Identification of Tetraspanin-7 as a Target of Autoantibodies in Type 1 Diabetes

Running title: Tetraspanin-7 in Type 1 diabetes

Kerry A. McLaughlin[1], Carolyn C. Richardson[1,2], Aarthi Ravishankar[1], Christina Brigatti[3], Daniela Liberati[4], Vito Lampasona[4], Lorenzo Piemonti[3], Diana Morgan[5], Richard G. Feltbower[5] and Michael R. Christie[1,2]

[1]Diabetes Research Group, Division of Diabetes & Nutritional Sciences, King's College London, London, U.K.

[2]School of Life Sciences, University of Lincoln, Lincoln, U.K.

[3]Diabetes Research Institute, IRCCS San Raffaele Scientific Institute, Milan, Italy

[4]Division of Genetics and Cellular Biology, IRCCS San Raffaele Scientific Institute, Milan, Italy

[5]Division of Epidemiology & Biostatistics, School of Medicine, University of Leeds, Leeds, UK

Corresponding author: Dr Michael R Christie, School of Life Sciences, Joseph Banks Laboratories, University of Lincoln, Lincoln LN6 7DL, United Kingdom

Phone: +44 1522 837434

Email: mchristie@lincoln.ac.uk

Word count of abstract: 199

Word count of main text: 3,998

Number of figures: 4.

One Supplementary Table

ABSTRACT

The presence of autoantibodies to multiple islet autoantigens confers high risk for development of Type 1 diabetes. Four major autoantigens are established (insulin, glutamate decarboxylase, IA-2, and zinc transporter-8), but the molecular identity of a fifth, a 38kDa membrane glycoprotein (Glima), is unknown. Glima antibodies have been detectable only by immunoprecipitation from extracts of radiolabeled islet or neuronal cells. We sought to identify Glima to enable efficient assay of these autoantibodies. Mouse brain and lung were shown to express Glima. Membrane glycoproteins from extracts of these organs were enriched by detergent phase separation, lectin affinity chromatography and SDS-PAGE. Proteins were also immunoaffinity purified from brain extracts using autoantibodies from diabetic patients' sera before SDS-PAGE. Eluates from gel regions equivalent to 38kDa were analyzed by LC-MS/MS for protein identification. Three proteins were detected in samples from the brain and lung extracts, and in the immunoaffinity purified sample, but not the negative control. Only tetraspanin-7, a multipass transmembrane glycoprotein with neuroendocrine expression, had physical characteristics expected of Glima. Tetraspanin-7 was confirmed as an autoantigen by demonstrating binding to autoantibodies in Type 1 diabetes. We identify tetraspanin-7 as a target of autoimmunity in diabetes, allowing its exploitation for diabetes prediction and immunotherapy.

INTRODUCTION

Detection of circulating autoantibodies to pancreatic islets (1), and identification of their molecular targets (2), has allowed development of high throughput autoantibody assays for clinical diagnosis of Type 1 diabetes and identification of individuals at risk for disease. Evidence from both animal studies and human trials indicate that Type 1 diabetes may be prevented in individuals at risk (3; 4). Hence, a range of therapies to interfere with immune responses has proved effective in preventing disease development in animal models of diabetes (5) and in slowing the loss of beta cell function occurring in the months following disease diagnosis in man (6-8). There is now a focus on development of procedures to interfere specifically in immune responses that cause Type 1 diabetes, requiring knowledge of the major targets of the autoimmune response. There is no single common autoimmune target and individuals differ in antigen specificity of autoimmune responses they develop. Four major humoral autoantigens have been identified in Type 1 diabetes by defining the specificity of autoantibodies in the disease: insulin (9), glutamate decarboxylase (10), IA-2 (11), and zinc transporter-8 (ZnT8) (12). Autoantibodies to a fifth, a 38kDa glycosylated membrane protein (Glima), have been detected in 19-38% of Type 1 diabetic patients, with significantly higher prevalence (up to 50%) in children (13-15). The molecular identity of Glima has for many years proved elusive, hampering the characterization of autoimmunity to the protein and the development of sensitive, specific autoantibody assays. Glima is expressed in pancreatic beta and neuronal cell lines, is hydrophobic, heavily N-glycosylated, having affinity for the lectin wheat germ agglutinin, and has a core protein backbone of approximately 22kDa (13-15). The aim of

this study was to take advantage of these known physical properties to prepare Glima-enriched extracts for identification of the autoantigen by mass spectrometry.

RESEARCH DESIGN AND METHODS

Patients. Serum samples were obtained from 40 Type 1 diabetic patients (12-26 y) within 6 months of diagnosis from clinics in West Yorkshire with informed consent for screening for high-titer Glima antibodies, 94 additional patients (12-63 y) for assay verification and 52 non-diabetic individuals as negative controls. Approval for analysis of autoantibodies in sera from these individuals was obtained from the Yorkshire and the Humber – Bradford Leeds Research Ethics Committee.

