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Research Article

Immunoprecipitating IgG autoantibodies to glutamic acid decarboxylase, GAD65, and/or a tyrosine phosphatase, IA2, are present in the majority of individuals experiencing pancreatic beta cell destruction and development of type 1 diabetes. Here we identify a third islet cell autoantigen, a novel 38-kD protein, which is specifically immunoprecipitated with sera from a subset of prediabetic individuals and newly diagnosed type 1 diabetic patients. The 38-kD autoantigen, named glima 38, is an amphiphilic membrane glycoprotein, specifically expressed in islet and neuronal cell lines, and thus shares the neuroendocrine expression patterns of GAD65 and IA2. Removal of N-linked carbohydrates results in a protein of 22,000 M(r). Glima 38 autoantibodies were detected in 16/86 (19%) of newly diagnosed patients, including three very young children, who had a rapid onset of disease, and in 6/44 (14%) of prediabetic individuals up to several years before clinical onset. The cumulative incidence of GAD65 and glima 38 antibodies in these two groups was 83 and 80%, respectively, and the cumulative incidence of GAD65, glima 38, and IA2 antibodies in the same groups was 91 and 84%, respectively. GAD65, IA2, and glima 38 represent three distinct targets of immunoprecipitating IgG autoantibodies associated with beta cell destruction and type 1 diabetes.

Identification and Characterization of Glima 38, a Glycosylated Islet Cell Membrane Antigen, Which Together with GAD_{65} and IA2 Marks the Early Phases of Autoimmune Response in Type 1 Diabetes

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Abstract

Immunoprecipitating IgG autoantibodies to glutamic acid decarboxylase, GAD_{65}, and/or a tyrosine phosphatase, IA2, are present in the majority of individuals experiencing pancreatic β cell destruction and development of type 1 diabetes. Here we identify a third islet cell autoantigen, a novel 38-kD protein, which is specifically immunoprecipitated with sera from a subset of prediabetic individuals and newly diagnosed type 1 diabetic patients. The 38-kD autoantigen, named glima 38, is an amphiphilic membrane glycoprotein, specifically expressed in islet and neuronal cell lines, and thus shares the neuroendocrine expression patterns of GAD_{65} and IA2. Removal of N-linked carbohydrates results in a protein of 22,000 M_r. Glima 38 autoantibodies were detected in 16/86 (19%) of newly diagnosed patients, including three very young children, who had a rapid onset of disease, and in 6/44 (14%) of prediabetic individuals up to several years before clinical onset. The cumulative incidence of GAD_{65} and glima 38 antibodies in these two groups was 83 and 80%, respectively, and the cumulative incidence of GAD_{65}, glima 38, and IA2 antibodies in the same groups was 91 and 84%, respectively. GAD_{65}, IA2, and glima 38 represent three distinct targets of immunoprecipitating IgG autoantibodies associated with β cell destruction and type 1 diabetes. (*J. Clin. Invest.* 1996. 97:2772–2783.) Key words: insulin-dependent diabetes mellitus • islet • autoantibodies • autoantigen • membrane glycoprotein

Introduction

Pancreatic β cells in islets of Langerhans can be destroyed by autoimmune processes resulting in insulin-dependent or type 1 diabetes (1). The destruction often proceeds over a long period of time before the clinical symptoms develop (2, 3). The gradual loss of β cells is accompanied by circulating islet cell antibodies demonstrated by indirect immunofluorescence staining of frozen sections of human pancreas (ICA)[1] (2, 3). Although β cell destruction is believed to be mediated by T cells (4), islet cell antibodies detected in the early phases of β cell destruction are likely to be directed to the same antigen(s) as pathogenic T cells. Furthermore, antigen-specific B lymphocytes may play an important role in presentation of rare β cell autoantigens to maintain a chronic autoimmune response that gradually depletes the β cell pool. ICA epitopes are usually only detected on frozen but not on fixed pancreatic tissue, consistent with their conformational nature. Immunoprecipitation of islet cell lysates in the presence of nonionic detergents, which preserve the conformation of proteins, has identified the smaller form of the neuroendocrine enzyme glutamic acid decarboxylase (GAD_{65}) (5, 6) and a 40-kD tryptic fragment of the tyrosine phosphatase (IA2) (7–9) as targets of islet cell antibodies associated with early as well as late stages of β cell destruction in 70–80% and 50–80% of patients, respectively (5–8, and references therein). Sequence analyses of the immunoglobulin genes encoding GAD_{65} autoantibodies have provided convincing evidence that the development of these antibodies is antigen driven and can involve many rounds of antigen selection (10). Reactivity with denatured carboxypeptidase H (11), a 52-kD protein (12), and a 69-kD protein with homology to BSA (13) on Western blots has also been reported in some

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The first phase of this work was carried out at the Hagedorn Research Laboratory, Gentofte, Denmark (by H.-J. Aanstoot and S. Bækkeskov).

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1. *Abbreviations used in this paper:* GABA, γ-amino butyric acid; GAD, glutamic acid decarboxylase; ICA, islet cell cytoplasmic antibodies measured by indirect immunofluorescence analysis; JDF, Juvenile Diabetes Foundation.

diabetic sera. However, the proteins identified by immunoblotting are unlikely to be targets of ICA in the majority of sera which only react on frozen (nondenaturing conditions) but not fixed (denaturing conditions) sections of human pancreas.

GAD_{65} is the synthesizing enzyme for the major inhibitory neurotransmitter γ-amino butyric acid (GABA) and is expressed in significant amounts in β cells and in GABA-ergic neurons (14). GABA may function as a paracrine signaling molecule in pancreatic islets (14). The diabetes-associated epitopes in GAD_{65} are predominantly conformational and only include a linear epitope in very rare cases (5, 15, 16). IA2 belongs to a family of membrane-spanning tyrosine phosphatases, but its membrane compartment in β cells has not yet been identified (8, 9). Autoantibodies in insulin-dependent diabetes mellitus are directed to conformational epitopes in the COOH-terminal half of the protein, which is likely to be cytosolic (8, 9). This region of the protein encompasses the 40-kD tryptic fragment, which was originally identified in trypsinated immunoprecipitates of rat islet cell proteins using type 1 diabetic sera (7). A 37-kD tryptic fragment detected in concert with the 40-kD fragment is derived from a cross-reactive tyrosine phosphatase (9) but does not seem to constitute an independent autoantigen. The amino acid sequence of IA2 predicts a molecular mass of 105 kD, but the precursor of the 40-kD tryptic fragment in islets seems to migrate as a diffuse band, at a much lower relative molecular mass of ~ 64,000 D(8). The reason for this difference in the predicted and apparent size of the islet protein on SDS gels is not clear and accurate identification of the IA2 antigen in SDS-PAGE analysis of immunoprecipitates of islet cell lysates with diabetic sera requires trypsinization. A partial cDNA clone of IA2 (52 kD) was independently identified by antibody screening of an islet cell cDNA library using a serum from a diabetic patient (17). The COOH-terminal region of IA2 also contains the single tentative catalytic tyrosine phosphatase domain. This domain has nonconserved amino acid substitutions in residues critical for enzyme activity, and tyrosine phosphatase activity of IA2 remains to be demonstrated (9). IA2 is expressed in pancreatic islets and in brain (9). Thus both GAD_{65} and IA2 are neuroendocrine enzymes.

We have sporadically detected an antigen with a molecular mass of 38 kD in immunoprecipitates of nontrypsinated rat islet cell proteins using human type 1 diabetic sera and native conditions (our unpublished results). Antibodies to a protein of a similar relative molecular mass have been reported in the BB rat, an animal model of type 1 diabetes (18). T cell reactivities to a 38-kD rat insulinoma protein, imogen 38 (19), and to the 38-kD nuclear transcription factor jun-B (20) have also been detected in newly diagnosed diabetic patients. We now report that a vigorous extraction of islet cell proteins in detergent results in a consistent detection of a 38-kD β cell antigen in immunoprecipitates with a subgroup of type 1 diabetic sera. The difficulty in extracting the 38-kD protein indicates that it is relatively insoluble and thus has escaped detection in many previous immunoprecipitation analyses with type 1 diabetic sera. Using an improved extraction method for solubilizing islet cell proteins, we have analyzed the incidence of 38-kD autoantibodies in newly diagnosed and prediabetic individuals and determined different characteristics of the 38-kD antigen. The results demonstrate that the 38-kD protein is a novel target of autoantibodies in a subset of prediabetic and diabetic patients and is distinct from both imogen 38 and jun B.

