G S Q S I R N S P Y D A L F I A L L V D K K H R Y R I L R G G F D G I | 319 |
| Cricetulus_griseus | L L T P S G S Q S V R N S P Y D G L F I A L L V E E K H K N D I L Q S G F D G I | 320 |
| Homo_sapiens       | Y T Y F A T N G F T Y G S S H Q N W A S L K L F C D K Y N L I F I P S V G P G Y | 360 |
| Bos_taurus         | Y T Y F A T N G F T Y G S S Y E N W A K I K Y F C D Q F D L M F I P S V G P G Y | 359 |
| Cricetulus_griseus | Y T Y F A T N G F T Y G S S H Q N W G N L K S F C D K N N L I F I P S V G P G Y | 360 |
| Homo_sapiens       | I D T S I R P W N T Q N T R N R I N G K Y Y E I G L S A A L Q T R P S L I S I T | 400 |
| Bos_taurus         | I D T S I R P W N S H N T R N R I N G K Y Y E T A L S A A L Q A H P S I I S I T | 399 |
| Cricetulus_griseus | I D T S I R P W N T Q N T R N R I N G K Y . ÿ . . ÿ . . ÿ .                     | .   |
| Homo_sapiens       | S F N E W H E G T Q I E K A V P K R T S N T V Y L D Y R P H K P G L Y L E L T R | 440 |
| Bos_taurus         | S F N E W H E G T Q I E S A V P K R T S N I V Y L D Y R P H K P S L Y L E L T R | 439 |
| Cricetulus_griseus | S F N E W H E G T Q I E K A V P K R T T T T V Y L D Y R P H K P S Y Y L E L T R | 440 |
| Homo_sapiens       | K W S E K Y S K E R A T Y A L D R Q L P V S - - - -                             | 462 |
| Bos_taurus         | K W S E K Y R K E R A I Y A L G H Q E L S T Q P V S                             | 465 |
| Cricetulus_griseus | K W P E K Y S K E R K T Y A L D Q Q L P T S - - - -                             | 462 |

Fig. 4.6. **Sequence alignment of human, bovine and chinese hamster (CHO-K1) endomannosidase.** Bovine endomannosidase ORF (GenBank JN385276) was sequenced as described in the text. The sequence of Syrian hamster (BHK-21) endomannosidase is unknown. Asterisk indicates conserved Trp-188 (Trp-187 in *B. taurus*) mutated in CHO-K1 endomannosidase, resulting in loss of enzymatic activity (Torossi et al. 2007).