Screen of type 1 diabetic patient sera for Glima antibodies. Glima antibodies were detected by a modification of immunoprecipitation assays previously described (13-15) using the neuronal mouse cell line GT1.7 as source of antigen. Endogenous proteins in GT1.7 cells were labeled by incubation in methionine-free DMEM medium containing 4 MBq/ml ^{35}S-methionine for 7 h at 37°C. Cells were washed with Hepes buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM benzamidine) and stored at -80°C. Frozen cell pellets were extracted in Hepes buffer containing 2% Triton X-100 for 2 h on ice and insoluble material removed by centrifugation at 15,000 *g* for 15 min at 4°C. Membrane glycoproteins were isolated by incubating cell extracts with wheat germ agglutinin-agarose on ice for 30 min and, after washing in Hepes buffer containing 0.5 mM methionine, 100 mg/l bovine serum albumin and 0.5% Triton X-100, eluted in the same buffer containing 0.5 M N-acetyl glucosamine. Aliquots (20 µl) of eluate containing 3 ×

10^5 cpm of radiolabeled protein were incubated with 5 µl of test sera for 18 hours at 4°C and immune complexes captured on 5 µl of protein A-Sepharose. Immunoprecipitated proteins were eluted in 15 µl of SDS-PAGE Loading Buffer (Novex, Life Technologies, Paisley, UK) with heating at 90°C for 5 min and subjected to SDS-PAGE on 12% polyacrylamide gels. After electrophoresis, gels were incubated in 40% v/v methanol, 2.5% v/v acetic acid and subsequently in Enlightning autoradiographic enhancer (Perkin-Elmer, Coventry, UK), each for 30 min. Gels were dried and contacted with X-ray film (BioMax MR film; Kodak, Watford, UK) for up to two weeks. After exposure, X-ray film was developed to detect radiolabeled proteins specifically immunoprecipitated by sera from Type 1 diabetic patients, with bands detected in the 38,000 relative molecular mass (M_r) region indicating positivity for Glima antibodies.

Tissue expression screen. To identify mouse organs containing highest levels of Glima for use in antigen purification, competitive binding studies were performed using detergent extracts of organs as unlabeled competitors with ^{35}S-methionine-labeled Glima for binding to Glima antibodies in serum from a high-titer Glima antibody positive patient. Mouse kidney, brain, heart, liver, thyroid, muscle, salivary gland, thymus, pancreas, spleen, adrenal, pituitary, and lung were dissected, frozen in liquid nitrogen and stored at -80°C before extraction. Tissues were homogenized in homogenization buffer (10 mM Hepes, pH 7.4, 0.25 M sucrose, 10 mM benzamidine), and membrane fractions sedimented by centrifugation at 15,000 *g* for 30 min at 4°C. Supernatants were removed and pellets extracted in 2% Triton X-100 extraction buffer for 2 h on ice. Extracts were centrifuged at 15,000 *g* for 30 min at 4°C and supernatants collected. The protein

concentrations of extracts were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Loughborough, UK).

For the competition assay, wheat germ agglutinin agarose eluates from extracts of ^{35}S-methionine-labeled GT1.7 cells were prepared as described above. Aliquots (20 µl) of GT1.7 cell glycoproteins containing 3×10^5 cpm of radiolabeled proteins were incubated for 18 h at 4°C with 5 µl of serum from patient 029 alone, or with 10 µl of detergent extracts containing 100 µg of extracted protein from each mouse tissue. Immune complexes were captured on protein A-Sepharose and processed for SDS-PAGE and autoradiography.

Partial purification of Glima from mouse brain and lung. Mouse brain and lung, shown to express Glima in the tissue screen (see Results), were homogenized in ice-cold homogenization buffer in a Dounce homogenizer and cell debris removed by centrifugation at 500 *g* for 5 min at 4°C. A membrane fraction was prepared by centrifugation of the supernatant at 10,000 *g* for 15 min at 4°C, and the pellet washed and extracted in 2% Triton X-114 extraction buffer for 2 h at 4°C. Insoluble material was removed by centrifugation at 10,000 *g* for 15 min at 4°C and a detergent phase prepared by heat-induced phase-separation as previously described (13). Fractions were added to wheat germ agglutinin-agarose at a ratio of 100 µl lectin-agarose to 5 mg total protein and incubated overnight at 4°C with gentle mixing. The beads were washed twice with Hepes buffer containing 0.5% Triton X-100 and twice in NOG buffer (1% n-octyl-

glucopyroside in Hepes buffer). Wheat germ agglutinin-binding proteins were eluted in 0.5 M n-acetyl-glucosamine in NOG buffer.

Eluates were concentrated using the SDS-PAGE Sample Preparation kit (Pierce, Life Technologies, Paisley, UK), solubilized in SDS-PAGE Loading Buffer (Novex, Life Technologies) for 10 minutes at 60°C and electrophoresed on 12% Bis-Tris gels in MOPS running buffer. Gels were stained with Brilliant Blue G-Colloidal Coomassie (Sigma-Aldrich, Poole, UK) and gel slices corresponding to the 38,000 M_r region excised for mass spectrometry.