Methods

Preparation of islet cell extracts and immunoprecipitation analysis of GAD_{65} and 38-kD antibodies. Neonatal rat islets were isolated and labeled with [^{35}S]methionine as described (21). Islets were swollen on ice for 10 min in HEMAP buffer (10 mM Hepes, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM aminoethyl-isothiouronium bromide hydrobromide, and 0.2 mM pyridoxal phosphate), followed by homogenization by 20 strokes in a glass homogenizer. The homogenate was centrifuged at 100,000 *g* for 1 h to obtain a cytosol and a particulate membrane fraction. The particulate membrane fraction was extracted in HEMAP buffer with 2% Triton X-114 for 2 h by repeated dispersion through a bent constricted pipette tip, followed by centrifugation at 100,000 *g* to remove debris. Amphiphilic proteins in both the cytosol fraction and the membrane extract were purified by temperature-induced Triton X-114 phase separation (5). The detergent phase of either membrane or cytosol fractions was precleared with a normal human serum before immunoprecipitation with the indicated sera (21). Extracts of 250–500 rat islets were used per immunoprecipitate. Immunoprecipitates were analyzed by SDS-PAGE using 15% gels and processed for fluorography (21). A quantitative estimate of the GAD_{65} and 38-kD protein immunoreactivity of sera (antibody index) was obtained by densitometric scanning of bands corresponding to the proteins on autoradiograms using a video densitometer (model 620) with 1D Analyst II and version 3.10 software (BioRad Laboratories, Richmond, CA). Serum I_{15} was used as an internal 38-kD antibody positive control in all analyses and its value arbitrarily set at 10. 38-kD antibody indexes in other sera were calculated from integrated peak areas by the formula: index = 10 × (value for unknown serum − value for negative control serum)/(value for serum I_{15} − value for negative control serum). Similarly GAD_{65} antibody indexes were expressed in relation to a standard positive control serum which is a Juvenile Diabetes Foundation (JDF) world standard for ICA analyses and is also used as a standard for quantitative analyses of GAD_{65} autoantibodies (22, 23). This serum was also used as a standard positive control serum for analysis of IA2 antibodies (see below). The values of GAD_{65} antibodies and IA2 antibodies, respectively, in this serum were each arbitrarily set at 10. GAD_{65} antibodies in sera were also analyzed by immunoprecipitation of [^{35}S]methionine-labeled recombinant human GAD_{65} expressed in COS-7 cells (23), an assay which had a 100% sensitivity and 100% specificity in the First International GAD Antibody Workshop (24). There was a complete correlation between sera scored positive and negative, respectively, in the two assays.

Analysis of IA2 antibodies. Analyses of IA2 antibodies using immunoprecipitation of rat islet cell extracts require trypsinization of either islet cell lysates or immunoprecipitates, which was not compatible with analyses of GAD_{65} and the 38-kD antigen in the immunoprecipitates. Therefore, IA2 antibodies were assayed by immunoprecipitation of [^{35}S]methionine-labeled fragment of human IA2 generated by in vitro transcription and translation of a partial cDNA clone IA2ic in a pGEM-4Z vector containing an SP6 promoter (8) (a gift of Dr. M. Christie, King's College, London, United Kingdom). IA2ic encodes the COOH-terminal region of IA2, encompassing the 40-kD tryptic fragment recognized by diabetes-associated antibodies.

IA2ic was transcribed and translated in vitro in the presence of [^{35}S]methionine (Amersham International, Little Chalfont, United Kingdom) using a kit from Promega Corp. (Madison, WI). 25–50 × 10^3 cpm of labeled protein in 20 μl IMP buffer (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 0.5 mM methionine, 10 mM benzamidine/HCl, 0.1 mg/ml BSA, 5 mM EDTA, 0.5% Triton X-114) was incubated with 5 μl of serum overnight at 4°C, followed by absorption to 5 μg of preswelled protein A–Sepharose in the same buffer for 1 h. The PAS was washed five times in IMP buffer and once in H_2O. Immunocomplexes were eluted in 20 μl of SDS sample buffer, analyzed by SDS-PAGE and autoradiography. Immunoprecipitated IA2c was quantitated by phosphorimaging and by liquid scintillation counting of 10 μl of the eluate. IA2 antibody indexes are expressed in relation to a

standard positive control serum (see above). This assay had a sensitivity of 69% in type 1 diabetic patients and a specificity of 97% in the 1995 Immunology of Diabetes Antibody Workshop.

Cell lines. The βTC3 cell line was derived from a transgenic insulinoma (25). The βHC16 cell line was derived from hyperplastic mouse islets (26). αTC1 and αTC2 are two cell lines derived from two independent transgenic mouse glucagonomas and show some expression of insulin in addition to glucagon (reference 27 and our unpublished results). The αTC1-6 cell line was derived from a single cell clone of αTC1, selected for its α cell phenotype (glucagon expression). Both αTC1-6 and αTC2 were a gift from Dr. D. Hanahan (University of California San Francisco). The GT1.1 and GT1.7 cell lines were derived from two independent single cell clones of a gonadotropin-releasing hormone secreting tumor cell line, GT1, procured from a transgenic mouse (28). The GT 1.1 and GT 1.7 were selected based on a strong neuronal phenotype in culture and were a gift from Dr. R. Weiner (University of California San Francisco). The human neuroblastoma cell line SKNSH (29) was a gift from Dr. W. Sadee (University of California San Francisco). The rat hepatoma cell line HTC (30) was a gift from Dr. A. Reuser (Erasmus University). The human melanoma cell line OMM1 was a gift from T. Luider (Erasmus University). All other cell lines (Table I) were obtained from the American Tissue Culture Collection (Rockville, MD). The αTC and βTC cell lines were cultured as described earlier (25, 26). The SKNSH, Kelly, GT1.1, and GT1.7 cell lines were cultured in RPMI 1640, supplemented with 10% FCS, 100 μg streptomycin/ml, and 100 IU penicillin/ml. The SKNSH cells were grown with and without 10 μM retinoic acid (Sigma Immunochemicals, St. Louis, MO), which has been shown to enhance the neuroblastoid phenotype in this cell line (29). All other cell lines were cultured in DME, supplemented with 10% FCS, 100 μg streptomycin/ml, and 100 IU penicillin/ml. The culture medium for the C6 cell line was supplemented with 10 μM retinoic acid. Cells were grown to 70–80% confluency and labeled with [^{35}S]methionine according to established methods (21). Membrane extracts were prepared, extracted, and subjected to Triton X-114 phase separation. Detergent phase aliquots corresponding to 1×10^6 cpm of labeled protein were used for each immunoprecipitate. Individual cell lines were immunoprecipitated as described for neonatal rat islets using serum I_{15} and serum C_1. The immunoprecipitates were analyzed by SDS-PAGE and fluorography (21).

Table I. Analysis of Expression of the 38-kD Protein in Different Cell Lines

Cell line	Origin	Expression
βTC3 (ref. 25)	Mouse pancreatic insulinoma	pos
βHC16 (ref. 26)	Mouse hyperplastic islets	pos
αTC-2 (ref. 27)	Mouse pancreatic glucagonoma	pos
αTC1.6 (ref. 27)	Mouse pancreatic glucagonoma	neg
GT1.1 (ref. 28)	GnRH neuronal tumor	pos
GT1.7 (ref. 28)	GnRH neuronal tumor	pos
Neuro 2A*	Mouse neuroblastoma	pos
C6*	Rat glial cells	pos
SKNSH (ref. 29)	Human neuroblastoma	neg
Kelly*	Human neuroblastoma	(pos)
Bowes*	Human melanoma	neg
Omm1*	Human melanoma	neg
CHO*	Chinese hamster ovary	neg
HeLa*	Human ovarian adenocarcinoma	neg
T47D*	Human ductal breast carcinoma	neg
Sk-NEP-1*	Human nephroblastoma	neg
Cos-1*	Monkey kidney tumor	neg
CV-1*	Precursor of Cos-1	neg
HepG2*	Human hepatocellular carcinoma	neg
BHK-21*	Baby hamster kidney	neg
HTC (ref. 30)	Rat hepatoma	neg
TERA-2*	Human teratocarcinoma	neg
CCD-118Sk*	Human fibroblast	neg

Aliquots of Triton X-114 detergent phase purified membrane protein fraction of each cell line, corresponding to 1×10^6 cpm per immunoprecipitate, were immunoprecipitated with serum I_{15} and C_1, respectively. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. A strong 38-kD band was detected in the positive cell lines after a 3-d exposure. In contrast, the 38-kD protein was not detected in any of the other cell lines after a 1-mo exposure. *American Type Culture Collection. *GnRH*, gonatotropin-releasing hormone.

