Original Articles

An Islet-Cell Protein Tyrosine Phosphatase Is a Likely Precursor to the 37-kDa Autoantigen in Type 1 Diabetes: Human and Macaque Sequences, Tissue Distribution, Unique and Shared Epitopes, and Predictive Autoantibodies

James LaGasse,* Laura Jelinek,† Shannon Sexson,† Cathy Lofton-Day,† John Breininger,* Paul Sheppard,† Wayne Kindsvogel,† and William A. Hagopian*

*Department of Medicine, University of Washington, Seattle, Washington, U.S.A.

†ZymoGenetics, Inc., Seattle, Washington, U.S.A.

ABSTRACT

Background: We sought to identify novel islet-cell autoantigens to better understand the pathogenesis, prediction, and immunotherapy of type 1 diabetes.

Materials and Methods: Macaque and human islet cDNA libraries expressed in mammalian cells were screened with human diabetes sera. A positive clone was sequenced directly and after 5′ rapid amplification of cDNA ends (RACE). Northern blotting and in situ hybridization revealed the tissue distribution of the corresponding protein. Antigen, expressed by in vitro translation, and tryptic peptides were analyzed by SDS–PAGE. For the immunoprecipitations, 183 diabetic, 60 prediabetic, and 91 control sera were used. Truncated antigens were used in immunoprecipitations for epitope mapping. Recombinant antigen expressed in transfected fibroblasts was used in competition assays.

Results: Sequencing yielded an 111-kDa, 1,013 amino acid, transmembrane protein (M1851) containing consensus protein tyrosine phosphatase (PTPase) sequence. M1851 was 77% identical in the intracellular domain, but only 31% identical extracellularly, to the islet-cell autoantigen ICA512. mRNA localized to brain, prostate, pancreatic islets, and adrenal medulla. After limited trypsinization, the in vitro translated antigen was 37 kDa. M1851 was recognized by 47% of prediabetes sera, 31% of new diabetes sera, but only 1% of healthy controls. Only 1/73 sera binding M1851 failed to bind ICA512, whereas 42/114 binding ICA512 did not bind M1851. M1851 reactivity was not fully displaced by ICA512 in 24/49 sera. Removing the C-terminal 27, 80, or 160 amino acids of M1851 decreased reactivity by 70%, 90%, and 100%, respectively.

Conclusions: This new islet-cell PTPase is likely to be the precursor to the 37-kDa tryptic fragment antigen. It is structurally related to ICA512 but has distinct diabetes autoantibody epitopes located at the C terminus.

INTRODUCTION

Insulin-dependent diabetes is an organ-specific autoimmune disease resulting from aberrant immune response to specific β-cell autoantigens (1). Humoral autoimmunity in type I diabetes (IDDM) is generally described in terms of the classic islet-cell antigen (ICA) assay (2) using frozen sections of human pancreas as assay substrate. Serum autoantibodies are detected using a fluorescent second antibody and indirect fluorescence microscopy. Multiple antigens are targets, including glutamic acid decarboxylase (GAD) (3) and the putative protein tyrosine phosphatase

Address correspondence and reprint requests to: William Hagopian, Department of Medicine, Box 357710, University of Washington, Seattle, WA 98195, U.S.A. Phone: 206-685-4775; Fax: 206-543-3169; E-mail: wah@u.washington.edu

The macaque sequence reported in this paper has been deposited in the GenBank data base (accession no. U91574).

(PTPase) ICA512 (4). Of new IDDM sera, 28/969 had ICA reactivity but were negative for autoantibodies to GAD, insulin, and ICA512 (W.A. Hagopian, unpublished observation), which suggests that other antigens are also recognized by islet-cell autoantibodies.

Identification of these additional β-cell antigens could greatly improve IDDM prediction, especially in those patients without a diabetic relative, where most new cases occur. The general population has a low prevalence of IDDM, so high test specificity is critical in achieving predictive values sufficient for clinical immunotherapy. Use of independent, recombinant antigens in radiobinding assays has proven to be highly specific. For example, combining GADab and insulin autoantibodies (IAA) led to a 99.7% specificity (5). However, only 41% of the subjects were positive for both tests. Adding additional independent antibody tests, and then requiring at least two to be positive, should preserve specificity but improve sensitivity, so that more prediabetics can participate in intervention trials. Identification of β-cell autoantigens in IDDM also has an important role in potential antigen-based immunointervention therapy. Proposed therapies include vaccination, intravenous monomeric antigen or altered peptide ligands, oral tolerance, and peptide–HLA constructs (6).

One important but elusive antigen has been the precursor to the 37-kDa tryptic fragment first described by Christie et al. (7). Although the parent antigen to this tryptic fragment ran as a diffuse band at about 64 kDa on SDS–PAGE, it was distinct from GAD (8). Presence of autoantibodies to the 37-kDa fragment were shown to be highly predictive of progression to clinical IDDM in both polyendocrine patients (9) and in first-degree relatives of current diabetic patients (10). The combination of 37-kDa reactivity and GAD reactivity was especially predictive (11,12).