Immunoaffinity purification with Glima antibodies. For immunoaffinity purification, 250 µl of pooled sera from three patients with high Glima antibodies were used with 250 µl sera from three antibody-negative individuals as negative control. Sera were incubated with Protein A-Sepharose (250 µl) for 1 h at room temperature with rolling and washed three times in borate buffer (100 mM boric acid, pH 8.3). Antibodies were cross-linked to Protein A-Sepharose with 20 mM dimethylpimelidate in borate buffer for 1 h. Unreacted sites were blocked with 20 mM ethanolamine for 10 min and washed before use.

Triton X-114 detergent phase-purified amphiphilic proteins from mouse brains prepared as above were added to the Glima antibody-positive and -negative-beads and incubated overnight at 4°C with mixing. Beads were washed with 0.5% Triton X-100 in Hepes buffer prior to elution in 2% SDS at 90°C for 10 min. The eluate was concentrated to 20 µl using the SDS-PAGE Sample Preparation Kit, subjected to SDS-PAGE and Colloidal

Coomassie gel staining as above, and gel slices in the 38,000 M_r region excised for mass spectrometry.

In-gel trypsin digestion and mass spectrometry of 38,000 M_r proteins. Gel slices representing 38,000 M_r regions of all samples were processed using the In-Gel Tryptic Digestion kit (Pierce) according to the manufacturer's instructions and the trypsin-treated extracts vacuum-dried and stored at -20°C prior to mass spectrometry. Samples were reconstituted in 30 µl of 50 mM ammonium bicarbonate for 30 min at room temperature and centrifuged at 15,000 *g* for 15 min to remove insoluble material. Samples were transferred to autosampler tubes and 10 µl of each analyzed by LC-MS/MS. Peptides were resolved by reversed phase chromatography on a 75 µm C18 EASY column using a linear gradient of acetonitrile in 0.1% formic acid at a flow rate of 300 nL/min over 50 min on an EASY Nano LC system (Thermo Fisher Scientific). The eluate was ionized by electrospray ionization using an Orbitrap Velos Pro machine (Thermo Fisher Scientific) operating under Xcalibur (version 2.2; Thermo Fisher Scientific) and precursor ions selected based on their intensity for sequencing by collision-induced fragmentation. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the mass-to-charge ratio (m/z) and the charge state of the peptide.

Tandem mass spectra were processed into peak lists using Proteome Discoverer (version 1.3; Thermo Fisher Scientific). All MS/MS samples were analyzed using Mascot (version 2.2.06; Matrix Science, London, UK) searching the Uniprot Mus musculus database, assuming digestion with trypsin. Mascot was searched with a fragment ion mass

tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM, with oxidation of methionine and carbamidomethylation of cysteine as variable modifications. Each dataset was analyzed with a reverse FASTA database acting as a decoy.

Scaffold (version 4.3.2; Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications, which were assigned by Peptide Prophet algorithms (16; 17) and accepted at > 95.0% probability. The UniProt database was manually searched for physical characteristics of the proteins identified, including molecular weight, tissue distribution, and glycosylation. Of those identified, only one, tetraspanin-7 (Tspan7), matched the known properties of Glima (see Results) and was characterized further.

Immunohistochemistry. Tspan7 localization in rodent tissues was performed by immunohistochemistry. Sections of formalin-fixed paraffin-embedded rat brain, pituitary, pancreas, adrenal gland, lung, muscle, heart, liver, kidney, spleen, and thymus were de-waxed and subjected to epitope retrieval in a microwave pressure cooker in 10 mM citric acid pH 6.0, 0.05% Tween 20. Endogenous peroxidase activity was inhibited with 0.3% H_2O_2 and non-specific binding blocked with 25% non-immune swine serum in PBS. Primary antibody to Tspan7 (Anti-TM4SF2, Sigma-Aldrich; HPA003140) was applied at 1:1,000 dilution and incubated overnight at 4°C. Antibody labeling was detected with the Envision kit (Dako, Ely, UK) according to the manufacturer's instructions, sections counterstained in Meyers Haematoxylin (Sigma-Aldrich) and visualized by microscopy.

Cloning and expression of recombinant Tspan7. cDNA for the coding region of mouse Tspan7 was amplified by RT-PCR from the mouse islet cell line Min6 using primers (5′- GAATTCATGGCATCGAGGAGAATGG-3′ and 5′-AGATCTCACCATCTCATACTGATTGGC-3′) that introduce *Eco*R1 and *Bgl*II sites at the 5′ and 3′ ends, respectively, with the native stop codon removed to allow expression as a fusion protein with a C-terminal purification tag. The PCR product was cloned into the pFLAG-CTS expression vector for protein expression in *E. coli* BL21 cells after induction with IPTG. Expressed protein was extracted from cells with Hen Egg Lysozyme (1 mg/ml) in PBS containing 10 mM benzamidine, 1 mM PMSF for 30 min at room temperature, followed by incubation in Triton X-100 (0.1%) for 5 min and DNase (1 µg/ml) for 10 min. The lysate was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant used in immunoprecipitation assays.

