Abstract

Cell-mediated autoimmune attack directed against islet proteins of ~38 kD in size has been associated with type 1 diabetes. A novel murine cDNA encoding an antigen of this size was cloned using a screening procedure based on the proliferative response of a human diabetic T cell clone (1C6) to a recombinant antigen epitope library. Membrane preparations from COS 7 cells transfected with the full-length 1,267-bp cDNA elicited a proliferative response from the reporter T cells comparable to that of the defined peptide epitope and native insulinoma antigen. In vitro translation and transfection experiments suggested that the protein is initially synthesized as a 44-kD protein and then processed to the native 38-kD form through the proteolytic removal of a 54-aa NH2-terminal mitochondrial targeting sequence. Differential centrifugation, Percoll density gradient centrifugation, and immunofluorescence studies confirmed localization of the antigen to mitochondria. Northern blot, Western blot, and 1C6 T cell proliferation assays showed that, although imogen 38 was more highly expressed in β cell than α cell lines, it was also present in other tissues. It is concluded that imogen 38 may be a target for bystander autoimmune attack in diabetes rather than a primary autoantigen. (J. Clin. Invest. 1996, 97:551–561.) Key words: diabetes • autoimmunity • insulin-dependent diabetes mellitus • mitochondria • islet

Introduction

Type 1 diabetes (insulin-dependent diabetes mellitus) results from cell-mediated autoimmune attack directed towards the islets of Langerhans, which culminates in the specific destruction of the pancreatic β cell. Serological studies on newly diagnosed type 1 diabetic patients and prediabetic genetically susceptible experimental animals indicate that multiple molecular targets for autoreactive antibodies exist (1). These include insulin (2) and carboxypeptidase H (3), which are localized to the secretory granule lumen; glutamate decarboxylase (GAD) (4) and ICA69 (5), which have a cytosolic or peripheral membrane association; ICA 512 (6) and the non-GAD 64-kD antigen (7), which are integral membrane proteins; and peripherin (8), a cytoskeletal component. To what extent the diversity in molecular character and intracellular localization of the autoantigens is a reflection of determinant spreading or secondary autoimmune phenomena is unclear.

The molecular targets of autoreactive T cells in diabetes overlap to some extent with those recognized by autoantibodies, as indicated by the finding that T cell antigen recall responses can be elicited to purified GAD, insulin, and carboxypeptidase H (9–12). The majority of T cell responses in spontaneously diabetic animals or humans, however, appears to be to unidentified proteins of a broad range of molecular sizes (13–16). Subcellular fractionation studies have suggested that the insulin-secretory granule may be a major source of such antigens (17–20).

We have shown that peripheral T cells from a large proportion of newly diagnosed type 1 human diabetes respond to crude insulinoma-secretory granule (ISG) preparations (16) and that membrane antigens in the size range of 36–40 kD may be important to this response (21). The presence of circulating antibodies to proteins of 38 kD in human (7) (22–24) and experimental diabetes (25) and the production of 38-kD responsive T cell lines from spontaneously diabetic nonobese diabetic mice (19) further suggest that autoantigens of this molecular size are significant. We have generated a cytotoxic 38-kD reactive CD4+ T cell clone (1C6) from PBMCs of a newly diagnosed type 1 diabetic patient using several rounds of in vitro stimulation with a crude membrane fraction of Rin m5F insulinoma cells (18). The epitope seen by clone 1C6 was recently mapped to a 70-aa fragment of an unidentified insulinoma protein by screening a subtracted cDNA fragment expression library (26). Subsequent fine mapping with synthetic peptides and molecular modeling have defined the minimal epitope as the 11-aa sequence, SLWEIEFAKQL.

We report here the isolation of the corresponding full-length cDNA from a mouse βTC3 insulinoma cDNA library, demonstrate its authenticity based upon its expression in prokaryote and eukaryote systems, and document its tissue and subcellular distribution using antibodies generated to the recombinant antigen. The antigen, which is termed imogen 38, appears to be a novel minor mitochondrial protein of variable but broad tissue distribution.