To find new β-cell protein antigens such as the precursor to the 37-kDa antigen, we constructed a highly representative islet cDNA library from pure isolated macaque islets (13). Unlike rodent islets, they are highly comparable to human islets in antigen expression (13) and have highly homologous sequences (14) of sufficient similarity to be useful in designing gene-specific primers for amplifications from human insulinoma cDNA template. The macaque library was constructed in a vector optimized for high-level transient expression in mammalian cells (15) favoring appropriate post-translational processing. Finally, screening utilized IgG from IDDM sera carefully selected for high-titer ICA not attributable to known islet antigens. New islet antigens detected in this way were expected to be disease-relevant.

A reactive clone (M1851) had a sequence similar but distinct from the putative PTPase ICA512 (16,17), also known as IA-2 (18). ICA512 is itself highly recognized by IDDM sera (19). We here define the structure of M1851, its tissue distribution, antigenic properties, and predictive role in IDDM.

MATERIALS AND METHODS

Highly purified islets were isolated from *Macaca nemestrina* (pigtail macaque) pancreata by collagenase digestion and density gradient centrifugation as described (13). Purified mRNA from 10^5 islets was used to construct a library representative of message at all abundance levels using techniques optimizing synthesis of full-length cDNA (15). cDNA were ligated into the vector pZCEP (15) containing a colE1 site and an SV40 promoter, for bacterial replication and mammalian expression, respectively. The library was electroporated into *Escherichia coli* DH10B cells (Gibco BRL, Gaithersburg, MD), selected on agar plates containing ampicillin, harvested, and split into 100 pools each of 10^5 recombinants each for storage as glycerol stocks at −80°C. Pools were grown separately on agar plates containing ampicillin prior to purification of plasmid DNA by phenol-chloroform extraction.

Sera from new-onset IDDM patients with ICA titer above 50 JDFU were adsorbed with excess recombinant human GAD65 (20), insulin (NovoNordisk, Bagsvaerd Denmark), and ICA512 (kind gift of Dr. D. Rabin). Two adsorbed sera had ICA titers remaining over 50 JDFU. IgG was purified using protein A-Sepharose (Zymed, South San Francisco, CA), precleared over formalin-fixed COS-7 cells, and stored at −80°C until use.

COS-7 cells grown to 70% confluence on glass slides were transfected with 1 μg of pool plasmid DNA using Lipofectamine (Gibco BRL). After growth for a further 72 hr, cells were fixed and permeabilized with equal parts ethanol and acetone. Slides were incubated overnight at 25°C with human diabetes IgG (2 mg/ml), washed, developed using biotinylated rabbit anti-human IgG, streptavidin-gold, and silver enhancer (Amersham, Arlington Heights, IL), and screened by microscopy. Three of 100 pools were positive,

with one or more single cells stained in each of three experiments.

The glycerol stock representing one of the positive pools was plated onto agar containing ampicillin. Colonies were probed with ^{32}P-labeled cDNA coding for the intracellular domain of ICA512 obtained by polymerase chain reaction (PCR) from human glioblastoma (18). Isolated plasmid DNA from a positive colony was sequenced, yielding a sequence similar but not identical to human ICA512 (17,18). Using plasmid pools from the original macaque library, a vector-specific upstream primer, and gene-specific antisense primers 5′-CTCTGTGGTCCATGCCTTG C-3′ and 5′-GCGCGATGAACTTGGTGGAGTCTT CTTGCTCCGCCTGA-3′, the remaining 5′ sequence was obtained by the PCR technique termed 5′ RACE (rapid amplification of cDNA ends) (21) (Marathon, Clontech, Palo Alto, CA). Using cDNA previously synthesized from human insulinoma mRNA, and primers based on the macaque sequence, 2,456 bases of the human homologue were also amplified by PCR and sequenced.

The second 5′ RACE primer above is based on M1851 antisense sequence, just external to the transmembrane region, which is distinct between M1851 and ICA512. This oligonucleotide probe was radiolabeled with γ^{32}P-ATP using T4 polynucleotide kinase (Gibco BRL). Northern analysis of RNA from 23 human tissues distributed over 3 commercially prepared blots (Clontech) each separately hybridized once. Ten milliliters of fresh Express-Hyb buffer (Clontech) containing 5×10^6 cpm/ml of labeled probe was incubated with the blots overnight at 37°C. Blots were then washed with 6× SSC, 0.1% SDS at 25°C, 50°C, and 60°C, and with 2× SSC, 0.05% SDS at 68°C, prior to autoradiography.