Tspan7 binding to autoantibodies in Type 1 diabetes. Individual Glima antibody-positive and -negative human sera (15 µl) were incubated with protein A-Sepharose (15 µl), and the immunoglobulin captured was cross-linked to beads with dimethyl pimelimidate (18). Bead-bound antibodies were incubated overnight at 4°C with Triton X-100 extracts of mouse brain, or with lysates of *E. coli* expressing recombinant mouse Tspan7. Beads were washed three times in 0.5% Triton X-100 in Hepes buffer and captured proteins subjected to SDS-PAGE and Western blotting using rabbit anti-Tspan7 antibody (anti-TM4SF; Sigma-Aldrich HPA003140) at 1:250 dilution overnight at 4°C. Immunoprecipitated Tspan7 was detected with goat anti-rabbit IgG-peroxidase (product

A0545; Sigma-Aldrich;) and SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific).

Luminescent immunoprecipitation assay for detection of Tspan7 antibodies

The coding region of human Tspan7 was cloned into the pCMVTnT vector as a fusion with Nanoluciferase (Promega, Southampton, UK) at the 3' end. The construct was transfected into HEK 293 cells with ExpiFectamine (Thermo Fisher Scientific) for transient expression of antigen. Transfected cells were extracted in either 2% Triton-X114 or passive lysis buffer (Promega) and insoluble material removed by centrifugation. Triton-X-114 extracts were subjected to phase separation as described above and analysed for fusion protein by Western blotting with rabbit antibodies to Nanoluciferase (gift of Promega UK), or Tspan7 (anti-TM4SF). Luciferase expression in the cell extracts was quantified by luminometry using Nano-Glo assay reagent (Promega) and aliquots of passive lysis buffer extracts containing 10^6 light units of antigen were incubated with 5 µl of serum at 4°C for 16 h prior to capture of antibody complexes on protein A Sepharose. Mouse brain extracts (150 µg protein) or lysates of E coli expressing Tspan7 (250 µg protein) were added to reactions as sources of Tspan7 to compete for antibody binding. Complexes were washed and luciferase activity immunoprecipitated determined by luminometry with Nano-Glo assay reagent.

RESULTS

Selection of Glima antibody-positive sera. To identify patients with high levels of Glima antibodies for immunoaffinity purification, sera from 40 recent-onset Type 1 diabetic patients were screened by immunoprecipitation using radiolabelled mouse GT1.7 cell extracts (Fig 1). Intense diffuse 38,000 M_r bands typical of Glima were detected for three patients, indicative of high levels of Glima antibodies (029, 037 and 110 in Fig. 1). These three sera were used for subsequent Glima characterization and purification. Weaker 38,000 M_r bands indicative of Glima antibody positivity were detected in an additional 11 patients (Fig 1).

Tissue specificity of Glima expression. To identify large organs in which Glima is expressed at suitably high levels for antigen purification, competitive binding studies were performed in which detergent extracts of normal mouse tissues acted as unlabeled competitors with radiolabeled Glima from GT1.7 cell lysates for binding to antibodies in serum from the strong Glima antibody-positive patient, 029. Extracts of brain, pituitary, and lung reduced the intensity of radiolabeled 38,000 M_r protein, indicative of Glima immunoreactivity in these tissues (Fig. 2).

Identification of Glima candidate proteins by mass spectrometry. Extracts enriched for glycosylated membrane proteins from both brain and lung were prepared using Triton X-114 phase separation of amphiphilic membrane proteins followed by wheat germ agglutinin affinity purification. Proteins migrating at 38,000 M_r by SDS-PAGE were

trypsinized and analyzed by LC-MS/MS. A total of 65 candidates in brain and 25 in lung were identified, of which 20 were common to both samples, (Supplementary Table 1). Glycosylated membrane proteins immunoprecipitated from brain extracts by antibodies in the high Glima antibody titer patients' serum pool were also subjected to SDS-PAGE and LC-MS/MS analysis and three of the 20 protein candidates common to brain and lung were present in the Glima antibody-positive sample, but not the negative control (Supplementary Table 1). These were: 1) cytoplasmic actin-1 (Actb), a ubiquitous non-glycosylated cytoskeletal protein with a predicted molecular weight of 42 kDa; 2) guanine nucleotide-binding protein G(i) subunit alpha-2 (Gnai2), a non-glycosylated membrane associated 40 kDa protein with a wide tissue distribution; and 3) tetraspanin-7 (Tspan7), a hydrophobic four-transmembrane domain protein with core molecular weight 27.5 kDa, five putative N-glycosylation sites and a neuroendocrine distribution. Tspan7 closely matched the known properties of Glima and additional studies were performed to compare properties and validate Tspan7 as the autoantigen.