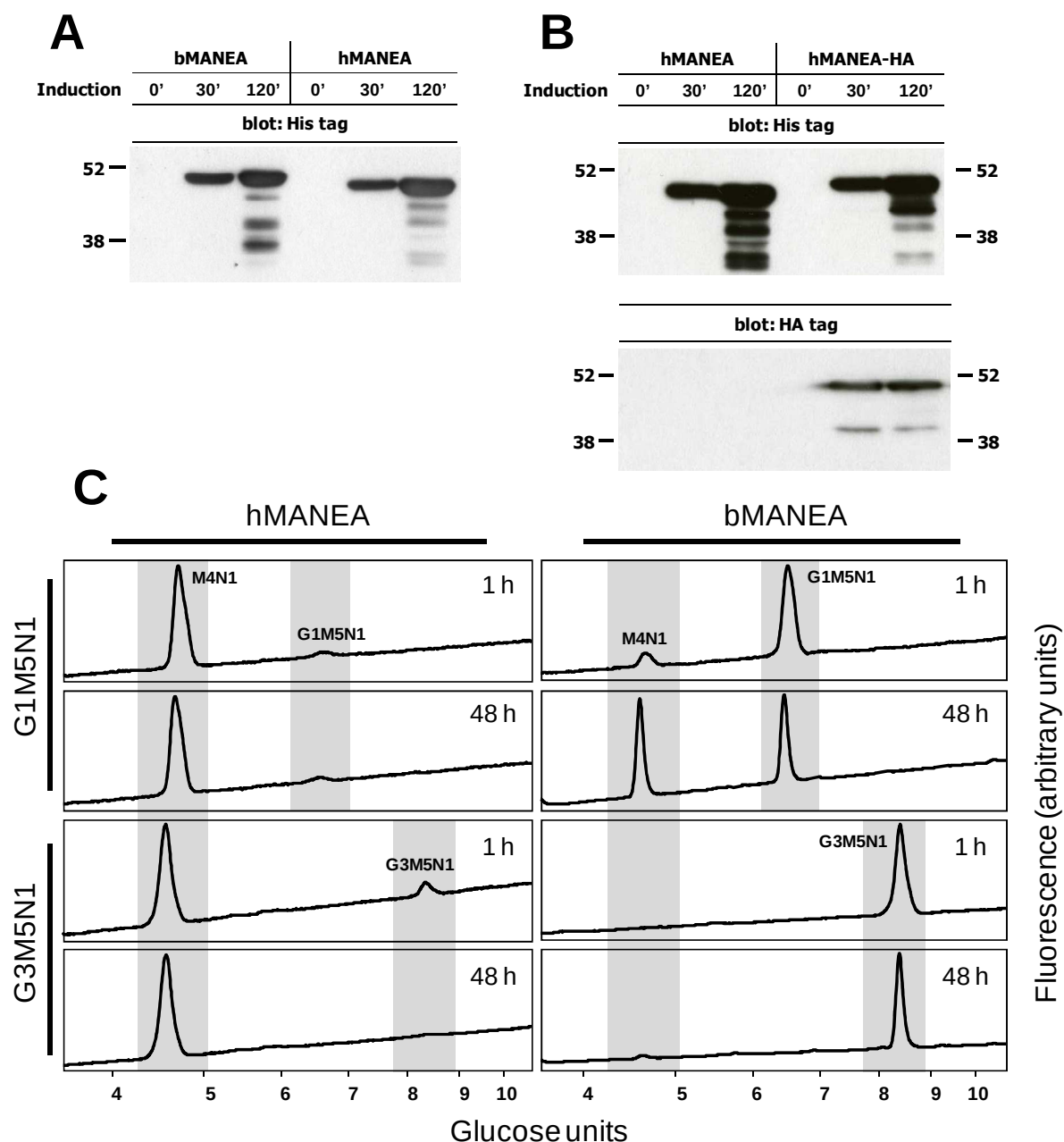


**Fig. 4.7. Expression of bMANEA and hMANEA in *E. Coli*.** A, immunoblotting of BL21/DE3 *E. Coli* cell lysates following transformation with pET15b-bMANEA or pET15b-hMANEA and induction with IPTG for the time periods indicated. B, same, following transformation with pET15b-hMANEA or pET15b-hMANEA-HA. C, activity assay of endomannosidase variants expressed in *E. Coli*. hMANEA or bMANEA (10 ng/reaction) were incubated with 2-AA labeled Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> or Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> (1 pmol/reaction) for the time periods indicated. Reaction products were filtered and analysed by NP-HPLC.

Large-scale expression of hMANEA/bMANEA followed by inclusion body isolation was then carried out. Proteins dissolved in GdHCl (6M) were purified on a Ni<sup>2+</sup>-affinity matrix and refolded as described in Materials and Methods. Following buffer exchange to 0.1M NaMES buffer (pH 6.5) and concentration, protein preparations (10 ng/reaction) were assayed

for activity using Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> or Glc<sub>3</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> (1 pmol/reaction). hMANEA was highly active and rapidly digested both the mono- and the triglucosylated substrate (Fig. 4.7, C). By contrast, bMANEA demonstrated low enzymatic activity and only processed Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub>, confirming the hypothesis that the substrate specificity of endomannosidase in MDBK cells is encoded in the amino acid sequence.

## **Defective endomannosidase is upregulated in MDBK cells**

While the substrate specificity of bMANEA agreed with that demonstrated for endomannosidase in MDBK extracts, the level of enzymatic activity appeared to be greatly diminished when compared to that of hMANEA. In cell extracts, however, the rates of Glc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>1</sub> hydrolysis did not differ significantly between MDBK and HepG2 cell lines (Fig. 4.5). In MDBK cells, it could be speculated that a low enzymatic activity of the defective endomannosidase was compensated for by upregulating the enzyme. Testing this hypothesis on a post-translational level was problematic due to the unavailability of robust antibodies (see Chapter 3) and the difficulty of quantitatively staining the enzyme variants from two different cell lines. The latter difficulty also applied to standard qPCR methods, since employing housekeeping genes (e.g., GAPDH) RNA as internal controls did not provide reliable means of standardisation between different cell lines. It was therefore decided to assay cellular levels of endomannosidase not in absolute units, but rather relative to those of glucosidase II – an enzyme acting as the counterpart of endomannosidase in removing the innermost glucose residues from N-linked oligosaccharides. Glucosidase II  $\alpha$ -subunit (GANAB) RNA was therefore used as a “functional” internal standard.

Semiquantitative RT-PCR was first carried out in order to optimise reaction conditions and primer specificity. Relative band intensities following amplification of MANEA or GANAB transcript fragments confirmed that the endomannosidase/glucosidase II ratios were elevated

in MDBK cells compared to HepG2 cells (Fig. 4.8, A). The degree of elevation was then measured by qPCR using the optimised amplification conditions. The ratio between the two deglycosylating enzymes was shown to be shifted towards endomannosidase approx. 6.6-fold (Fig. 4.8, B). It is therefore concluded that in MDBK cells, a defective variant of endomannosidase with reduced activity and limited specificity is upregulated, resulting in an unaltered processing of glucosylated N-linked oligosaccharides generated in physiological conditions.