The same antisense probe, as well as the corresponding ICA512 antisense probe (5′-AGC TGCCTCCTCCCTCTGTCCCACTCCTGTCTGCAA GA-3′) were separately hybridized in situ with 14-μm frozen sections from macaque pancreas, adrenal gland, and muscle. Sections fixed in 4% paraformaldehyde were acetylated with acetic anhydride and delipidated in chloroform prior to use. Probes were end labeled with ^{33}P-dATP (New England Nuclear) using terminal deoxytransferase (Gibco BRL). Probes (2 pmol/ml) were incubated on sections overnight at 45°C, washed twice in 1× SSC at 60°C for 30 min, dehydrated in ethanol, and apposed to autoradiography film (Hyperfilm Betamax, Amersham) for 2–6 days. In situ hybridizations were performed three times on each tissue with similar results.

Using primers 5′-CCACCATGCGCCATAGCT CTCAGCACAGGCTGAA-3′ (sense) and 5′-TCA CTGGGGCAGGGCCTTGAG-3′ (antisense) based on the human sequence, the complete 1,131-nucleotide sequence of intracellular M1851 was amplified from human insulinoma cDNA, ligated into vector pcDNAII (Invitrogen, San Diego, CA), and confirmed by sequencing in both directions. Coupled transcription and translation (TNT, Promega, Madison, WI) proceeded in the presence of ^{35}S-methionine. Limited trypsinization (Sigma, St. Louis, MO) was performed at 4°C for 15 min by the method of Christie et al. (7).

Immunoassays each used 4 μl of human serum in duplicate incubated overnight at 4°C with radiolabeled icM1851, precipitated with protein A-Sepharose, washed, counted by scintigraphy, and expressed as an M1851 index = (unknown − negative control)/(positive control − negative control). The positive control serum is the world ICA standard used to calculate JDF units. This serum immunoprecipitates GAD, ICA512, and M1851 (5,22). Serum samples included 91 healthy control sera (median age 22 years, 51% females), 183 unselected type 1 diabetic patients sampled at onset (median age 11 years, 49% females), and 60 type 1 diabetic patients sampled a mean of 2.0 years before onset (median age 12 years, 42% females). Assay cutoff was an index of 0.04, determined as the mean + 3 standard deviations of indices from the 91 control sera. Sensitivity and predictive value were calculated as described previously (5). Parallel autoantibody assays used radiolabled icICA512.

To obtain unlabeled icICA512 antigen for competition studies, PCR-generated cDNA sequence for the icICA512 (18) was ligated into the expression vector pZEM219b containing the SV40 promoter (22) and confirmed by sequencing. This vector also constitutively expresses dihydrofolate reductase, allowing methotrexate selection of baby hamster kidney fibroblast transfectants. Cellular icICA512 expression was confirmed by immunocytochemistry (data not shown) using rabbit polyclonal antiserum to ICA512.1 (kindly provided by Dr. D. Rabin) (17). Cells were homognized in 0.25% Triton X-114 and 10 mM benzamidine in PBS at pH 7.4. icICA512 concentration was estimated at 7 μg/ml of cell extract by Western blotting, using as standard recombinant ICA512.1 (kindly provided by Dr. D. Rabin; data not shown). Competition immunoprecipitations used radiolabeled

icM1851 in the presence of 0.5 μg of unlabeled icICA512 per microliter of patient serum. For icM1851 autoantibodies not fully blocked, competitions were repeated at 2.5-fold greater unlabeled icICA512 with no change in results. Extracts from nontransfected fibroblasts had no effect on icM1851 immunoprecipitation.

PCR-amplified sequences based on intracellular M1851 but containing mutations resulting in the deletion of the C-terminal 27, 80, and 160 amino acids (AA) of the 376 AA intracellular domain, were ligated into pcDNAII, radiolabeled during in vitro expression, and used for duplicate immunoprecipitation assays as described above for intact icM1851.

RESULTS

The cloning strategy for M1851 in macaque and human is shown in Fig. 1A. Starting with the original macaque library M1851 clone extending from the midextracellular sequence to the 3′ end (sequence 1), successive 5′ RACE amplifications yielded sequences 3 through 7. A longer clone was also identified by screening the library with one of these products, to obtain confirmatory sequence (sequence 2). Signal peptide, tribasic site, glycosylation site, transmembrane span, PTPase core sequence, stop codon, and poly-A tail are underlined. The partial human sequence was obtained by PCR using gene-specific primers (sequences 9 to 11) and 3′ RACE (sequence 8).

The 3,042-nucleotide cDNA sequence and 1,013 residues of primary protein structure of macaque M1851, as well as 2,430 nucleotides and 809 residues of the human sequence, are shown in Fig. 1B. The protein was mildly acidic (predicted pI 5.70) with a calculated molecular mass of 111,190 Da. Where human sequence was available, the human and macaque M1851 sequences were highly homologous (2362/2456 = 96.2% nucleotide identity; 773/817 = 94.6% AA identity). As expected, the rat and macaque M1851 sequences were much less similar (75.7% nucleotide and 69.6% AA identity).