Two-dimensional gel electrophoresis and determination of isoelectric points. Two-dimensional gel electrophoresis using isoelectric focusing or nonequilibrium pH-gradient gel electrophoresis in the first dimension and SDS-PAGE in the second dimension was carried out as described (21, 31). Gels were exposed to phosphorimaging plates for 3–21 d and read at high amplification using an AGFA clinical phosphor storage plate reader. The resulting 16-bit images were converted to 8-bit images with a final spatial resolution of ~ 200 μM (1,024 × 1,024 pixels). The radioactive spots were analyzed by image scanning using the Bio-Image software version 4.6 (Millipore, Bedford, MA). The isoelectric points were determined by coelectrophoresis with carbamylated creatin phosphokinase charge chain markers (BDH, Poole, Dorset, United Kingdom) and with total HeLa cellular proteins containing several hundred marker proteins of known relative molecular mass, pI, and location in the two-dimensional pattern.

Description of patient groups and sera. Sera were collected after informed consent from the following groups of individuals: (*a*) 86 newly diagnosed Dutch, Finnish, and Swedish type 1 diabetic patients ($I_{1–86}$), including 15 that developed diabetes at < 2 yr of age, and 71 that developed diabetes at > 2 yr of age; (*b*) 65 Dutch and Finnish control individuals ($C_{1–65}$) including 15 at < 2 yr of age, 50 at > 2 yr of age; and (*c*) 44 North-American and Dutch prediabetic individuals (age 2.6–49.9 yr) ($P_{1–44}$). The first (and sometimes only) serum available from the individuals in this group was sampled 3–85 mo before clinical onset of type 1 diabetes.

ICA and immunofluorescence analysis. ICA were analyzed by indirect immunofluorescence of frozen sections of human pancreas from cadaveric kidney donors of blood group 0 (32–34). Samples were titrated and end point titers were defined as the highest titer of detectable ICA staining. Positive samples were expressed in JDF units by comparing their end point dilution to a standard calibration curve using the international JDF reference serum provided by the Immunology of Diabetes Workshops (35). The test laboratories are participants of the ICA proficiency program conducted under the auspices of the Workshops (36). A titer of 10 JDF units was considered positive.

For double immunofluorescence analysis of human pancreas, frozen sections were air dried for 20 min and incubated with one of the following sera: I_{14}, I_{15}, or I_{31}, at different dilutions overnight. After a 15-min wash in PBS, 3% BSA, the slides were incubated for 30 min in a mixture of rabbit antiglucagon (Dako, Glostrup, Denmark) and rabbit antisomatostatin (Dako) both diluted 1:20 in PBS. After several wash steps of 15 min each in PBS, 3% BSA, the slides were incubated with a mixture of FITC-labeled swine anti–human IgG (Dako) and tetramethyl rhodamine isothiocyanate–labeled goat anti–rabbit IgG (Dako) at dilution 1:60 and 1:15, respectively. After a final wash, the slides were covered in Vectashield (Vector, Burlingame, CA) and viewed on an Aristoplan immunofluorescence microscope (Leica Instruments, Wetzlar, Germany).

Deglycosylation of the 38-kD antigen. The 38-kD antigen was isolated from Triton X-114 detergent phase fractions of rat islet membranes by immunoprecipitation with I_{15}. The protein was eluted from the protein A–Sepharose pellet by boiling for 3 min in 0.4 M Na-

phosphate, pH 7.9, 1% SDS, and 0.7% βME, diluted twofold, supplemented with Nonidet P-40 (Calbiochem-Novabiochem, San Diego, CA) to a final concentration of 2.5%, and divided into three aliquots. One aliquot was frozen immediately, two aliquots were incubated with and without *N*-glycanase (Genzyme, Cambridge, MA) at 37°C for 18 h. All three samples were boiled in SDS sample buffer and analyzed by SDS-PAGE and fluorography using a Bio Max film (Eastman Kodak Co., Rochester, NY).

Results

Solubilization of the 38-kD protein. In the course of analyzing sera from type 1 diabetic patients for GAD_{65} autoantibodies using Triton X-114 detergent phase purified membrane protein fractions from islets of Langerhans, we discovered reactivity to a second membrane protein of 38 kD (see Fig. 1, lanes *I_4, I_9, I_{14}–I_{16}*). We have used these sera to characterize this 38-kD species and to establish reliable conditions for its extraction, which has facilitated screens to establish the incidence of 38-kD autoantibodies in prediabetic and recent onset diabetic patients.

Extraction of the GAD_{65} antigen from islets of Langerhans is complete after 30 min in 1% nonionic detergents. In contrast, the 38-kD protein is only sporadically detected in such extracts by immunoprecipitation with a serum from a newly diagnosed type 1 diabetic patient, I_{15}. In preliminary experiments, several detergents (CHAPS, β-octyl-glucoside, sodium deoxycholate, Triton X-114) were tested for their ability to extract the 38-kD protein. A 2% concentration of each detergent effectively solubilized the 38-kD protein during a vigorous 2-h extraction of islet cell membranes. Triton X-114 was selected for all further experiments to facilitate a partial purification of the 38-kD protein by a temperature-induced phase transition and separation of amphiphilic membrane proteins into the Triton X-114 detergent phase.

The 38-kD protein is an amphiphilic β cell membrane glycoprotein of pI 5.6–6.1. The relative insolubility of the 38-kD protein suggested that it was membrane bound. Cytosolic and membrane proteins were subjected to a Triton X-114 phase separation to assess the amphiphilicity of the 38-kD protein (Fig. 1). In contrast to the GAD_{65} autoantigen which is found as a soluble hydrophilic, a soluble amphiphilic, and a membrane bound amphiphilic form (37, 38) (Fig. 1, compare lanes *16* and *18*), the 38-kD protein was only detected in the particulate fraction, where it partitioned into the detergent phase (Fig. 1, lanes *15* and *16*). Thus, the 38-kD protein is an amphiphilic membrane protein. The relative insolubility of the 38-kD protein suggests that it is an integral membrane protein in contrast to GAD_{65}, which is anchored to membranes via lipid residues (38).

The 38-kD protein was detected as a broad band on fluorograms of SDS gels, suggesting heterogeneity in size and/or charge (Fig. 1). Two-dimensional gel electrophoresis using isoelectric focusing in the first dimension and SDS-PAGE in the second dimension (21, 31) revealed seven spots of similar relative molecular weight and isoelectric points of 5.6–6.1 (results not shown).

The 38-kD protein is expressed in cells of neuroendocrine origin. Immunofluorescence analysis of frozen sections of human pancreas, using serum I_{15} which had the strongest immunoreactivity to the 38-kD protein of all sera analyzed in this study, showed bright staining of pancreatic islet β cells in the islet β cell core and weak staining of some glucagon- and/or somatostatin-positive cells in the periphery (Fig. 2). The staining pattern of sera I_{14} and I_{31} both of which had a weaker immunoreactivity to the 38-kD protein in immunoprecipitation experiments was similar. Exocrine cells were negative. Human type 1 diabetic sera often contain antibodies to several antigen specificities, thus sera I_{14} and I_{15} were positive for antibodies to IA2 as well as the 38-kD protein. Although serum I_{31} was negative for IA2 and GAD_{65} antibodies, it may still contain antibodies to other ICA antigens. Therefore, the immunofluorescence experiments only show that the antigens recognized by these sera are islet cell specific in the pancreas, but do not directly address the distribution of the target antigen(s) within the islet.

Because of the limitations of the immunofluorescence analysis for cellular localization studies, using polyspecific human sera, we analyzed the expression of the 38-kD antigen in cell lines derived from different tissues by immunoprecipitation.