Localization of Tspan7 in rat tissues. The tissue distribution of Tspan7 was determined by immunohistochemistry for comparison with patterns of Glima expression in competition experiments above. Strong immunolabeling for Tspan7 was detected in the rat brain, in particular the cerebral cortex, hippocampus, cerebellum, striatum and thalamus (Fig. 3A), in the pancreatic islets (Fig. 3B), in the anterior pituitary (Fig. 3C), and in epithelial cells lining the alveoli in the lung (Fig. 3D). These observations agree with the Glima immunoreactivity described above, and previously (13, 15). Weak Tspan7 immunolabelling was also found in cells of the adrenal gland (Fig. 3E). No evidence of

Tspan7 expression was found in the exocrine pancreas (Fig. 3B), muscle, heart, liver, kidney, spleen, or thymus (not shown).

Immunoprecipitation of Tspan7 by antibodies in Type 1 diabetes. To demonstrate that Tspan7 is a target for autoantibodies in Type 1 diabetes, extracts of mouse brain and lysates of *E. coli* expressing recombinant mouse Tspan7 were subject to immunoprecipitation with Glima antibody-positive and -negative sera followed by Western blotting with a rabbit antibody to Tspan7. A 38,000 M_r band representing Tspan7 was selectively immunoprecipitated from mouse brain detergent extract by three Glima antibody-positive sera, but not by control samples. The bands detected co-migrated with the brain lysate control (Fig. 4A). Antibodies in Type 1 diabetic patients' sera also specifically immunoprecipitated Tspan7 from bacterial lysates containing recombinant protein (Fig 4B). Here, the protein migrated at approximately 22,000 M_r, consistent with a lack of glycosylation in bacteria (Fig. 4B). The results confirm Tspan7 as a target of autoantibodies in Type 1 diabetes.

Analysis of Tspan7 antibodies by luminescence immunoprecipitation assay

Patients screened for Glima antibodies were analyzed for Tspan antibodies by immunoprecipitation of recombinant Nanoluciferase-tagged human Tspan7. Western blotting with rabbit polyclonal antibodies to both Nanolucoferase and Tspan7 detected diffuse 38,000 M_r bands (the expected size of the non-glycosylated fusion protein) as the dominant immunoreactivity in cells transfected with the construct, with additional bands at approximately 80,000 M_r (Fig 4C). The 38,000 Mr protein partitioned into the

detergent on temperature-induced phase separation in Triton X-114. Transfected cell extracts were used in immunoprecipitation studies with normal control sera or with sera from Glima antibody-positive and -negative Type 1 diabetic patients. All but one (V015) of the controls (n=52) had low Tspan7 antibodies (Fig 4D). Four patients with high Glima antibodies (Fig 1) also immunoprecipitated high luciferase activity in the Tspan7 antibody assay (Fig 4D) and significantly higher levels of Tspan7 antibodies were found in Glima antibody-positive patients than those Glima antibody negative ($p<0.0001$; Mann Whitney U test). In competition assays, natural or recombinant Tspan7 in brain or E coli extracts partially (control sample V015), or completely (Glima antibody positive Type 1 diabetic patients) blocked antibody binding to the Nanoluciferase-Tspan7 construct (Fig 4E). Control V015 did not bind Tspan7 from mouse brain extracts when tested in the Western blotting assay. A second set of 94 recent onset Type 1 diabetic patients were also tested in the Tspan7 antibody assay. Using a cut-off of mean + 3SD of controls (omitting the outlier), 40 (43%) were positive for Tspan7 antibodies (Fig 4D).

DISCUSSION

Autoantibodies to "Glima" in Type 1 diabetes were first reported in 1996 (13), but its molecular identity has since then remained unknown. We used mass spectrometry of Glima-enriched fractions of brain and lung to search for likely candidates for Glima. LC-MS/MS analysis identified 65 proteins in 38,000 M_r gel samples of amphiphilic membrane glycoproteins from brain and 25 from lung, of which 20 were common to both (Supplementary Table 1). Additional LC-MS/MS analysis of immunoaffinity-purified proteins isolated from brain extracts using protein A Sepharose-coupled immunoglobulins from Glima antibody-positive sera (with similar preparations from Glima antibody-negative sera as a negative control) further narrowed down potential candidates for testing of autoantigenicity. Only six proteins detected in the brain or lung extracts were also present in the immunoaffinity purified sample but absent in the negative control. Of these, five (Actb, Gnai2, Sfxn5, Kctd12 and Tuba1b) had considerably higher predicted molecular weight (>36kDa) than expected for the non-glycosylated Glima protein (approximately 22kDa; reference 15). Furthermore, Actb, Gnai2 and Tub1a are ubiquitous cytoplasmic proteins lacking the amphiphilic characteristics expected of Glima. Sfxn5 and Kctd12 were not detected in the lung extract. Tspan7 was consequently the most promising candidate for Glima identified in the LS-MS/MS analyses.