1. Abbreviations used in this paper: GAD, glutamic acid decarboxylase; GST, glutathione S-transferase; ISG, insulinoma-secretory granule.
Methods

Unless otherwise stated, all molecular cloning procedures were performed by standard protocols (27). Initial cloning of a cDNA fragment encoding the 38-kD antigenic target was achieved by using the proliferative response of the T cell clone 1C6 to screen a cDNA expression library prepared from a subtraction of mouse insulinoma βTC3 with mouse glucagonoma αTC2 cDNA (26, 28). The 211-bp insert in the positive clone (31G) was radiolabeled by random priming (oligolabeling kit; Pharmacia, Uppsala, Sweden) with [α-32P]dCTP (Amersham International, Little Chalfont, UK) and used to screen a full-length cDNA library prepared from mouse RIPTag insulinomas in the plasmid vector pSVSPORT (29, 30). Sequencing was performed in both directions by the Sanger dideoxy chain termination method on double-stranded templates using synthetic oligonucleotide primers (33).

An EcoRI fragment spanning residues 9 to 946 of the cDNA (aa 1–315) (Fig. 1) was inserted into the pGEX-1 vector (32) to generate a glutathione S-transferase (GST) fusion protein. The full-length protein was also expressed in Escherichia coli as an NH2-terminal His6-tagged construct in pREP7 B (Invitrogen Corp., Abingdon, UK) and in COS7 cells using a cytomegalovirus promoter construct in pCDNAs (Invitrogen Corp.). In both instances, the KpnI and HindIII sites in the original pSVSPORT vector were used for subcloning. Bacterial GST fusion proteins were purified by preparation of inclusion bodies (33) followed by SDS-PAGE and electroelution with an Extranphor apparatus (LKB, Stockholm, Sweden). Bacterial His6-tagged proteins were purified by metal chelate chromatography (Ni2+ nitrilotriacetic acid agarose; QIAGEN Inc., Chatsworth, CA) under denaturing conditions (8 M urea, 30 mM Tris, 10 mM mercaptoethanol, pH 8), and elution was achieved with 150 mM imidazole in the same buffer. The protein was extensively dialyzed, initially against PBS containing 1 mM mercaptoethanol and then against PBS alone.

Rat insulinomas were propagated by subcutaneous transplantation in New England Deaconess Hospital rats and homogenized with a Dounce homogenizer in 0.3 M sucrose, 10 mM MES KCl, 1 mM EDTA, and 1 mM MgSO4, pH 6.5, at 4°C as previously described (34). The homogenate was centrifuged at 500 g for 10 min, the pellet was rehomogenized twice and centrifuged under the same conditions, and the pooled supernatants were then subjected to sequential centrifugation at 4°C at 1,700 g for 10 min, 3,000 g for 20 min, 30,000 g for 20 min, and 300,000 g for 60 min. The 30,000-g pellet was resuspended in 6 ml homogenization medium, and 2.5 ml was mixed with 7.5 ml 36% (vol/vol) Percoll (Sigma Chemical Co., St. Louis, MO) containing 2 mM EGTA, 1 mM MgSO4, and 10 mM MES K+, pH 6.5, and centrifuged for 45 min at 25,000 g in a rotor (Rotavir T1270; Dupont, Stevenage, UK). The gradient was divided into 10 fractions, and particulate material was recovered by repeated dilution of each fraction with 3 vol homogenization medium followed by centrifugation at 30,000 g for 20 min in a rotor (SW50.1; Beckman Instruments, Inc., Spinco Division, Palo Alto, CA). The differential and density gradient centrifugation fractions were assayed for 1C6 T cell proliferative activity and for a series of organelle marker proteins, namely insulin (granules) (34), cytochrome oxidase (mitochondria) (35), aryl sulphatase (lysosomes) (36), and alkaline phosphatase (plasma membrane) (37). Western blot analyses were performed using antisera to either carnitine (lysosomes) (36), and alkaline phosphatase (plasma membrane) (37).

Results

Cloning and expression of the 38-kD antigen. 13 independent clones (7 between 1,231–1,267 bp) were obtained from screen-
ing 2 × 10^5 colonies. All of these were judged to be derived from the same mRNA based from the sequence at their 5' and 3' ends (150–250 bp) and by restriction mapping with a panel of six cutter restriction endonucleases. The longest clone (1,267 bp; Fig. 1) was sufficiently large with the addition of a poly A tail (typically 150 bp) to account for the size of the endogenous mRNA (1,500 bp; see Fig 8). The first AUG codon in this sequence bore a consensus Kozak motif and was shown to be a functional start site for in vitro translation (Fig. 2). The translational product was of sufficient size to incorporate the sequence of the native antigen (see below). From these data and structural features of the open reading frame described below, the sequenced clone was judged to be full length.