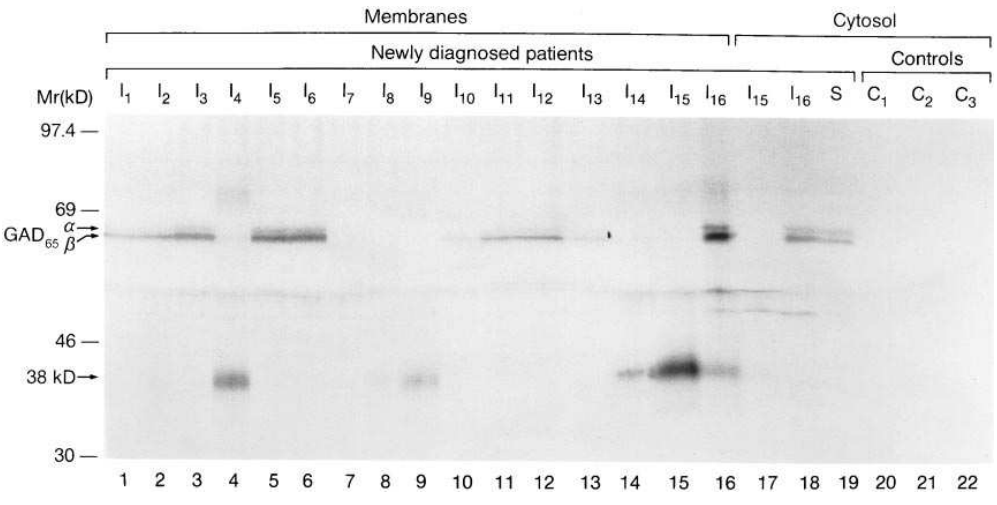

Figure 1. Immunoprecipitation of the 38-kD protein from Triton X-114 detergent phase purified particulate and cytosol fractions of islet cells by diabetic sera. Fluorogram of an SDS-PAGE showing immunoprecipitation of membrane and cytosol fractions of [^{35}S]methionine-labeled islet cell proteins with sera from newly diagnosed diabetic patients I_1–I_{16} (lanes *1–18*), a stiff-man syndrome serum (lane *19*), and sera from healthy controls C_1–C_3 (lanes *20–22*). GAD_{65} which splits into two bands, α and β, can be seen in immunoprecipitates from both membrane and cytosol fractions with serum from patient I_{16} (lanes *16* and *18*) whereas the 38-kD protein is only detected in immunoprecipitates from the membrane fraction with serum from patient I_{15} and I_{16} (compare lanes *15* and *16* with lanes *17* and *18*).

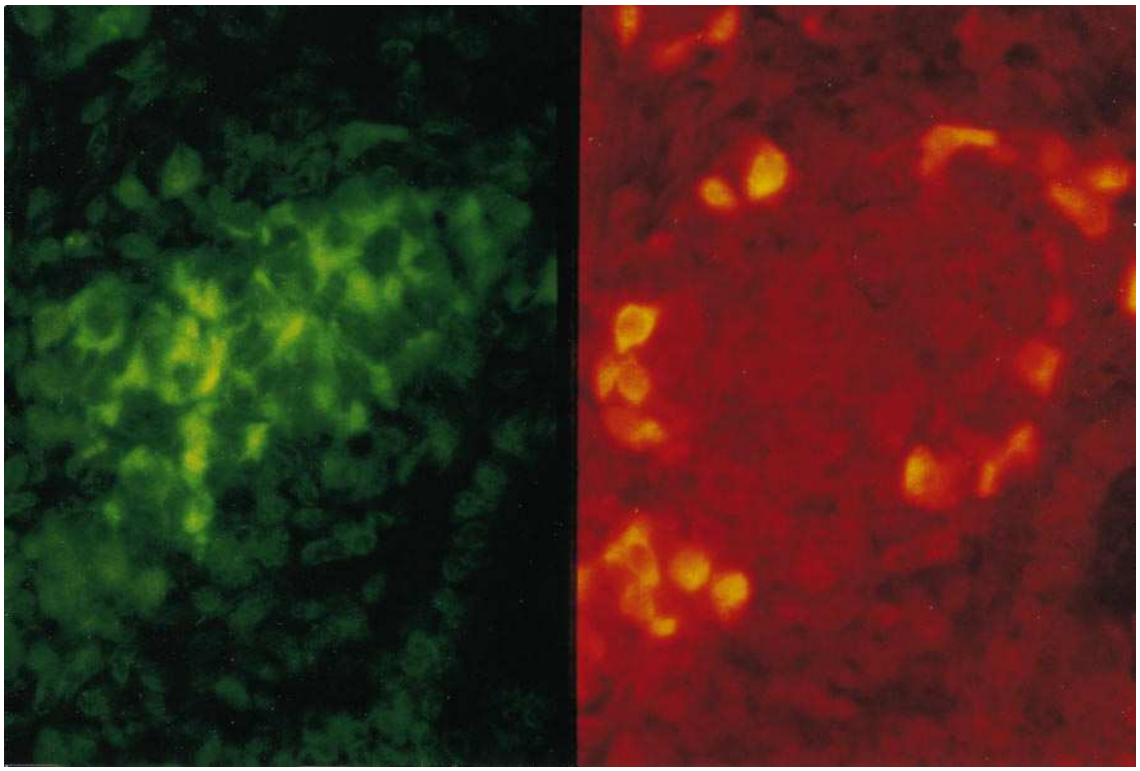

Figure 2. Immunofluorescence analysis of human pancreas using a 38-kD antibody positive serum. Double immunofluorescence analysis of a frozen section of human pancreas stained with human type 1 diabetes serum I_{15} (dilution 1:100) and a mixture of rabbit antiglucagon and rabbit antisomatostatin antibodies. FITC-labeled secondary antiserum was used to visualize human IgG (*left*) and tetramethyl rhodamine isothiocyanate–labeled secondary antiserum was used to visualize rabbit IgG (*right*).

These analyses detected expression of the 38-kD antigen in the majority of islet- and brain-derived cell lines, but not in a variety of cell lines from other tissues (Fig. 3 and Table I). The positive cell lines included the βTC3 cell line derived from a transgenic mouse β cell tumor (25), βHC16 cell line derived from hyperplastic mouse islets (26), one of two αTC cell lines, derived from mouse glucagonomas (27), two cell lines GT1.1 and GT1.7 derived by single cell cloning from a mouse gonadotropin-releasing hormone neuronal tumor (28), and having the highest degree of neuronal phenotype of the cell lines analyzed, Neuro 2A, a mouse neuroblastoma cell line, and C6, a rat glial cell line, which had a neuronal phenotype when cultured in the presence of retinoic acid. The human neuroblastoma cell line, Kelly, was borderline positive (Fig. 3). All other cell lines were negative (Fig. 3 and Table I). Thus, expression of the 38-kD protein seems to be restricted to cells of pancreatic islet and neuronal/glial origin.

The electrophoretic mobility of the 38-kD antigen varied in different cell lines. Although the 38-kD protein had a similar mobility in islets and islet-derived cell lines, the mobility was lower in some neuronal cell lines (Fig. 3, lanes *5* and *11*), suggesting a variability in posttranslational modification(s) between different cell lines.

The 38-kD protein is an N-asp–linked glycoprotein and the nonglycosylated core has a molecular mass of 22 kD. The broad mobility of the 38-kD antigen on SDS gels, and its heterogeneity on two-dimensional gels, suggested that the protein undergoes posttranslational modification(s). To address whether the 38-kD antigen is *N*-asp glycosylated, the protein was purified from rat islets by immunoprecipitation and incubated with the enzyme *N*-glycanase, which cleaves mature *N*-aspartic acid–linked carbohydrate moieties. Fig. 4 shows that treatment of the 38-kD antigen with *N*-glycanase results in a protein with a molecular mass of 22 kD, suggesting that the protein core of the 38-kD protein is a molecule of 22 kD, which undergoes an extensive *N*-asp glycosylation resulting in an increase in molecular mass of $\sim$ 11 kD for the protein expressed in islets. The variation in mobility on SDS gels observed for the 38-kD protein in neuronal and islet cell lines (Fig. 3) is likely to reflect differences in glycosylation.