Tspan7 is a member of the tetraspanin family, members of which share structural characteristics of four transmembrane domains, with one short (EC1) and one long (EC2)

extracellular loop (19). Four of the putative N-glycosylation sites are contained within the EC2 domain with a further site located in EC1. The presence of multiple transmembrane domains and N-glycosylation sites within Tspan7, is consistent with the hydrophobic properties and heavy N-glycosylation previously reported for Glima (13,15). There are four amino acid differences between mouse and human Tspan7, all being located in the long extracellular loop. The tissue distribution of Tspan7 expression has not been widely investigated but analysis of transcriptional activity of the Tspan7 gene has shown restricted tissue distribution with high levels being detected in regions of the adult mouse brain and lung (20). In the pancreas, Tspan7 is found specifically in the islets of Langerhans (21). Functionally, tetraspanin family members are involved in mediating signal transduction events and have been noted to regulate cell development, activation, growth, and motility through the trafficking of other transmembrane proteins (22). Both the extracellular and intracellular domains are able to interact with other proteins, and a number of tetraspanins bind integrins, thereby forming links with the actin cytoskeleton (23). Mutations in the Tpan7 gene are associated with X-linked mental retardation and neuropsychiatric diseases, potentially as a result of impaired ability of the actin cytoskeleton to drive neurite outgrowth (24). The ability of tetraspanins to form complexes with other membrane and cytosolic proteins may explain co-purification of multiple protein fragments identified in the LC-MS/MS analysis.

Proteins immunoprecipitated by Glima antibody-positive sera from brain extracts, or from bacterial extracts containing recombinant Tspan7, also bound rabbit antibodies to Tspan7 by Western blotting, confirming Tspan7 as the target of the antibodies. Glima

autoantibodies bound both the glycosylated natural 38,000 M_r Tspan7 in brain and the 22,000 M_r non-glycosylated form of the protein expressed in *E. coli.* A luminescence-based immunoprecipitation system (LIPS; 25) using Nanoluciferase-tagged human Tspan7 expressed in mammalian cells showed that patients with high levels of Glima autoantibodies were also strongly positive in the anti-Tspan7 LIPS. The relationship between Glima- and Tspan-autoreactivity is imperfect, which may in part be the consequence of difficulties in ascertaining whether or not diffuse Glima bands are present on autoradiographs of immunoprecipitation reactions (see Fig 1). Non-diabetic individuals had low levels of Tspan7 antibodies, with the exception of one strongly positive control. Antibodies in this control serum bound poorly to natural Tspan7 from mouse brain extracts suggesting that antibodies in this particular sample may bind epitopes not displayed on the natural protein. The SDS-PAGE gel migration of the fusion protein indicated that the majority of recombinant luciferase-tagged protein was not subject to heavy glycosylation found on the natural protein, indicative of incorrect membrane insertion, protein folding or intracellular targeting of the fusion protein required for appropriate post-translational modification. Incorrect folding or lack of glycosylation may reveal antibody epitopes not normally displayed on the natural Tspan7. Further optimization of Tspan7 expression should permit the development of high throughput assays for the detection of diabetes-associated Tpan7 autoantibodies with high sensitivity and specificity.

Autoimmunity to major autoantigens in Type 1 diabetes appears within the first five years of life in at-risk children (26), with individual immune responses developing

sequentially rather than simultaneously (27). Autoimmunity in the disease is therefore progressive, with the order of appearance of autoimmune responses to individual antigens differing between individuals, and diversification of the immune response being essential for disease progression; individuals who develop autoimmunity to only single autoantigens rarely develop disease (28). The optimum strategy currently adopted for assessing disease risk is to screen individuals for the presence of autoantibodies to multiple islet autoantigens. The inclusion of Tspan7 antibodies in the screen may improve sensitivity and specificity of disease prediction in large populations, and will provide a fuller description of the major autoimmune responses that are developing in that individual, necessary for guiding the selection of autoantigen-specific immunotherapeutics to prevent the disease.

AUTHOR CONTRIBUTIONS

KAM designed the study, researched and analyzed data and wrote the manuscript; CCR, AR, CB, DL, VL, LP, DM and RF researched and analyzed data. MRC designed the study, researched and analyzed data and contributed to the writing of the manuscript. All authors reviewed and edited the manuscript and approved the final version for submission. MRC is guarantor of the study and, as such, had full access to all data and takes responsibility for the integrity and accuracy of the data analysis.

ACKNOWLEDGMENTS

This study was funded by a research grant from Diabetes UK (grant 11/0004297) and by a Society for Endocrinology Early Career Award to KAM. Research by C.B. D.L. V.L. and L.P. was conducted within the framework of the Italian Ministry of Research's project “Ivascomar project, Cluster Tecnologico Nazionale Scienze della Vita ALISEI”. CCR was supported by a PhD Studentship from King’s College London Graduate School. Part of this study was presented as a late breaking poster at the 75th Scientific Sessions of the American Diabetes Association in Boston in June 2015. The authors confirm that there are no conflicts of interest relevant to the contents of this article.