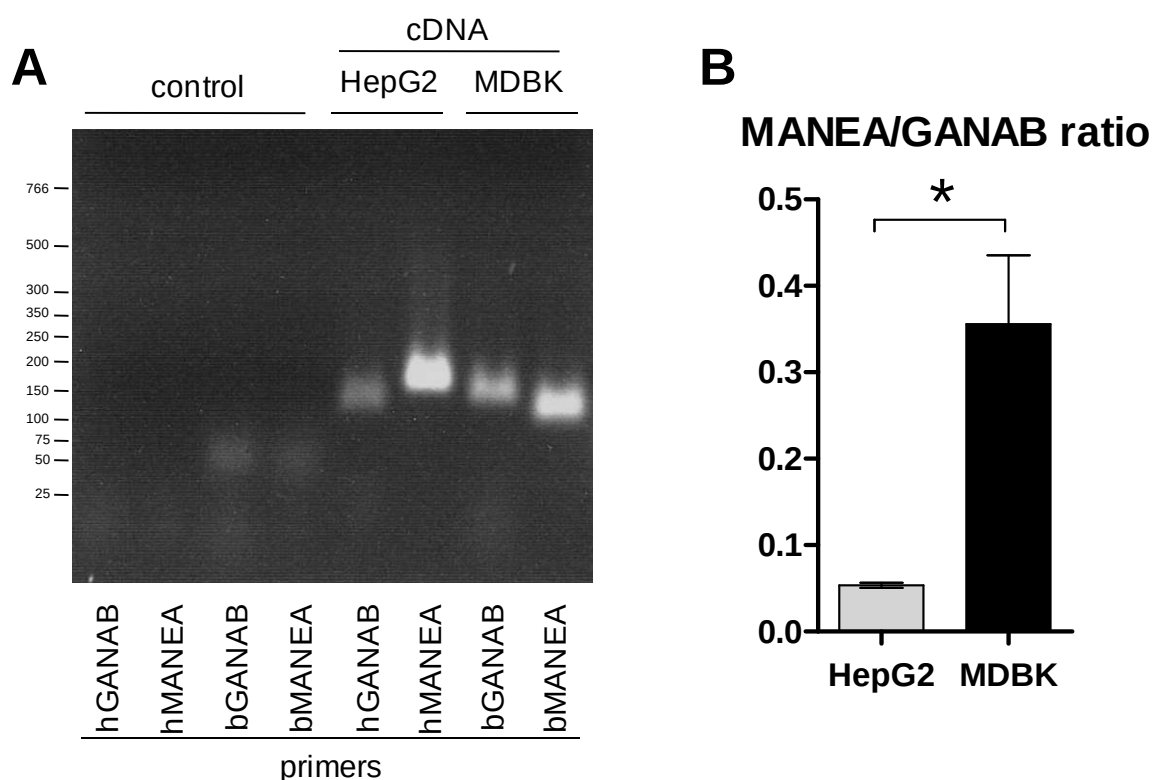


**Fig. 4.8. MDBK endomannosidase levels are elevated relative to glucosidase II.** A, semiquantitative PCR showing elevated MANEA/GANAB ratios in MDBK cells. cDNA was generated from total RNA extracted from HepG2 or MDBK cells, and fragments of either glucosidase II  $\alpha$ -subunit or endomannosidase transcripts were amplified. Template-free reactions were used as controls. B, MANEA/GANAB cDNA ratios assessed by qPCR. Total cDNA was generated as above, and qPCR was carried out using the optimised amplification conditions. Asterisk indicates  $p < 0.05$  in a two-tailed unpaired Student's t-test ( $n=3$ ).

## Crystallography trials

In order to establish the structural basis for the differences in specificity of endomannosidase variants, crystal structures of hMANEA and bMANEA were sought. However, purification and crystallisation of truncated endomannosidase has proven to be challenging.

Optimisation of  $\text{Ni}^{2+}$ -affinity purification in denaturing conditions yielded a >95% pure full-length protein as determined by SDS-PAGE. However, following refolding and dialysis, successful separation of monomeric endomannosidase using size exclusion chromatography was not possible. Several types of resin, including Superose 200, Sephacryl and TSK 55-S, as well as different buffer systems and detergents were tested. In all cases, endomannosidase appeared to be completely absent in the eluate. Separation of refolded proteins from isolated inclusion bodies (without  $\text{Ni}^{2+}$ -affinity purification), followed by SDS-PAGE of obtained fractions, showed that only the full-length endomannosidase product was retained on the column, as opposed to cleavage products and contaminant proteins, indicating that the retention could be specific rather than caused by protein aggregation.