The 376 AA intracellular domain of human M1851 and the 379 AA intracellular domain of human ICA512 (18) had 73.4% identity (278/379 residues). However, even after optimal alignment, the 612 AA extracellular domain of macaque/human M1851 was in large part distinct (31.2% identity, 191/612 residues) from the 576 AA extracellular domain of human ICA512 (Fig. 1C). Greatest similarity in the extracellular region occurred at the N-terminal 100 residues. In addition to the rat and mouse homologues, Genbank searches revealed greatest homology of M1851 with many protein tyrosine phosphatases, including in decreasing similarity, ICA512 (IA2, PTP35, ICA105, IA2a, pheochromocytoma), liver protein tyrosine phosphatase, and leukocyte common antigen (CD45).

Tissue distribution of M1851 was studied using an oligonucleotide probe based on unique sequence just extracellular to the transmembrane domain. Northern analysis using mRNA from 23 human tissues detected strong 5.5-Kb and weaker 3.3-Kb messages in brain, pancreas, and prostate, with still lesser signals in the spinal cord, thyroid, adrenal, and gastrointestinal (GI) tract (Fig. 2). To further define tissue localization, in situ hybridization was performed on macaque pancreas, adrenal gland, and muscle (Fig. 3). Specific signal was demonstrated in pancreatic islets and adrenal medulla, but not muscle. An ICA512 probe and an insulin B-chain probe also hybridized to pancreatic islets, as did the ICA512 probe to adrenal medulla. Unlabeled antisense probes displaced labeled probe binding, treatment of tissue with RNAse A abolished hybridization, and labeled sense oligonucleotides corresponding to each probe showed no hybridization.

The intracellular portion of M1851 was expressed and radiolabeled in vitro in the presence of ^{35}S-methionine. Polyacrylamide gel electrophoresis (PAGE) and autoradiography of the resulting polypeptide revealed a major 46-kDa band and a minor 33-kDa band, both immunoprecipitated by IDDM sera (Fig. 4, left panel). Limited trypsinization of the immunoprecipitated polypeptide followed by SDS–PAGE and autoradiography revealed a 37-kDa fragment from both macaque and human icM1851. This fragment was distinct from the 40-kDa fragment produced by trypsinization of the 379 AA intracellular domain of human ICA512 (Fig. 4, right panel).

Immunopreciptation assays using radiolabeled recombinant intracellular M1851 (icM1851) revealed autoantibodies in 56/183 (30.6%) newly diagnosed IDDM patients, 28/60 (46.7%) first-degree relatives later progressing to clinical diabetes, but only 1/91 (1.1%) healthy control subjects. M1851 antibody indices are presented in Fig. 5, left panel, for these three subject groups. For first-degree relatives, this represents a positive predictive value of 58% at 48% sensitivity in prediabetes (5). Prediabetics had a significantly higher prevalence of GADab than M1851 when tested more than 5