Autoantibodies to the 38-kD antigen are present in a subgroup of newly diagnosed diabetic individuals and complement GAD_{65} and IA2 autoantibodies. Using the improved method for solubilization of the 38-kD antigen, we analyzed 38-kD antibodies in 86 newly diagnosed type 1 diabetic patients and 65 healthy individuals by immunoprecipitation (Figs. 1 and 5 and Table II). 16 patients (19%) and none of the healthy individuals were positive for antibodies to the 38-kD protein. In comparison 65 (76%) patients and none of the controls were positive for GAD_{65} antibodies. Six patients (7%) were positive for 38-kD antibodies but negative for GAD_{65} antibodies (Table III). Thus, the cumulative incidence of GAD_{65} and/or 38-kD antibodies in newly diagnosed type 1 diabetic patients was 83%. The incidence of ICA was 81%.

The 21 sera from newly diagnosed type 1 diabetic patients which were negative for both 38-kD and GAD_{65} antibodies were analyzed for antibodies to IA2 (Fig. 6, lanes *1–14*). Seven of the double negative sera were positive for IA2 antibodies.

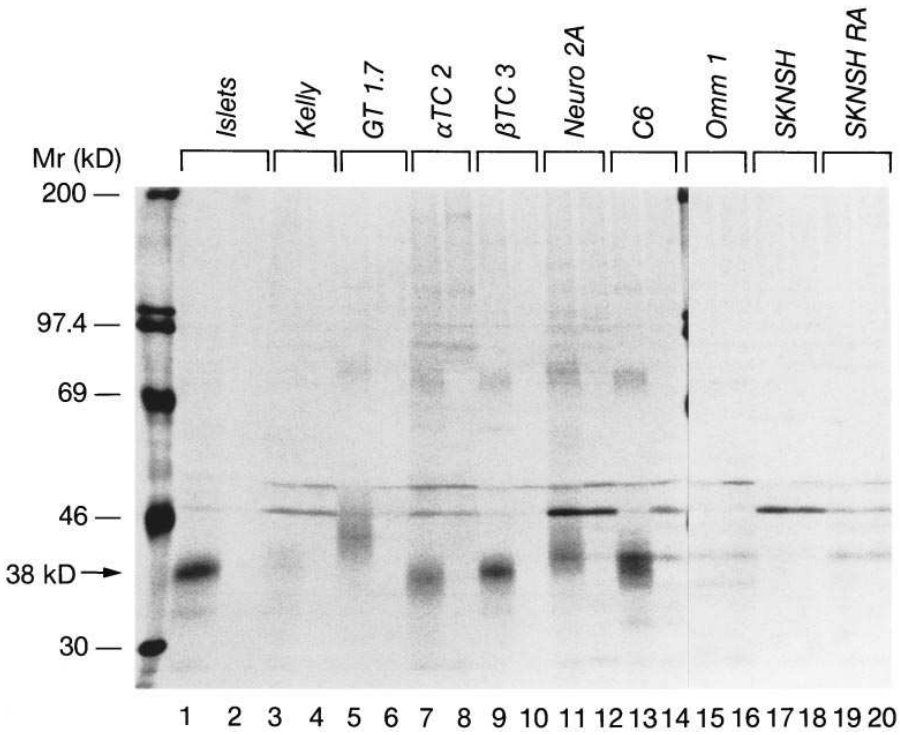

Figure 3. Expression of the 38-kD protein in islet and neuronal cell lines. Fluorogram of an SDS-PAGE analysis of immunoprecipitates of Triton X-114 detergent phase purified membrane protein fractions of the indicated cell lines using serum I_{15} (lanes with odd numbers) and serum C_1 (lanes with even numbers). Equal numbers of cpm's were used for each immunoprecipitate.

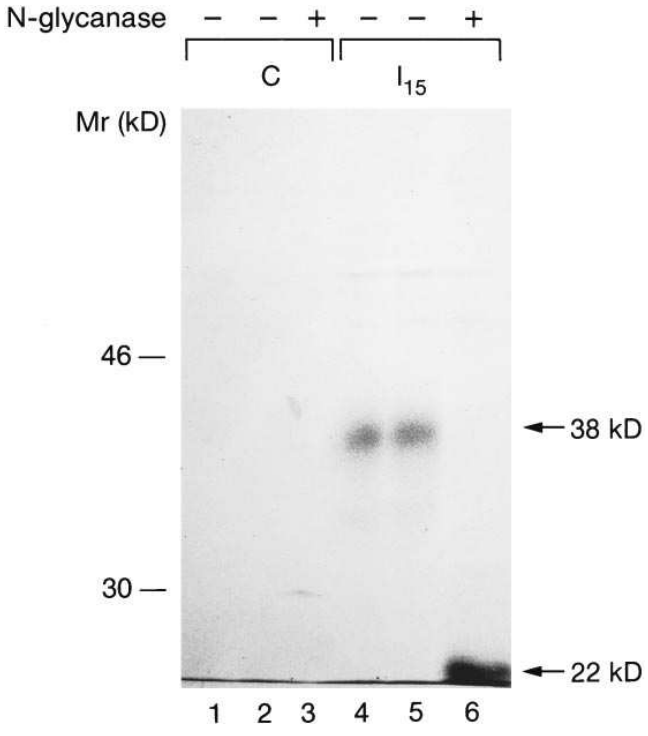

Figure 4. The 38-kD antigen is an *N*-asp–glycosylated protein. Fluorogram of an SDS-PAGE analysis of immunoprecipitated 38-kD protein (lanes *4–6*) incubated with (lane *6*) and without (lane *5*) *N*-glycanase at 37°C for 18 h. Lane *4* is an untreated sample stored at −20°C before SDS-PAGE analysis. Lanes *1–3* show a control immunoprecipitate treated identically.

Thus 78/86 (91%) of patients were positive for antibodies to either one or more of the three autoantigens (Table II).

β cell destruction can progress for several years before the first clinical symptoms of type 1 diabetes appear. It is conceivable that the prolonged autoimmune destruction may result in secondary autoimmune responses to molecules which are released from damaged β cells. Since the destruction of pancreatic β cells in children who develop type 1 diabetes at a very young age must be rapid, we speculated that the antibody specificities in such individuals may better reflect the primary immune responses involved in β cell destruction than is the case in individuals who have experienced many years of autoimmune response to β cells before the clinical onset. In addition to 38-kD and GAD_{65} antibodies, IA2 antibodies were analyzed in all individuals, who developed diabetes < 2 yr of age. Among the 15 patients in this age group, three children (20%), who had a clinical onset of disease at 1.3, 1.6, and 1.8 yr of age, respectively, were positive for 38-kD autoantibodies (Table III). Of these three patients, two were positive for both IA2 and GAD_{65} antibodies, and one was positive for GAD_{65} autoantibodies but negative for IA2 autoantibodies (Table III). 14 patients (93%) were GAD_{65} antibody positive and 12 patients (80%) were positive for IA2 antibodies. The youngest patient, who had a clinical onset of disease at 0.8 yr of age, was positive for both GAD_{65} and IA2 antibodies. Only one patient (1.7 yr) was negative for all three antibodies, and he was also negative for ICA. Thus, all three antigens are targets of autoantibodies in these very young children.

The 38-kD antigen as well as GAD_{65} and IA2 are targets of early B cell responses associated with β cell destruction. To further assess whether the 38-kD antigen is a target of early rather than late immune responses in type 1 diabetes we analyzed 38-kD antibodies in a group of 44 individuals (age 2.6–49.9 yr at clinical onset) from whom sera were available 3–85