Crystallography trials were therefore attempted without employing SEC. Using dynamic light scattering, it was confirmed that >99.9% hMANEA remained in the non-aggregated form in the concentrated solution. Initial crystallisation was achieved at OD<sub>280</sub>=20 (approx. 11 mg/ml protein) in 50 mM Tris-HCl buffer, pH 8.0, 4°C, using the sitting drop vapour diffusion method and the following buffer systems at the ratio of 1:1:

1. 40% PEG300, phosphate/citrate buffer 0.1 M, pH 4.2 (*NB: crystals obtained were later shown to be small molecule-derived*)
2. 14% 2-propanol, 30% glycerol, 0.14 M  $\text{CaCl}_2$ , 0.07 M sodium acetate buffer, pH 4.6

3. 15% PEG6000, 0.1 M Tris, pH 7.5
4. 12% PEG8000, 0.1 M calcium acetate, 0.1 M sodium cacodylate buffer, pH 5.5

However, crystallisation failed in optimisation trials using any of the conditions. This may have been due to variable levels of aggregation in different enzyme preparations, which was unavoidable due to the unavailability of SEC. For the same reason, cleavage of the His-tag was not performed, which posed an additional challenge for crystallisation.

## Discussion

In the present chapter, clarification of the conflicting data on the cellular activity of endomannosidase was sought. In certain cell lines such as MDBK, endomannosidase is incapable of compensating for glucosidase inhibition imposed by CST or NB-DNJ. It does, however, remain active in *in vitro* experiments. The apparent contradiction between these two findings raised questions, particularly intriguing in light of the finding that endomannosidase could interact with glycoproteins prior to their retrograde transport to the ER followed by degradation (Chapter 3). Since endomannosidase appeared to be conserved independently of its enzymatic function, it was tempting to speculate that the protein could have some additional non-glycosidase role. On the other hand, the fact that MDBK endomannosidase failed to act on at least some glucose-bearing glycoproteins in the secretory pathway was puzzling and further challenged the established view on endomannosidase as a non-selective post-ER deglycosylation system.

The resolution of the apparent contradiction lies in substrate specificity. Following glucosidase inhibition, the majority of newly synthesised glycoproteins remain in a triglycosylated state. Although most variants of endomannosidase appear to be capable of processing such

substrates, MDBK endomannosidase demonstrates genetically determined strict specificity for monoglucosylated glycans. The latter were used as substrates in the previously reported *in vitro* experiments. The fact that MDBK endomannosidase is incapable of deglucosylating glycoproteins in glucosidase-inhibited cells does not therefore point to cell-specific differences in protein function or localisation, but merely reflects the triglucosylated state of ER-exported substrates.

It is noteworthy that the number of glucose residues is not the only limiting factor for MDBK endomannosidase, since only substrates with fewer than 8 mannose residues were processed by the enzyme. What is the biological significance of such an extreme substrate specificity? Endomannosidase is mainly distributed between cis- and medial Golgi. At this stage the vast majority of glucosylated oligosaccharides have been processed by ER and Golgi mannosidases to the end structure of  $\text{Glc}_n\text{Man}_7\text{GlcNAc}_2$ . In cells treated with glucosidase inhibitors this takes the form of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ , the major oligosaccharide on secreted proteins (see Chapter 2). However, the conditions of glucosidase inhibition are artificially imposed and do not reflect the normal state of N-glycosylation within the secretory pathway. In physiological conditions, the majority of glycoproteins are rapidly processed to a monoglucosylated form by ER glucosidases I and II, followed by the rate-limiting deglucosylation step catalysed by glucosidase II. Endomannosidase is only required for deglucosylation of a small fraction of glycoproteins that escape the ER without complete processing by glucosidase II. Since the first two deglucosylation steps occur rapidly and immediately after translation, the vast majority of endomannosidase substrates in normal conditions would be represented by monoglucosylated oligosaccharides, specifically  $\text{Glc}_1\text{Man}_7\text{GlcNAc}_2$ . It is therefore unlikely that evolutionary pressure would affect the ability of endomannosidase to process glycans of higher molecular weight.