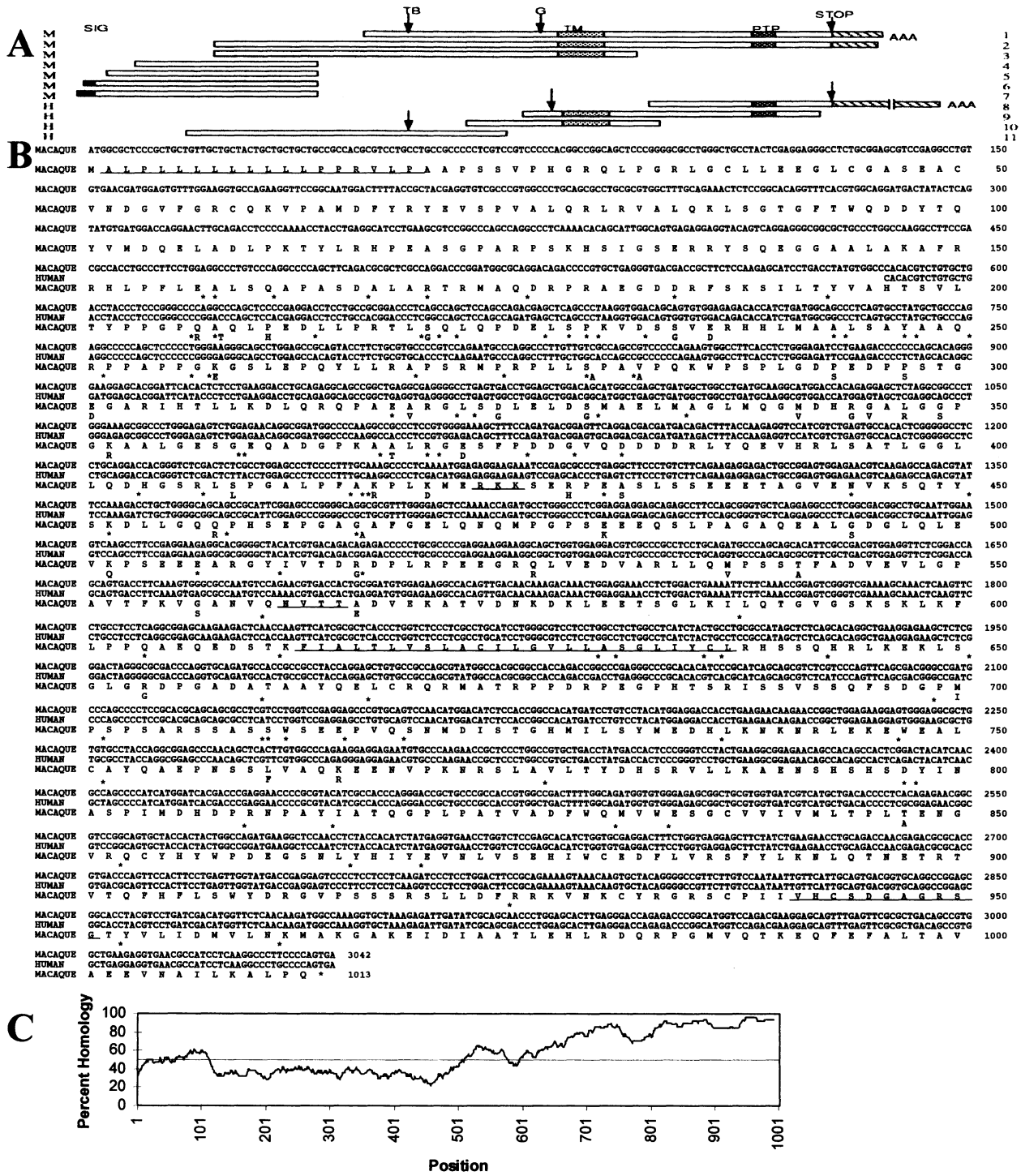

FIG. 1. Cloning strategy, macaque and human sequences, and homology to ICA512/IA2

(A) Cloning strategy for macaque (M, top 7 sequences) and human (H, bottom 4 sequences) M1851. Specific products described in the text are indicated by numbers to the right in the figure. (B) The 3,042-nucleotide cDNA sequence and 1,014 amino acid primary structure of macaque islet M1851. A partial sequence (2,456 nucleotides) from human M1851 is also aligned. The 11-residue tyrosine phosphatase core sequence, the 25-residue transmembrane region, an extracellular tribasic site (RKK) and an asparagine glycosylation site (NVTT) of possible post-translational processing, and the 18-residue signal peptide, are underlined. Asterisks are placed above differences between human and macaque nucleotide sequences, and human amino acids differing from the macaque sequence are noted directly below the corresponding macaque residue. (C) Homology between human ICA512 and human/macaque M1851 proteins. Both identical and conservative changes are allowed. The graph shows homology over a 25-residue window. The horizontal line represents 50% homology.

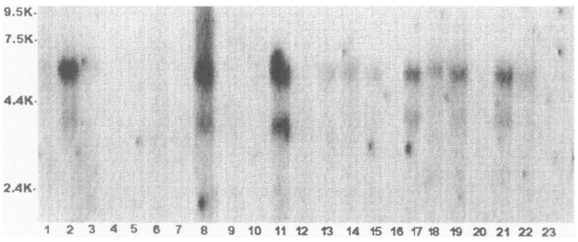

FIG. 2. Northern analysis using an M1851-specific oligonucleotide probe on RNA from 20 human tissues

Tissues are as labeled: 1, heart; 2, brain; 3, placenta; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, pancreas; 9, spleen; 10, thymus; 11, prostate; 12, testes; 13, uterus; 14, small intestine; 15, colon; 16, peripheral lymphocytes; 17, stomach; 18, thyroid; 19, spinal cord; 20, lymph node; 21, trachea; 22, adrenal; and 23, bone marrow. Strong 5.5-kb and weaker 3.3-kb mRNAs (arrows) are present in lanes representing brain, pancreas, and prostate, with lesser signals in spinal cord, adrenal, thyroid, and GI tract.

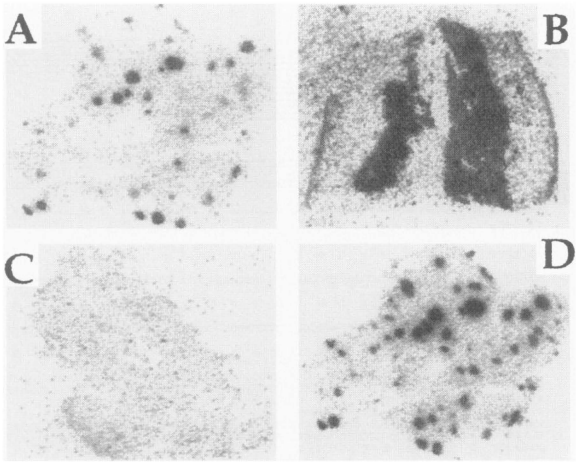

FIG. 3. In situ hybridization in macaque pancreas, adrenal, and muscle using an M1851-specific antisense oligonucleotide probe

Strong hybridization was detected in pancreatic islets (A) and adrenal medulla (B) but not in muscle (C). An ICA512-specific antisense probe (D) also hybridized to islets.

years before diagnosis (Fig. 5, right panel). However, this difference disappeared completely near the time of diagnosis, which suggests that M1851 autoantibodies appear later in prediabetes than GADab.