REFERENCES

1. Bottazzo GF, Doniach D: Islet-cell antibodies (ICA) in diabetes mellitus (evidence of an autoantigen common to all cells in the islet of Langerhans). Ric Clin Lab 1978;8:29-38

2. Weenink SM, Christie MR: Autoantibodies in Diabetes. In Autoantibodies and Autoimmunity: Molecular Mechanisms in Health and Disease. Pollard M, Ed. Weinheim, VCH Verlag GmbH & Co, 2005, p. 321-349

3. Skyler JS: Primary and secondary prevention of Type 1 diabetes. Diabetic Medicine 2013;30:161-169

4. Michels A, Zhang L, Khadra A, Kushner JA, Redondo MJ, Pietropaolo M: Prediction and prevention of type 1 diabetes: update on success of prediction and struggles at prevention. Pediatric Diabetes 2015:n/a-n/a

5. Luo X, Herold KC, Miller SD: Immunotherapy of type 1 diabetes: where are we and where should we be going? Immunity 2010;32:488-499

6. Keymeulen B, Vandemeulebroucke E, Ziegler AG, Mathieu C, Kaufman L, Hale G, Gorus F, Goldman M, Walter M, Candon S, Schandene L, Crenier L, De Block C, Seigneurin JM, De Pauw P, Pierard D, Weets I, Rebello P, Bird P, Berrie E, Frewin M, Waldmann H, Bach JF, Pipeleers D, Chatenoud L: Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. N Engl J Med 2005;352:2598-2608

7. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R, Gottlieb PA, Marks JB, McGee PF, Moran AM, Raskin P, Rodriguez H, Schatz DA, Wherrett D, Wilson DM, Lachin JM, Skyler JS: Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361:2143-2152

8. Feutren G, Papoz L, Assan R, Vialettes B, Karsenty G, Vexiau P, Du Rostu H, Rodier M, Sirmai J, Lallemand A, et al.: Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset. Results of a multicentre double-blind trial. Lancet 1986;2:119-124

9. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL: Insulin antibodies in insulin-dependent diabetics before insulin treatment. Science 1983;222:1337-1339

10. Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, De Camilli P: Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 1990;347:151-156

11. Payton MA, Hawkes CJ, Christie MR: Relationship of the 37,000- and 40,000-MI tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512). J Clin Invest 1995;96:1506-1511

12. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW, Hutton JC: The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A 2007;104:17040-17045

13. Aanstoot HJ, Kang SM, Kim J, Lindsay LA, Roll U, Knip M, Atkinson M, Mose-Larsen P, Fey S, Ludvigsson J, Landin L, Bruining J, Maclaren N, Akerblom HK, Baekkeskov S: Identification and characterization of glima 38, a glycosylated islet cell membrane antigen, which together with GAD65 and IA2 marks the early phases of autoimmune response in type 1 diabetes. J Clin Invest 1996;97:2772-2783

14. Winnock F, Christie MR, Batstra MR, Aanstoot HJ, Weets I, Decochez K, Jopart P, Nicolaij D, Gorus FK: Autoantibodies to a 38-kDa glycosylated islet cell membrane-associated antigen in (pre)type 1 diabetes: association with IA-2 and islet cell autoantibodies. Diabetes Care 2001;24:1181-1186

15. Roll U, Turck CW, Gitelman SE, Rosenthal SM, Nolte MS, Masharani U, Ziegler AG, Baekkeskov S: Peptide mapping and characterisation of glycation patterns of the glima 38 antigen recognised by autoantibodies in Type I diabetic patients. Diabetologia 2000;43:598-608

16. Keller A, Nesvizhskii AI, Kolker E, Aebersold R: Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Anal Chem 2002;74:5383-5392

17. Nesvizhskii AI, Keller A, Kolker E, Aebersold R: A statistical model for identifying proteins by tandem mass spectrometry. Anal Chem 2003;75:4646-4658

18. Harlow E, Lane D: *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, 1988

19. Hemler ME: Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol 2005;6:801-811

20. Lizio M, Harshbarger J, Shimoji H, Severin J, Kasukawa T, Sahin S, Abugessaisa I, Fukuda S, Hori F, Ishikawa-Kato S, Mungall CJ, Arner E, Baillie JK, Bertin N, Bono H, de Hoon M, Diehl AD, Dimont E, Tom C Freeman TC, Fujieda K, Hide W, Kaliyaperumal R, Katayama T, Lassmann T, Meehan TF, Nishikata K, Ono H, Rehli M, Sandelin A, Schultes EA, 't Hoen PAC, Tatum Z, Thompson M, Toyoda T, Wright DW, Daub CO, Itoh M, Carninci P, Hayashizaki Y, Forrest ARR, Kawaji H, FANTOM consortium:

Gateways to the FANTOM5 promoter level mammalian expression atlas. Genome Biol 2015;16:22; http://www.ebi.ac.uk/gxa/genes/ENSMUSG00000058254

21. Hald J, Galbo T, Rescan C, Radzikowski L, Sprinkel AE, Heimberg H, Ahnfelt-Rønne J, Jensen J, Scharfmann R, Gradwohl G, Kaestner KH, Stoeckert C Jr, Jensen JN, Madsen OD: Pancreatic islet and progenitor cell surface markers with cell sorting potential. Diabetologia 2012 55:154-65