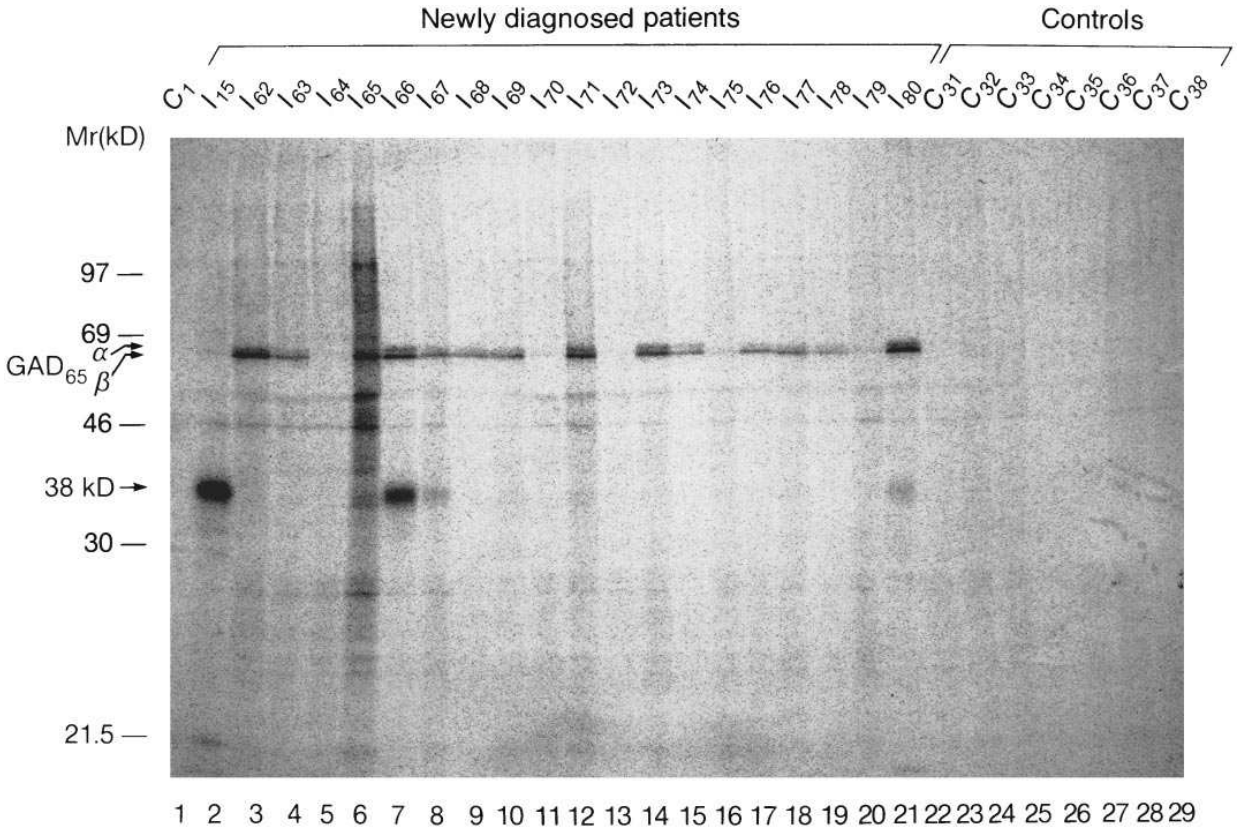

Figure 5. Analyses of 38-kD and GAD_{65} autoantibodies in newly diagnosed type 1 diabetic sera. Immunoprecipitation of membrane fractions of [^{35}S]methionine-labeled islet cell proteins with sera from newly diagnosed diabetic patients I_{15} and I_{62}–I_{80} (lanes *3–21*) and healthy controls C_1 and C_{31}–C_{38}. The diabetic sera recognize either the 38-kD protein alone, the GAD_{65} protein alone, both proteins, or no specific protein.

mo before clinical onset of type 1 diabetes (Figs. 7 and 8 and Table II). The sera were also analyzed for GAD_{65} and IA2 autoantibodies (Table II). 6 of the 44 prediabetic individuals (14%) were positive for 38-kD antibodies in the first serum sample available, which was 3, 9, 25, 33, 53, and 74 mo, respectively, before clinical onset of type 1 diabetes. Fig. 8 (lanes *3–6*) shows the analysis of 38-kD antibodies in an individual followed from 53 to 20 mo before clinical onset.

Table II. Incidence of Autoantibodies to a 38-kD β Cell Membrane Protein in Type 1 Diabetes and Comparison with GAD_{65} ab, IA2 ab, and ICA

Group	*n*	Average age at diagnosis or sampling of sera (controls)	Range	F/M	ICA	Inc. of 38-kD ab	Inc. of GAD_{65} ab	Cumulative inc. of 38-kD and/or GAD_{65} ab	Cumulative inc. of 38-kD GAD_{65} and/or IA2 ab
		yr	*yr*						
Newly diagnosed diabetic patients	86	8.8±8.7	0.8–57.0	35/51 (0.7)	70*/86 (81%)	16‡/86 (19%)	65/86 (76%)	71/86 (83%)	78/86 (91%)
Healthy controls	63	9.8±8.2	0.9–54.2	28/35 (0.8)	0/63 (0%)	0/63 (0%)	0/63 (0%)	0/63 (0%)	N/A
Prediabetic individuals 3–85 mo before clinical onset	44	19.2±12.5	2.6–49.9	14/30 (0.5)	28§/44 (64%)	6¶/44 (14%)	33/44 (75%)	35/44 (80%)	37/44 (84%)

*10 of whom were both GAD_{65} and 38-kD antibody negative, 3 of whom were GAD_{65}, 38-kD, and IA2 ab negative. ‡14 of whom had GAD_{65} autoantibodies, IA2 autoantibodies, or both. §One of whom was both GAD_{65} and 38-kD antibody negative, none of whom was GAD_{65}, 38-kD, and IA2 ab negative. ¶All of whom had GAD_{65} autoantibodies, IA2 autoantibodies, or both. *Inc.*, Incidence; *ab*, antibodies.

Table III. Summary of Data on 38-kD Antibody Positive Individuals

No.	Sex	Age at onset	ICA (JDF units)	GAD_{65} ab index	38-kD ab index	IA2 ab index	Prediabetic samples months before onset	HLA-DR
I_4	F	6.1	40	Neg	7.9	10.8		
I_9	M	5.7	Neg	Neg	4.5	Neg		
I_{14}	F	4.8	40	Neg	1.3	5.8		
I_{15}	M	15.9	8	Neg	10.0	6.1		3, 4
I_{16}	F	17.3	53	12.3	4.8	6.6		4, 4
I_{17}	M	12.8	53	2.2	1.7	4.3		3, 4
I_{18}	M	7.9	Neg	2.7	1.8	Neg		4, 4
I_{28}	F	6.0	20	11.0	2.5	Neg		
I_{29}	M	12.1	> 1200	7.2	2.8	11.6		
I_{30}	M	33.6	400	Neg	1.8	4.5		
I_{31}	M	7.2	320	Neg	3.0	Neg		
I_{44}	F	10.9	40	14.0	1.7	Neg		
I_{48}	M	12.1	160	9.6	2.8	Neg		
I_{66}	F	1.6	610	6.5	8.8	1.0		4, 7
I_{67}	F	1.3	275	4.6	1.6	Neg		1, 4
I_{80}	M	1.8	285	8.7	1.7	5.1		7, 9
P_5	F	5.5	160	Neg	4.1*	7.2	3	2, 3
P_8	M	18.7	80	Neg	2.0*	8.7	33, 26	4, 4
P_{17}	M	15.1	160	11.3	9.5*	Neg	53, 41, 31, 20, 18, 3	4, 4
P_{18}	M	14.1	160	7.0	5.8*	5.6	74, 18	3, 4
P_{33}	M	5.1	80	4.3	3.9*	Neg	9	
P_{37}	M	18.0	42	7.0	2.0*	8.6	25	1, 4

*38-kD ab index in first available sample.

Among the 38-kD antibody positive prediabetic individuals, two were positive for both GAD_{65} and IA2 antibodies, two were positive for GAD_{65} but not IA2 antibodies, and two were positive for IA2 but not GAD_{65} antibodies (Table III). GAD_{65} antibodies were detected in a total of 33 (75%) of the prediabetic patients in the first sample available 3–85 mo before clinical onset of disease, a result consistent with earlier studies (39, 40). IA2 antibodies were detected in 20/44 (45%) of the prediabetic individuals. Thus the 38-kD as well as IA2 and GAD_{65} antibodies can be detected up to several years before clinical onset. The cumulative incidence of 38-kD, GAD_{65}, and IA2 autoantibodies was 84% in the prediabetic individuals. The incidence of ICA in this group was 64% (Table II).