**Figure 1.** Nucleotide and deduced amino acid sequence of cloned 38-kD antigen. The putative mitochondrial import sequence is indicated by a dotted underline with the position of positively charged residues double underlined. Potential cleavage sites of the mitochondrial matrix-processing peptidase are also shown (I). The start (nt 626) and end (nt 835) of the 211-bp probe fragment 31G are indicated above the nucleotide sequence, and the minimal antigenic epitope is underlined. The starting positions of two sequences subcloned into prokaryote and eukaryote expression vectors are indicated above the nucleotide sequence. The nucleotide sequence information has been deposited with European Bioinformatics Institute (EBI) for inclusion in Genbank and EMBL databases and is available under accession number Z46966.
acids (4 Arg, 1 His), 16 hydrophobic residues, and a single negatively charged residue at position 25. The positioning of the various Arg residues argued against this sequence functioning as a secretory protein signal sequence but was compatible with a mitochondrial import signal, which is typified by a positively charged amphipathic sequence and the general absence of acidic residues (41). A potential cleavage site for a mitochondrial processing peptidase (Arg<sub>41</sub> Asp<sub>42</sub>) was located at the COOH terminus of this sequence. This was followed by a hydrophobic stretch spanning 14 aa and further potential cleavage sites for the mitochondrial processing peptidase complex at Arg<sub>55</sub>, Arg His<sub>56</sub>, or Arg Ser<sub>57</sub>. The sequence of the 31G probe that was used to screen the cDNA library appeared in the central region of the molecule (nt 625–835) flanked by Rsal and AluI sites consistent with the original subtraction cloning procedure (42), which used PCR amplification of doubly digested fragments of bTC3 cDNA. The corresponding sequence of the full-length clone was identical to that reported previously except at nucleotide positions 629 (G → A), 675 (T → C), 702 (A → T), and 740 (T → C), which resulted in amino acid changes at positions 211 (Arg → Lys), 234 (Arg → Ser), and 245 (Ile → Thr). These differences may be the result of either natural polymorphisms or infidelities in the multiple PCR reactions used in the original subtraction cloning procedure. The fact that the 5′ and 3′ sequences of the 13 independent clones appeared to be derived from the same mRNA argued against there being multiple isoforms of the protein within the β cell. The antigen epitope that had been mapped using synthetic peptides was in the expected reading frame and orientation in the cloned sequence (aa 266–275). Neither the nucleotide nor the protein sequence encoded by the cloned cDNA showed remarkable homology to cDNA sequences deposited in GENBANK, EMBL, PIR, or SWISSPROT data bases using FASTA, BLAST, and MOTIF algorithms.

In vitro translation of the sequence (clone 1C2; Fig. 1) generated a 44-kD protein consistent with the calculated Mr (43,887 D) of the 1.152-bp open-reading frame (Fig. 2). This product is significantly larger than the rat insulinoma protein (38 kD) identified on the basis of the T cell 1C6 proliferative response to electroeluted proteins or mouse antigen detected by Western blotting (see below). This raised the question of whether an alternate translation initiation site could be used or whether co- or posttranslational proteolysis of the protein occurs in vivo. The addition of dog pancreatic microsomes to an assay translating 1C2 cRNA did not affect the size of the translation product or protect it from digestion by proteinase K as added at the end of the translation reaction. It was therefore unlikely that the protein was translocated into the lumen of the endoplasmic reticulum. A 5′-truncated cRNA (clone 9B2) that contained the second AUG codon with a Kozak consensus sequence was translated but with a lower efficiency than clone 1C2. The resultant protein was smaller in size (33 kD) than the major form of the native antigen, although it did correspond in size to one of the minor immunoreactive forms of the antigen visualized by Western blot analyses (see below).