Of sera from 153 newly dignosed patients, 83 (54%) recognized icICA512 and 48 (31%) recognized icM1851. Only 1/48 (2%) sera recognizing icM1851 did not precipitate icICA512, but 35/83 (42%) reactive with icICA512 did not precipitate icM1851. Sera reactive to both antigens generally precipitated ICA512 to a greater extent than M1851, leading to greater calculated autoantibody indices for the former antigen (Fig. 6, left panel). However, it is not known whether greater specific autoantibody concentration or greater affinity accounted for this difference.

In order to test whether M1851 autoantibodies recognized only epitopes shared with ICA512, recombinant icICA512 antigen was expressed in baby hamster kidney fibroblasts. Extracts from these fibroblasts fully blocked icM1851 reactivity in 29/53 icM1851-positive sera tested, while a median of 21.4% (range 3–55%) of original immunoreactivity was retained in 24/53 sera (Fig. 6, right panel). Increasing icICA512 competitor concentration did not reduce this residual immunoreactivity in any of these 24 sera, suggesting that unique M1851 epitopes were being recognized. Of these sera, 14/24 had a M1851ab index greater than their ICA512ab index in the left panel of Fig. 6.

Mutants containing C-terminal deletions or mutations from the intact 376 AA icM1851 were constructed by PCR and expressed for immunoassays as above. Removal of the C-terminal 27 residues decreased reactivity by 32% from 19/53 to 13/53 IDDM sera. Importantly, antibody level was also greatly decreased, from a median index among positive sera of 0.49 to a median index of 0.04. Removal of the C-terminal 94 AA decreased reactivity further, to 6/53 IDDM sera (11%), a 68% decrease from the intact intracellular antigen. Median index decreased to 0.03. Finally, removal of the C-terminal 159 AA abolished all recognition by the 53 IDDM sera. Whether through primary sequence or complex conformational effects, these results emphasize the importance of the far C-terminal region in M1851 autoantibody epitopes.

DISCUSSION

Receptor forms of protein tyrosine phosphatases comprise a large superfamily of enzymes with multiple signaling functions regulating cell growth (23), differentiation (24), and adhesion (25). Marked variability in extracellular structure among the transmembrane PTPases is consistent with diverse ligands for adhesion. Intracellular catalytic domains necessarily retain greater homology. Notably, M1851, like ICA512 but unlike most members of the transmembrane PTPase

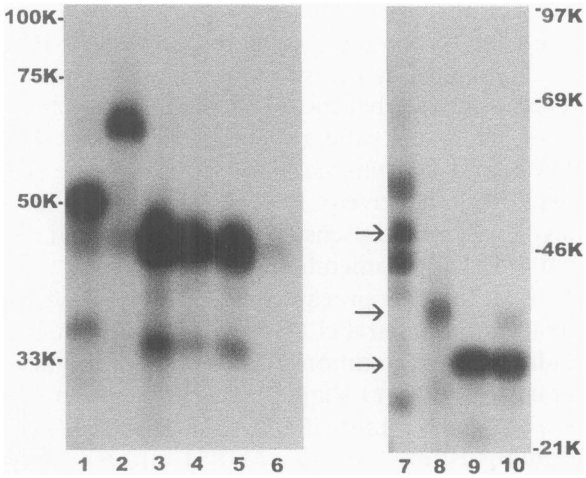

FIG. 4. Polyacrylamide gel electrophoresis and autoradiography of radiolabeled β-cell antigens

Human M1851 (intracellular domain, residues 637-1013) is shown as total translated protein (lane 3), and after precipitation with rabbit polyclonal anti-ICA512 (lane 4), with human IDDM serum (lane 5) and with human healthy control serum (lane 6). Human icICA512 (residues 602-979) (lane 1) and whole human GAD65 (residues 1-585) (lane 2) are shown for comparison. GAD65, icICA512, and icM1851 ran at 65 kDa, 50 kDa, and 45 kDa, respectively. The major products of limited trypsin digestion of human GAD65, human icICA512, macaque icM1851, and human icM1851 ran at 50 kDa, 40 kDa, 37 kDa, and 37 kDa, for lanes 7 to 10, respectively.

family, has a single putative catalytic domain (18). Like ICA512 and LCA (CD45), M1851 also has an Asp-for-Ala substitution in the active site (26). The physiological role of these PTPases in the β-cell remains to be determined, but the rat homologue of M1851 is associated with insulin secretory granules (26). Neither β-cell PTPase has yet been proven to be catalytically active (27).