At the same time, bovine endomannosidase has been shown to be characterised not only by limited specificity, but also by reduced activity. In MDBK cell extracts, however, the rates of Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> processing were unimpaired. This was likely due to increased expression of the enzyme in the cell, as evidenced by the increase in endomannosidase/glucosidase II mRNA ratio. Such increased expression enables two speculations to be made. First, a feedback mechanism must exist to ensure complete deglucosylation of N-linked oligosaccharides in the secretory pathway, since impaired enzymatic functionality of the bovine variant of endomannosidase leads to compensative transcriptional upregulation of the endomannosidase gene, which ultimately results in the normalisation of glycan processing rates. Second, post-ER deglucosylation of Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub> is necessary and sufficient for the correct progression of the secretory pathway. Both of these claims could be viewed from the perspective of cellular regulation as well as from the evolutionary viewpoint. Indeed, higher levels of expression could be determined genetically if evolutionary pressure exists to ensure a certain level of post-ER deglucosylating activity. At the same time, the substrate specificity of the bovine endomannosidase points to the absence of evolutionary pressure for the processing of glycans other than Glc<sub>1</sub>Man<sub>7</sub>GlcNAc<sub>2</sub>, again supporting the “necessary and sufficient” model. It is important however to take into account the function of each cell type used in the enzyme assays. Direct comparison of secretory pathway regulation in various cell lines is difficult due to the variability of protein load, which could affect the relative importance of specific biosynthetic enzymes in the ER or Golgi. Rates of substrate misprocessing and thus the importance of quality control and ERAD may also vary depending on the nature of the secretory cargo and the relative rates of flux.

Can a cell function without endomannosidase? It can, as evidenced by CHO-K1 cells, completely devoid of endomannosidase activity. However, the conservation of endomannosidase in higher eukaryotes, its function *in vivo* in the absence of glucosidase inhibitors, and the case

of MDBK/bovine endomannosidase strongly suggest that, in general, the function of the enzyme in post-ER deglycosylation of secreted proteins as well as ERAD substrates is beneficial in mammalian evolution.

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# Chapter 5. General discussion

## Free oligosaccharide analysis as a tool to study glycoprotein degradation

Glycoproteins constitute the majority of secretory cargo in eukaryotic cells (Varki et al. 1999). It is therefore vital that newly synthesised glycoproteins undergo stringent quality control to ensure only correctly folded substrates proceed in the multi-step process of anterograde trafficking from the ER to their final destination. Such control necessarily involves two tightly linked mechanisms: first, a system that assists correct folding and prevents the formation of misfolded intermediates and aggregates, and second, a system that degrades such aberrant cargo should it be generated (reviewed in Lederkremer 2009). Glycoprotein degradation is therefore one of the essential control mechanisms in the secretory pathway. The most well-known pathway for glycoprotein degradation is termed ER-associated degradation (ERAD) (reviewed in Vembar et al. 2008), a pathway through which both glucosylated and non-glucosylated proteins can be destroyed following retrotranslocation from the ER lumen/membrane into the cytosol (Wiertz et al. 1996). As part of the process, N-linked oligosaccharides are removed from the glycoproteins, resulting in free oligosaccharides (FOS) (reviewed in Suzuki 2007). The latter could be considered a by-product of ERAD.

The mechanism of N-glycosylation involves an *en bloc* cotranslational transfer of a pre-synthesised oligosaccharide onto the asparagine residues in a polypeptide chain. This is opposed to sequential addition of monosaccharides in O-glycosylation (Varki et al. 1999). N-

linked oligosaccharides therefore have an identical “starting point” irrespective of nature of the polypeptide. This initial form of the asparagine-linked glycan is subject to processing by enzymes throughout the secretory pathway: in the ER, glycosidases participate in quality control and ERAD targeting, whereas in the Golgi apparatus further trimming and modification of N-linked glycans results in the formation of a variety of functional carbohydrates. Although the nature of the protein can influence glycan processing, particularly in the late stages, carbohydrate analysis allows a dynamic and global view of the state of the secretory pathway to be gained. On the other hand, free oligosaccharide analysis presents an overview of ER-associated degradation in a similar manner (Alonzi et al. 2008; Kukushkin et al. 2011). Since in normal conditions the initial state of the N-linked oligosaccharides is the same for the vast majority of newly synthesised glycoproteins (reviewed in Kelleher et al. 2006), the pattern of free oligosaccharides present in the cell reflects processing of misfolded glycoproteins during quality control and degradation and is therefore a valuable tool for studying ERAD targeting and mechanisms.

FOS analysis does, however, have a number of limitations. First, the free oligosaccharides released from degrading misfolded proteins are rapidly processed by cytosolic and lysosomal enzymes, making it difficult to obtain and interpret data in a prolonged experiment. One way of overcoming this complication is by employing glycosidase inhibitors to stabilise FOS. In the experiments described in this work, cells were treated with N-butyldeoxynojirimycin (NB-DNJ), a competitive inhibitor of  $\alpha$ -glucosidases including ER glucosidases I and II (Karlsson et al. 1993). Treatment with NB-DNJ results in the failure of glucosidases to remove the three glucose residues from nascent N-linked oligosaccharides. This in turn results in (i) increased misfolding and ERAD due to the inability of tri- or diglucosylated glycoproteins to bind to ER chaperones calnexin or calreticulin (Branza-Nichita et al. 2001), and (ii) stabilisation of the released free oligosaccharides, which cannot enter the lysosome or escape the ER in the glucosylated form (Moore et al. 1994; Moore et al. 1995; Haga et al. 2009). Glucosidase inhi-

bition has some additional experimental advantages, such as the possibility to discriminate between FOS generated through the standard ERAD or through the endomannosidase-mediated pathway (see Chapter 3). Alternatively, cytosolic or lysosomal mannosidases can be inhibited to reduce the rates of post-ERAD processing of free oligosaccharides (Butters et al. 2009). However, it is important to understand the effect glycosidase inhibition may have on ERAD machinery itself, as well as on glycoprotein substrates in the secretory pathway.