Follow-up samples were available for 34 of the prediabetic individuals. None of the individuals changed from antibody negative to antibody positive status in later samples. Thus, antibody negative individuals remained negative and single antibody positive individuals did not become double or triple antibody positive in the observation period, suggesting that these antibodies are not a consequence of prolonged β cell destruc-

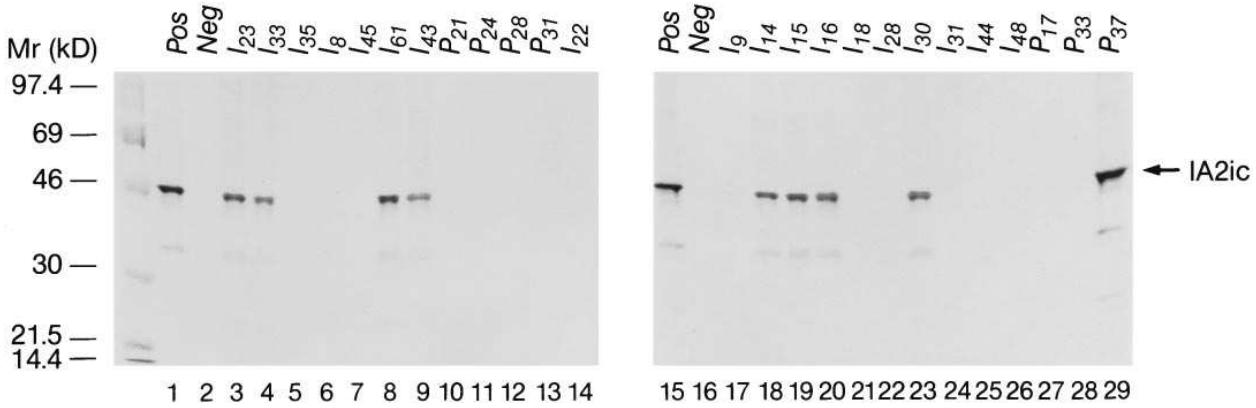

Figure 6. IA2 antibody analysis in 38-kD/GAD65 antibody negative individuals and in 38-kD antibody positive individuals. Fluorogram of SDS-PAGE analysis of immunoprecipitates of a COOH-terminal fragment of IA2, IA2ic, with 38-kD/GAD65 antibody negative sera (lanes *3–14*) and 38-kD antibody positive sera (lanes *17–29*). Lanes *1* and *15* show immunoprecipitates with a positive control serum. Lanes *2* and *16* show immunoprecipitates with a negative control serum. Several of the double antibody negative sera in lanes *3–14* are positive for IA2 antibodies, and several of the 38-kD antibody positive sera in lanes *17–29* are negative for IA2 antibodies.

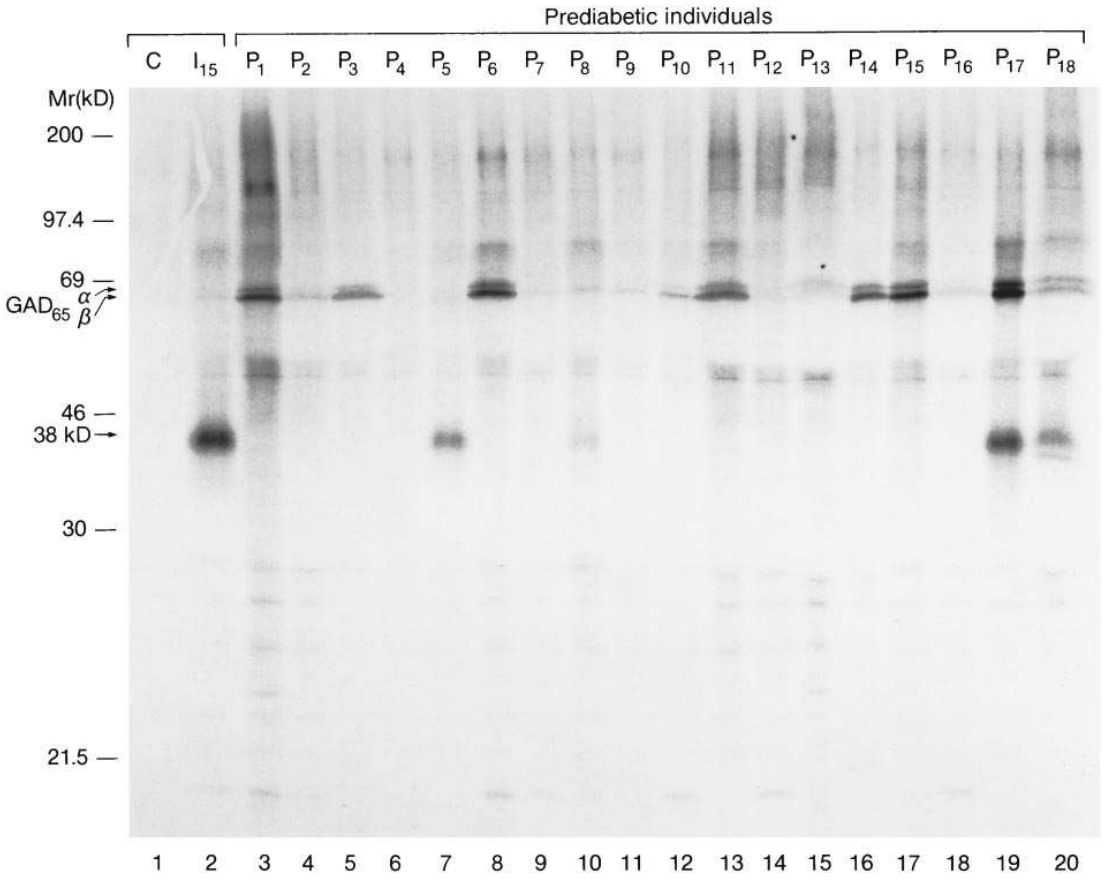

Figure 7. Analyses of 38-kD and GAD_{65} autoantibodies in prediabetic individuals. Immunoprecipitation of membrane fractions of [^{35}S]methionine-labeled islet cell proteins with sera from prediabetic individuals P_1–P_{18} (lanes *3–21*). The 38-kD protein is recognized by P_5, P_8, P_{17}, and P_{18}, 3, 33, 53, and 74 mo, respectively, before clinical onset of type 1 diabetes.

tion. Rather, they may be determined by genetic and/or environmental factors which are either present or absent at the onset of the autoimmune process.

Four of the 38-kD antibody positive individuals were followed regularly for a period of 30–48 mo after clinical onset. Two of these (I_{17} and I_{18}) were negative already 3 mo after clinical onset (results not shown). I_{15} and I_{16} were still strongly antibody positive at 3 mo after clinical onset, but became weakly

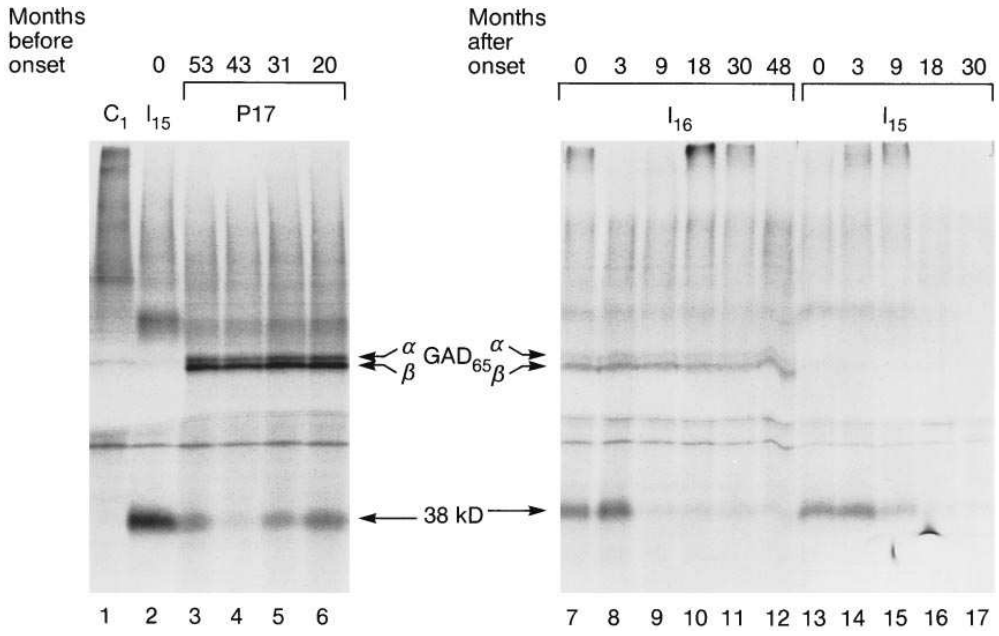

Figure 8. 38-kD antibodies before and after clinical onset of type 1 diabetes. Fluorogram of SDS-PAGE analysis of immunoprecipitates of membrane fractions of [^{35}S]methionine-labeled islet cell proteins with sera from different time points before (lanes *3–6*) and after (lanes *8–12* and *13–17*) clinical onset of type 1 diabetes.

positive at 9 mo (Fig. 8). I_{15} was antibody negative at 18 mo and onwards, whereas I_{16} remained weakly positive 18, 30, and 48 mo after clinical onset (Fig. 8). Thus, 38-kD antibodies in all four individuals decreased significantly shortly after clinical onset of diabetes.