The tissue distribution of M1851 is generally neuroendocrine in nature. Northern analysis here showed strong hybridization to human mRNA from brain and pancreas, and weaker hybridization in spinal cord, thyroid, adrenal, and GI tract. In situ hybridization using macaque tissues further localized expression to pancreatic islets and adrenal medulla. Mouse (24) and rat (26) homologues of M1851 have been recently described. Northern and Western analyses in the rat identified the homologue (termed phogrin) in islets, brain, pituitary, and gastric fundus, as well as insulinoma, glucagonoma, adrenal medullary, and pituitary corticotroph cell lines. The mouse homologue (termed PTP-NP) was identified in developing nervous system and endocrine pancreas (24). So far, the only exception to this neuroendocrine distribution is human prostate found positive in our Northern analysis.

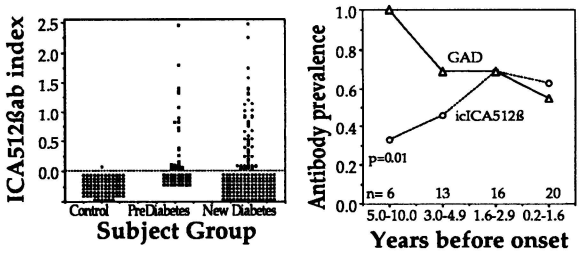

FIG. 5. Recombinant icM1851 autoantibody assay in diabetes and prediabetes

Left: Indices representing relative levels of autoantibodies to recombinant intracellular M1851 (icM1851), which were positive in 56/183 new IDDM patients, 28/60 first-degree relatives later progressing to clinical diabetes, and 1/91 healthy control subjects. The dotted line represents the cutoff for positivity, which is an index of 0.04. Right: Comparison of the prevalence of autoantibodies to GAD and to M1851 versus sampling time before the diagnosis of IDDM. Numbers of subjects in each time range are shown at the bottom of the panel.

Study of the rat homologue (26) detailed a number of basic biochemical features of the protein, most notably an insulin secretory granule membrane location, a dibasic cleavage site corresponding to residues 424-425 in Fig. 1B, and a putative glycosylation site corresponding to residues 562-565 in Fig. 1B. Evidence of expected

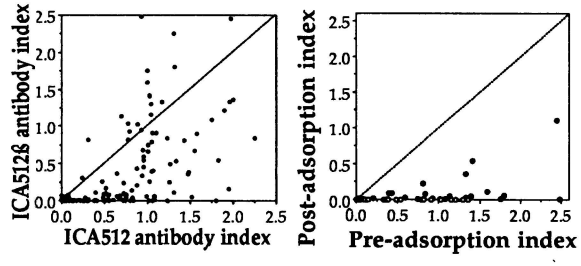

FIG. 6. Comparison of autoantibody epitopes between M1851 and ICA512

Left: Direct comparison of autoantibody indices to icM1851 versus icICA512. Right: Competition of binding of radiolabeled icM1851 by unlabeled recombinant icICA512. For 53 IDDM sera recognizing icM1851, icM1851ab index without competitor is plotted versus the same index in the presence of excess unlabelled icICA512. Reactivity was fully blocked in 29/53 sera (open symbols), while 24/53 retained partial ability to bind icM1851 (filled symbols). In both panels, the dotted line denotes equal indices.

sizes following limited proteolysis and endoglycosidase treatment supported the presence of these post-translational modifications. Although only 70% identical to that of the rodent, the primate M1851 sequence has preserved tribasic and glycosylation sites, and there is no reason to suspect that similar post-translational processing does not occur in humans. Additional common structural features include a cysteine-rich region near the amino terminus (AA 15-60) and multiple potential phosphorylation sites for casein kinase II and protein kinase C located intracellularly. Including the 1,680-bp human 3′ untranslated region (but not the 5′ untranslated region nor the polyadenosine tail), our estimated human M1851 sequence spans 4,722 nucleotides, compared with the 5.5-Kb mRNA observed by Northern blot. It is not known what accounts for the 2 message sizes (5.5 Kb and 3.3 Kb) observed in the human. However, the macaque M1851 homologue from islets had a 3′ UTR of only 209 bp, which could be consistent with a 3.3-Kb message.

Interestingly, features of both the ICA512 and M1851 PTPases are shared with the other two major targets for autoantibodies in IDDM, GAD and insulin. First, the antigens are at least in part membrane bound (28,29), which may result in more efficient immune recognition (30). Secondly, autoantibody epitopes are found in that part of the protein normally located in the cytosol. Whether these features are present in all autoantigens serving as humoral targets in autoimmunity to β-cells remains to be determined.