Another limitation of free oligosaccharide analysis is that although it provides a global view on glycoprotein degradation, the FOS profiles are essentially an average of a diverse population of carbohydrates released from different glycoproteins, and even within one glycoprotein, from different glycosylation sites. Since multiple degradation mechanisms may coexist in the cell, oversimplification must be avoided when interpreting FOS data.

Despite the limitations described, FOS analysis has proven to be a robust and versatile tool to study glycoprotein degradation in this and previously published work. To the author's knowledge, it is at present the only available method to analyse global ERAD in cells both quantitatively and qualitatively. In the present work, FOS analysis has helped uncover two previously unknown or ambiguous routes for glycoprotein degradation. The first one was characterised by luminal deglucosylation of misfolded glycoproteins as opposed to that at the cytosolic face of the ER membrane. The second one occurred downstream of processing by endomannosidase, i.e. involving anterograde transport to cis-/medial Golgi, followed by retrograde transport of misfolded substrates into the ER prior to degradation. The two described pathways are not mutually exclusive.

## **Luminal FOS: implications and limitations**

Canonical glycoprotein ERAD involves the action of the cytosolically oriented peptide:N-glycanase, which deglycosylates glycoproteins as part of the degradation process. This route

results in the production of cytosolic FOS. By analysing FOS in cells in which removal of glucose residues from nascent glycoproteins is inactivated pharmacologically (ER glucosidases I and II) and genetically (Golgi endomannosidase), it was shown that a separate population of FOS is generated in the lumen of the ER. These FOS were stable and mainly represented by an oligosaccharide with the structure  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$ . Previous experiments (Alonzi et al., unpublished) have confirmed that the latter is glycoprotein-derived and must therefore be generated as part of glycoprotein degradation. Further experiments revealed additional properties of the pathway, i.e. altered sensitivity to certain ERAD factors and, importantly, dependence on Golgi-ER trafficking. The latter was supported by the fact that glucosylated luminal FOS were only detectable in large amounts in cells deficient in Golgi endomannosidase activity.

Following glucosidase inhibition in CHO-K1 and MDBK cells, luminal oligosaccharides comprise up to 50% of the detectable glucosylated FOS. Whether or not this estimation could be extrapolated to determine the contribution of the newly defined ERAD pathway to overall glycoprotein degradation is debatable due to a number of reasons. Firstly, although the dependence of  $\text{Glc}_3\text{Man}_7\text{GlcNAc}_2$  free oligosaccharide production on PNGase activity supports the glycoprotein origin of luminal FOS, a fraction of the latter could be generated during glycoprotein biosynthesis from the dolichol-linked precursors. Since in this case the released FOS are chemically identical to ERAD-derived oligosaccharides, quantitation of the latter is hindered. However, preliminary data (Kukushkin et al., unpublished) indicate that glycoprotein- and lipid-derived luminal FOS may be biochemically discernable in pulse-chase experiments, most likely due to differences in sub-ER localisation leading to differential rates of efflux from the cell. This is supported by the stable accumulation of FOS sensitive to proteasomal inhibition by MG132, as opposed to the rapid secretion of MG132-insensitive FOS into the culture medium. These results suggest that while glucose-bearing glycoprotein-derived FOS continuously accumulate in the ER for the period of the treatment (24 h), lipid-

derived species are maintained at low levels due to steady clearance from the cell. These data raise intriguing questions regarding compartmentalisation of ER-associated degradation machinery. Indeed, accumulating evidence suggests that a separate compartment specialised for quality control and ERAD (ERQC) may exist in the perinuclear region of the ER (Kamhi-Nesher et al. 2001; Frenkel et al. 2003). It could therefore be speculated that the biogenesis of luminal FOS may be dependent of glycoprotein transport into the ERQC. Same could apply to the production of cytosolic FOS, as it is at present unclear at which stage of quality control/ERAD are the two pathways separated.