Among all of the 22 38-kD antibody positive individuals in the combined prediabetic and newly diagnosed groups of type 1 diabetic patients, 20 were positive for either GAD_{65} antibodies (14/22), IA2 antibodies (13/22), or both (7/22). There was no correlation between any two sets of antibodies in these individuals, suggesting that the antibodies to each of the three antigens are separate entities which do not cross-react with the other two antigens (Table III).

The 38-kD antibody positive sera did not recognize their target antigen on Western blots, suggesting that 38-kD antibodies, much as GAD_{65} and IA2 antibodies, are primarily directed toward conformational epitopes (results not shown).

Regarding immune recognition, it is well established that the MHC haplotype is influential. More than 90% of all individuals who develop type 1 diabetes are HLA-DR3 and/or DR4 positive (41). HLA data could only be obtained for 12 of the 22 38-kD antibody positive individuals (Table III). The DR4 haplotype was particularly abundant in those individuals (10/12 compared with 4/12 for DR3, and 2/12 for DR1 and DR7, respectively). However the data set is too small to analyze for statistical significance.

Discussion

We have identified an islet cell *N*-asp–glycosylated membrane protein, of 38,000 M_r, which is a target of immunoprecipitating IgG autoantibodies in a subgroup of type 1 diabetic patients and prediabetic individuals. The 38-kD protein is the third antigen identified by immunoprecipitation in this disease, if the tyrosine phosphatases that are precursors to the 37/40-kD tryptic fragments are counted as one entity. The 38-kD antigen shares the neuroendocrine expression pattern and conformational epitope characteristics of GAD_{65} and IA2.

Taken together among the 130 patients, who were analyzed either in the prediabetic period or at the clinical onset of disease, 22 (17%) were 38-kD antibody positive (Table III) compared with 98 (75%), who were GAD_{65} antibody positive. Two patients were positive for 38-kD antibodies only, whereas 20 had GAD_{65} and/or IA2 antibodies in addition to the 38-kD antibodies. Thus 106 (82%) were positive for antibodies to either 38-kD, GAD_{65}, or both antigens. Among the 24 double antibody negative patients, 9 were IA2 antibody positive. Thus, the cumulative incidence of all three antibodies was 91%. Both GAD_{65} and IA2 antibodies were detected at clinical onset in a child, who developed type 1 diabetes as early as 0.8 yr of age, and 38-kD antibodies were detected in a child who developed diabetes at 1.3 yr of age. Since the duration of β cell autoimmunity in those very young children must have been significantly shorter than is often the case in older individuals (39, 40), this result suggest that all three proteins may be targets of primary rather than secondary autoimmune processes directed to the β cell in the human disease. This notion is supported by the appearance of antibodies to all three antigens several years before the clinical onset of type 1 diabetes. Thus, antibodies to all three antigens, GAD_{65}, IA2, and the 38-kD protein mark periods of early β cell destruction.

In the prediabetic group, the GAD_{65} antibody assay alone detected 33/44 (75%) of the patients. A combination of GAD_{65} and 38-kD antibody assays detected 35/44 (80%), and a combination of IA2 and 38-kD antibody assays detected 22/44 (50%) of the individuals. A combination of GAD_{65} and IA2 antibody assays detected 37/44 (84%), which was the same as the cumulative sensitivity for all three antibody assays.

The incidence of ICA detected by immunofluorescence of frozen sections of human pancreas was 75% (98/130). In the prediabetic and newly diagnosed groups, the 38-kD, and/or GAD_{65}, and/or IA2 immunoprecipitation assays detected a total of 19 individuals negative for ICA by the immunofluorescence assay, indicative of a lower sensitivity of the ICA method to detect antibodies to these antigens. In the prediabetic group all ICA-positive individuals ($n = 28$) were positive for either GAD_{65} antibodies, IA2 antibodies, or both. A combination of GAD_{65} antibodies and 38-kD antibodies detected all these individuals except one, whereas a combination of 38-kD antibodies and IA2 only detected 15 of the ICA-positive individuals. Among the 70 ICA-positive patients in the newly diagnosed group, the cumulative antibody assays to all three antigens detected all but three individuals. In these three individuals the humoral immune response may have evolved to focus on other target molecules by the time of clinical onset. Immunoprecipitation analyses did not reveal islet cell protein(s) specifically recognized by those sera (results not shown). It is conceivable that ICA reactivity in those 38-kD and GAD_{65} antibody negative sera may be directed to nonprotein molecules like gangliosides (42). Finally, seven individuals in the prediabetic group and five individuals in the newly diagnosed group were negative for antibodies by both immunofluorescence and immunoprecipitation assays.

The 38-kD antigen is distinct from two diabetes-associated antigens of similar apparent molecular mass previously described, jun B and imogen 38. Its cellular expression pattern differs from that of imogen 38, which was identified as a target of a $CD4^+$ T cell clone derived from a type 1 diabetic patient and has a wide tissue distribution (19). Furthermore, imogen 38 is not *N*-asp glycosylated (19) and does not seem to be a target of humoral autoantibodies in type 1 diabetes.

Honeyman et al. (20) reported the isolation of a cDNA clone encoding jun-B by antibody screening of an expression library from islets and placenta using a human serum from a type 1 diabetic patient. They subsequently detected peripheral T cell responses to jun-B in some type 1 diabetic patients and their relatives and concluded that jun-B is a target autoantigen in type 1 diabetes. jun-B has a molecular mass of 38 kD.

jun-B is a nonglycosylated, soluble nuclear protein and therefore differs from the 38-kD protein described here. Furthermore, rat jun-B has a pI ≥ 9 on two-dimensional gels and the calculated pI for human jun-B based on the amino acid sequence (43) is 9.58. Finally, antibodies to jun-B do not immunoprecipitate the 38-kD antigen (our unpublished results). To distinguish the novel 38-kD autoantigen described here from jun B and imogen 38, we name it glima 38 (glycosylated islet membrane antigen of 38 kD).

The relation between glima 38 and a diabetes-associated (but as yet uncharacterized) antigen in the diabetes-prone BB rat (18) is unclear. The BB rat protein migrates as a sharp band on SDS gels, a characteristic inconsistent with the highly glycosylated nature of glima 38, and thus appears to be different. Glima 38 is not related to a 38-kD protein only detected in im-

munoprecipitates from one preparation of DR3 positive human islets, using GAD_{65} antibody positive sera, and described earlier (6), because none of these sera recognize glima 38. This human islet 38-kD protein migrated as a sharp nonglycosylated band and may have been a degradation product of GAD_{65} in this particular preparation of human islets.

We have extensively analyzed immunoprecipitates of detergent lysates of [^{35}S]methionine-labeled islets with diabetic and control sera by one- and two-dimensional gel electrophoresis in attempts to detect additional islet cell proteins that are specifically and consistently recognized by autoantibodies in type 1 diabetes under native conditions. The stringent conditions of immunoprecipitation require that antibodies must be of the IgG isotope (for binding to protein A–Sepharose) and must be of sufficient affinity and specificity to recognize their target protein in the midst of an abundance of other islet cell proteins. Whereas this assay clearly detects the $GAD_{65}\alpha/\beta$ doublet as well as the 38-kD protein, in nonprotease conditions, and the 40-kD IA2 and 37-kD tyrosine phosphatase fragments in protease conditions, it does not detect carboxypeptidase H, a 69-kD protein with homology to BSA, or insulin (reference 21 and our unpublished results) although reactivity of those proteins with antibodies in diabetic sera has been detected in other assays (11–13, 44). Thus, strong immunoprecipitating IgG responses to conformational protein epitopes may be limited to the GAD_{65}, 38-kD, and the tyrosine phosphatase antigens in the human disease. An outstanding question is whether GAD_{65}, IA2, and the 38-kD antigens are targets of pathogenic human T cells which are believed to mediate β cell destruction in type 1 diabetes (4), a result which would suggest that these proteins have a potential for antigen-specific immunotherapy aimed to eliminate or inhibit autoimmune T cells and prevent the disease in humans.

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