Limited trypsin digestion of the two protein tyrosine phosphatases yields a 40-kDa fragment from ICA512 and a 37-kDa fragment from M1851. This method of trypsin cleavage utilizes antigen bound to human autoantibodies, and yields distinct fragments remaining antibody-bound due to preservation of diabetes-relevant epitopes. Although we have not here proven that these fragments are identical to the tryptic fragments described by Christie et al. (7), a large body of evidence is consistent with this conclusion, which has also been reported by others for trypsin-treated human ICA512 (31) and for trypsin-treated mouse (32) and rat (26) homologues of M1851. The 37-kDa and 40-kDa tryptic fragments were described by Christie to derive from a faint 64-kDa band distinct from GAD that was soluble only in 8 M urea (8). ICA512 and M1851 contain extracellular (intralumenal) dibasic and tribasic residues, respectively, yielding 592 and 588 residue fragments C-terminal to the putative cleavage site, each still containing a hydrophobic transmembrane sequence (18). These are of the proper size to account for the poorly soluble and diffuse 64-kDa band described by Christie. Christie and co-workers also showed that the 40-kDa and 37-kDa fragment precursors were glycosylated (31) and that they bound to lectins via N-acetyl glucosamine and mannose, respectively. ICA512 and M1851 each have the proper consensus glycosylation site in the extracellular juxtamembrane sequence.

That immunoprecipitation of these two fragments is often parallel (7) is consistent with our findings of (*a*) common intracellular amino acid sequences, and (*b*) shared epitopes between the two antigens in most IDDM sera. That the far C terminus is important for M1851 autoreactivity is consistent with structural requirements described for two of four major icICA512 autoantibody epitopes (33). Taken together, the data that ICA512 is precipitated by many more sera than M1851, that C-terminal deletions abolish all M1851 reactivity, and that excess ICA512 only partly blocks M1851 reactivity, is consistent with M1851 lacking the juxtamembrane epitope(s) described for ICA512 but containing both unique and shared epitopes at the far C terminus.

Although many candidate β-cell autoantigens have been described for IDDM, remarkably few are actually demonstrated targets of the autoimmune response. Insulin autoantibodies (34) have been shown to be highly predictive, especially in combination with ICA and in the very young. T cell responses to human insulin have also been demonstrated (35,36). A 64-kDa autoantigen (37) identified as glutamate decarboxylase (GAD) is the target of highly predictive autoantibodies (22) and T cells (35,38). The putative protein tyrosine phosphatase ICA512 (16) is also a target of highly predictive antibodies (19) and T cells (35,36). Other proposed antigens have been less useful. Carboxypeptidase H antibodies were weakly associated with IDDM (12), ICA69 autoantibodies were not associated with IDDM (12), Imogen 38 is a mitochondrial protein which was not β-cell specific (39), and Glima 38 reactivity relies on heavy glycosylation and autoantibodies to it were uncommon in diabetes (40). Antibodies to bovine serum albumin (41) and heat shock protein (42) were nonpredictive in diabetes. The 105-kDa RIN antigen (43) and 52-kDa rubella-associated antigen (44) remain elusive, and predictivity has not been confirmed. Data on the reactivity of sulfatides (45) and gangliosides (46) remain limited since these nonprotein antigens are not easily used in immunoassays. That M1851 autoantibodies are

prevalent in diabetes and prediabetes and that M1851 contains unique autoantibody epitopes not present on ICA512, suggest that this is a new IDDM autoantigen. Given the limited (77%) amino acid identity in the intracellular domain, unique T cell epitopes are also possible.

The utility of M1851 autoantibodies in the screening and prediction of IDDM may be limited, since ICA512ab had greater sensitivity whereas only 0.7% of patients not detected by ICA512ab had M1851ab. Like ICA512ab, M1851 autoantibodies are most prevalent near disease onset, which suggests that M1851 is not primary in IDDM pathogenesis. Nevertheless, T lymphocyte help is required to form the high-affinity precipitating autoantibodies we have described. Cellular immunity developing late in prediabetes may be important, for example, in β-cell killing which has been suggested to occur primarily late in the pathogenesis (47).

Therapies based on such antigens involved late in the pathogenesis may be especially relevant in treating older IDDM patients identified by autoantibody testing at the time of clinical NIDDM diagnosis (22). These late onset IDDM patients are nearly as numerous as classical childhood IDDM (22,48). Late immuno-intervention therapy in these patients may be especially effective (49,50) perhaps because of the slowly progressive nature of their β-cell destruction. Given the high specificity of HLA–peptide interactions, differences in protein sequence between even homologous domains of ICA512 and M1851 could lead to different T cell activation by these molecules. Efforts to develop peptides (51) and peptide HLA conjugates (52) for immunotherapy require use of specific amino acid sequences from these or other β-cell autoantigens.

The presence of unique autoantibody epitopes on M1851, and its general neuroendocrine tissue expression, support M1851 as a new islet autoantigen in IDDM. Its dual roles as a β-cell insulin secretory granule protein and as a target of the autoimmune response in prediabetes may provide important insights into β-cell physiology as well as the pathogenesis and therapy of autoimmune diabetes.

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