Secondly, there are additional difficulties of estimating the proportion of glycoproteins degraded via either the “cytosolic FOS” or “luminal FOS” pathway, which stem from the limitations of FOS analysis described above. Since the effect of certain ERAD factors and treatments on the levels of the two populations of FOS is different, it is unlikely that the latter can originate from different glycosylation sites of the same glycoprotein molecules targeted for degradation. However, the possibility that some substrates are able to produce both types of FOS in a single degradation event cannot be excluded. Finally, the ability to generate relatively stable FOS populations, as well as to discern luminal and cytosolic FOS, is dependent on the use of glucosidase inhibitors. This leads to the perturbation of normal cellular pathways of folding and quality control. In particular, glucosidase inhibition disrupts calnexin-/calreticulin-dependent folding by preventing the association of nascent glycoproteins with the lectin chaperones. Inhibitor treatment could therefore have a profound impact on ERAD levels and is not unlikely to influence the ratio between luminal and cytosolic FOS. This is indirectly supported by the low levels of GlcNAc<sub>2</sub>-reducing terminating FOS in cells treated with NAP-DNJ at 5  $\mu$ M, which at this concentration predominantly inhibits the removal of the third but not the first two glucose residues, therefore not blocking glycoprotein interactions with calnexin and calreticulin.

## **Golgi apparatus and endomannosidase role in glycoprotein ERAD**

Several pieces of evidence have prompted an investigation of the involvement of Golgi endomannosidase in ERAD and specifically in the production of luminal FOS. Firstly, a large proportion of luminal FOS following glucosidase inhibition was only observed in cells deficient in endomannosidase activity. Secondly, luminal FOS were sensitive to the perturbation of Golgi-to-ER retrograde transport. However, overexpression of endomannosidase did not selectively affect luminal FOS, but instead had an impact on all types of glucosylated FOS. It was therefore concluded that an additional trafficking pathway preceding ERAD has been uncovered. This pathway had no correlation with the formation of either cytosolic or luminal FOS specifically, leading to a conclusion that at the stage of cis- to medial Golgi apparatus the fate of misfolded glycoproteins is undefined with respect to the mechanism of degradation. In an alternative explanation, overexpressed endomannosidase may be present in the endoplasmic reticulum to an extent sufficient to cause significant deglycosylation of ER-resident ERAD substrates. Such possibility cannot be discarded even though no ER-resident endomannosidase was detected by immunofluorescence, and additionally knockdown of endogenous (i.e., non-overexpressed) endomannosidase in HEK-293T cells led to a significant effect on the levels of glucosylated FOS.

Some evidence suggests that ERAD targeting in certain cases may be tightly linked to ER-Golgi and retrograde transport (see Chapter 1). Recently, ER mannosidase I, an extensively studied modulator of the glycan-based degradative signaling, has been shown to reside in the Golgi apparatus *in vivo* (Pan et al. 2011). It was additionally demonstrated that its ERAD-enhancing properties did not depend on ER or Golgi localisation, suggesting that cycling of misfolded substrates between the compartments is a ubiquitous process in mammalian cells. The experiments presented in this work, however, suggested differential dependence of the formation of luminal/cytosolic FOS on vesicular trafficking between Golgi and ER. It is

therefore not unlikely that although recycling of ERAD substrates between the ER and post-ER compartments may take place in most cases (as suggested by the effects of endomannosidase overexpression/knockdown on the levels of total FOS), the extent of such trafficking and consequentially Golgi residence time may differ. Such view provides an explanation for the absence of selectivity in the effect of endomannosidase on glucosylated FOS in our experiments. When overexpressed, endomannosidase could saturate the recycling route, deglucosylating substrates with even short Golgi residence time. This would result in equal effect on substrates destined for the “cytosolic FOS route” and the “luminal FOS route”, as opposed to the presumed *in vivo* situation in which low levels of the enzyme favour deglucosylation of substrates that are continuously recycled between compartments or have longer Golgi residence time. This model is supported by additional evidence showing that increased levels of luminal FOS are found following complete glucosidase inhibition rather than inhibition of glucose trimming at the monoglucosylated state. In the former case, failure of nascent glycoproteins to interact with ER-localised folding/quality control machinery likely results in increased bulk-flow trafficking to the Golgi apparatus. High levels of luminal FOS therefore correlate with decreased ER retention of glycoproteins.

Although ERAD was demonstrated to occur downstream of processing by endomannosidase in the ERGIC/Golgi (see Chapter 3), it is not the only available processing route for glycoproteins at this stage of the secretory pathway following glucosidase inhibition. A fraction of substrates, otherwise degraded with the production of luminal or cytosolic FOS, can evidently be rescued following deglucosylation by endomannosidase. This stabilisation of misfolded proteins by endomannosidase could be interpreted in different ways. Since protein misfolding is generally believed to be required for ERAD commitment independently of the glycan-based signalling (see Chapter 1), it is unlikely that the population of endomannosidase-rescued glycoproteins comprises substrates in the native conformation. Their stabilisation must therefore be a result of either abnormal anterograde transport, or abnormal post-ER quality control. The