22. Maecker HT, Todd SC, Levy S: The tetraspanin superfamily: molecular facilitators. FASEB J 1997;11:428-442

23. Berditchevski F: Complexes of tetraspanins with integrins: more than meets the eye. J Cell Sci 2001;114:4143-4151

24. Zemni R, Bienvenu T, Vinet MC, Sefiani A, Carrie A, Billuart P, McDonell N, Couvert P, Francis F, Chafey P, Fauchereau F, Friocourt G, Portes Vd, Cardona A, Frints S, Meindl A, Brandau O, Ronce N, Moraine C, Bokhoven HV, Ropers HH, Sudbrak R, Kahn A, Fryns JP, Beldjord C, Chelly J: A new gene involved in X-linked mental retardation identified by analysis of an X;2 balanced translocation. Nat Genet 2000;24:167-170

25. Burbelo, PD, Ching, KH, Mattson, TL, Light, JS, Bishop, LR, Kovacs, JA: Rapid antibody quantification and generation of whole proteome antibody response profiles using LIPS (luciferase immunoprecipitation systems). Biochem Biophys Res Comm 2007;352:889–895

26. Ziegler AG, Bonifacio E, Group B-BS: Age-related islet autoantibody incidence in offspring of patients with type 1 diabetes. Diabetologia 2012;55:1937-1943

27. Barker JM, Barriga KJ, Yu L, Miao D, Erlich HA, Norris JM, Eisenbarth GS, Rewers M: Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY). J Clin Endocrinol Metab 2004;89:3896-3902

28. Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, Winkler C, Ilonen J, Veijola R, Knip M, Bonifacio E, Eisenbarth GS: Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children. JAMA 2013;309:2473-2479

Figure legends

Figure 1. Autoradiogram showing a screen of serum samples from Type 1 diabetic patients for Glima antibodies by immunoprecipitation of the 38,000 M_r protein from extracts of GT1.7 cells with detection by SDS-PAGE and autoradiography. A normal control serum (-ve) and a Type 1 diabetic patient previously determined to be positive for Glima antibodies (SL) were included in the assay. The location of Glima on the autoradiograph is marked. An indication of whether the samples were determined to be negative (-) or positive (+) for Glima antibodies is shown under each lane of the figure.

Figure 2. Autoradiograph of tissue expression screen demonstrating competition for Glima antibody binding to Glima antibody positive serum "029" by proteins in Triton X-100 detergent (100 μg) extracts of normal mouse tissues. Serum from a non-diabetic individual was included as a negative control. Reduced intensity of the 38,000 M_r band is indicative of Glima immunoreactivity in that tissue.

Figure 3. Immunohistochemical analysis of Tspan7 expression in rat tissues. Sections of formalin fixed, paraffin-embedded rat tissues were labeled with rabbit antiserum to Tspan7 and labeling detected by a peroxidase/DAB-based system with positive labeling detected as brown staining under the microscope. Representative images of labeling of tissue sections of brain (A), pancreas (B), pituitary (C), lung (D), and adrenal (E) are shown.

Figure 4. A. Tspan7 labeling of Western blots of mouse brain proteins immunoprecipitated by antibodies in sera from Glima antibody-negative and positive

recent onset Type 1 diabetic patients. The migration of molecular weight markers (10^{-3} x M_r) on the gel and the localization of Tspan7-specific bands at 38,000 M_r are marked. **B.** Tspan7 labeling of Western blots of proteins from lysates of Tspan7-expressing *E.coli* immunoprecipitated by antibodies in sera from Glima antibody-negative and -positive recent onset Type 1 diabetic patients. The localization of Tspan7-specific bands at approximately 22,000 M_r is marked; migration of molecular weight markers as in A. In both panel A and B, IgG heavy chains (50,000 M_r), light chains (25,000 M_r) and cross-linked immunoglobulin (>100,000 Mr) from all serum samples were also detected on the blot as a consequence of cross-reactivity with the peroxidase-conjugated anti-rabbit detection antibody. C. Tspan7 was expressed as a fusion protein with Nanoluciferase and Triton X114 extracts of cells were subject to heat-induced phase separation. Detergent and aqueous phases were subject to SDS-PAGE and Western blotting with antibodies to Nanoluciferase (NanoLuc) or Tspan7. The migration of molecular weight markers are shown (10^{-3} x M_r). D. Detergent extracts of Nanoluciferase were immunoprecipitated with normal control sera (n=30), Glima antibody (Ab) positive Type 1 diabetic (T1D) patients' sera (n=15) and Glima antibody-negative Type 1 diabetic patients' sera and luciferase activity associated with each immunoprecipitate determined by luminometry. Data are plotted as luciferase activity immunoprecipitated in kilo light units (kLU) and sample codes for control or diabetic individuals with high levels of antibodies are shown. E. Samples from control individuals or Glima antibody-positive Type 1 diabetic patients were tested for competitive binding by natural or recombinant Tspan7 in the LIPS by performing the immunoprecipitations in the absence (black bars) or presence of 150 μg of mouse brain extract (white bars) or 250 μg of lysates of E coli expressing recombinant

Tspan7 (hatched bars). Assays were performed in triplicate. Addition of brain and E coli lysate significantly blocked antibody binding for all samples ($p<0.0001$; ANOVAR with Dunnett's correction for multiple comparisons), with the exception of control sample CH.