former seems likely due to the reasons discussed above (decreased ER retention following glucosidase inhibition), but disagrees with the assumption that most ERAD substrates routinely traffic between the ER and Golgi. On the other hand, post-ER quality control has been implicated to act in yeast but has not been defined in mammalian cells (see Chapter 1). Mutated variants of  $\alpha$ 1-antitrypsin were shown to be non-selectively processed by endomannosidase and secreted following glucosidase inhibition (Torossi et al. 2006). Theoretically, a post-endomannosidase quality control system could provide an explanation for the sorting of deglycosylated proteins either for retrograde transport and ERAD, or for maturation and secretion. Abnormal secretion of mutant  $\alpha$ 1-antitrypsin could be merely a result of quality control overflow. Indeed, the rate of misfolded proteins traffic through Golgi and their processing by endomannosidase would be greatly increased following glucosidase inhibition. Alternatively, sorting of proteins at this stage may not be conformation-based, but could depend on the nature of the polypeptide, e.g. the presence of ER retention signals. However, this model does not explain stabilisation of glycosylated glycoproteins by endomannosidase.

Overall, the dichotomy of glycoprotein fate that occurs downstream of processing by endomannosidase raises challenging questions regarding post-ER quality control. Using free oligosaccharide analysis to gain a global view of ER-associated degradation, a glycoprotein processing route that exists in the ERGIC/Golgi compartments was uncovered. The latter involves targeting of some glycoproteins for retrograde transport and disposal, while stabilising other substrates that are degraded in the absence of endomannosidase. Together with the data on the connection between luminal FOS and Golgi-to-ER trafficking, these results highlight the importance of post-ER processing of glycoproteins in ERAD.

## The necessary and sufficient activity of endomannosidase in mammalian cells

Although endomannosidase is conserved in all higher eukaryotes, the catalytic activity of the enzyme varies greatly between species and cell lines (Karaivanova et al. 1998). It was hereby shown that in certain cases, substrate specificity of endomannosidase can be restricted to truncated monoglucosylated glycans including  $\text{Glc}_1\text{Man}_7\text{GlcNAc}_2$ , which is likely to represent the major substrate of endomannosidase *in vivo* in the absence of glucosidase inhibitors. Such extreme substrate preference was demonstrated for bovine endomannosidase, and it is predicted that a similar limitation of specificity would be observed in canine cells, based on the reported *in vitro* and cellular properties of MDCK endomannosidase. Expression of bovine and human endomannosidase *E. Coli* has allowed us to establish that the differences in substrate specificity are encoded genetically and not conferred by additional factors that hinder processing of oligosaccharides of higher molecular weight. It was additionally demonstrated that in MDBK cells, endomannosidase has reduced activity, which is compensated for by increased levels of expression, ultimately resulting in normal processing phenotype. Taken together, the data presented suggest that endomannosidase ability to process monoglucosylated oligosaccharides is necessary and sufficient for evolutionary conservation.

It has been known for some time that in the absence of glucosidase inhibitors endomannosidase processes a considerable proportion of substrates trafficking through the secretory pathway (Weng et al. 1996). The data obtained here confirm the importance of this deglycosylation route in mammalian cells. Although the function of endomannosidase is generally seen as a backup deglycosylating system, it is not entirely clear why does such a significant fraction of N-linked oligosaccharides (>15%) fail to get deglycosylated in the ER, since the mechanisms of ER retention are directly dependent on monoglucosylated glycans. The interactions with calnexin or calreticulin should prevent the escape of glucosylated folding inter-

mediates into the post-ER compartments, unless the quality control machinery is saturated. One explanation for the failure of glucosidase II to ensure complete removal of glucose residues from substrates exiting the ER is differential processing of various glycosylation sites. While some of the latter favour interaction with calnexin and calreticulin, others are not involved in lectin binding, and could therefore have no retention capabilities in the monoglucosylated form (Caramelo et al. 2007; Tokhtaeva et al. 2010). Additionally, a recent report suggests that binding to calnexin is dependent on the conformation of the Glc(G1)—Man(D1) unit, with only the minor (30%) conformer being recognised by the lectin chaperone (Mackeen et al. 2009).

Another explanation involves the possibility of calreticulin trafficking into glucosidase II-negative regions of ERGIC and Golgi. The lectin has been shown to be incorporated into COPII vesicles and exported to post-ER organelles in complex with a glycoprotein substrate (Howe et al. 2009). It is also known to colocalise with endomannosidase, as well as copurify with the enzyme on an affinity matrix (Spiro et al. 1996; Zuber et al. 2000). Whether or not calreticulin recycling is a specific cellular adaptation or merely a consequence of the unspecific nature of the bulk flow, remains to be established. However, the possibility of “quality control export” raises the possibility that endomannosidase may act as a Golgi-localised functional analogue of glucosidase II in the ER (Roth et al. 2003).

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