A bacterially expressed protein of the cloned sequence generated as a GST hybrid (aa 1–315) stimulated the proliferation of the CD4<sup>+</sup> T cell clone 1C6 in the presence of HLA-DR1 antigen-presenting cells (Fig. 3). The response was of the same approximate magnitude and molar dose response as seen with a synthetic 20-mer peptide incorporating the epitope (aa 259–278) (26). A GST construct of clone 9B2 and a histidine-tagged full-length construct of 1C2 (aa 1–384) produced similar responses, whereas the corresponding fusion partners prepared from wild-type pGEX1 and pRSET B vectors were inactive in the assay (maximal stimulation index < 3; data not shown). Native antigen in the form of a crude secretory granule fraction from rat insulinoma (45,000-g pellet) produced a similar maximal proliferative response to the recombinant Histagged construct and the synthetic antigen. At submaximal concentrations, the response to the native antigen and synthetic peptide was additive (data not shown). It is concluded that the cloned sequence represented the authentic precursor of the 38-kD antigen.

Subcellular localization of the 38-kD antigen. Differential centrifugation analyses of an insulinoma homogenate prepared under isotonic conditions (Fig. 4) showed that the 1C6 T cell reactivity was pelleted completely at 30,000 g for 20 min and was recovered in high yield and specific activity in a frac-
38-kD Diabetic Autoantigen

Figure 4. Differential centrifugation analysis of the subcellular distribution of the 38-kD antigen in insulinoma tissue. A postnuclear supernatant from a rat insulinoma homogenate in isotonic medium was subjected to successive centrifugation steps to yield a 1,700-g × 10 min pellet (P1.7), a 3,000-g × 20 min pellet (P3), a 30,000-g × 20 min pellet (P30), and a 300,000-g × 60 min pellet (P300) and supernatant (S300). Each fraction was assayed for organelle marker proteins and 1C6 reactivity as indicated on the y-axes; Western blot analyses were performed with affinity-purified guinea pig anti–38-kD proteins. In the latter two assays, the quantity of protein used in each assay was proportionate to the total protein recovered in each fraction as shown in the uppermost panel.

Figure 5. Percoll density gradient centrifugation analysis of the 38-kD antigen in insulinoma tissue. A particulate fraction prepared from insulinoma homogenate at 30,000 g for 20 min was subjected to isopycnic density gradient centrifugation and recovered in 10 fractions of decreasing density. Each fraction was assayed for organelle marker proteins and 1C6 reactivity as indicated on the y-axes; Western blot analyses were performed with rabbit anti–carboxypeptidase H and affinity-purified guinea pig anti–38-kD protein. Equivalent volumes of each fraction were analyzed in each assay to ensure that the response reflected the distribution of the antigen rather than its specific activity.
which could be translated in vitro to a protein of lower
the vector pCDNA3. The shorter cDNA clone, 9B2 (Fig. 1),
in fractions of a lower buoyant density.
kaline phosphatase, though the latter was mostly concentrated
plasma membrane marker, and aryl sulphatase, a
marker of lysosomes.

Isopycnic density gradient centrifugation analysis of rat insulinoma tissue using Percoll in isotonic sucrose as the medium (Fig. 5) showed that the major peak of 1C6 reactivity coincided with the 38-kD immunoreactivity on Western blotting. Both paralleled the distribution of the mitochondrial marker cytochrome oxidase but differed from that of the lysosomal marker, aryl sulphatase, and the secretory granule markers, insulin (determined by immunoblot) or carboxypeptidase H (determined by Western blotting). The distribution of the antigen overlapped with that of the plasma membrane marker, alkaline phosphatase, a marker of lysosomes.

Table I. 1C6 T Cell Proliferative Response to Transfected COS 7 Cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>None</th>
<th>Vector alone</th>
<th>Furin</th>
<th>9B2</th>
<th>1C2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td>141±31</td>
<td>142±47</td>
<td>200±70</td>
<td>128±32</td>
<td>317±77</td>
</tr>
<tr>
<td>Particulate</td>
<td>121±28</td>
<td>90±32</td>
<td>113±35</td>
<td>102±27</td>
<td>10,476±1,562</td>
</tr>
<tr>
<td>Controls</td>
<td>PHA 23,915±5,695</td>
<td>IL-2 32,744±4,103</td>
<td>Medium 112±49</td>
<td>CMF 60,955±3,818</td>
<td></td>
</tr>
</tbody>
</table>

Cells were harvested 24 h after transfection, sonicated in a low-salt buffer, and separated into a soluble and particulate fraction by centrifugation. Results with untransfected and mock-transfected cells are shown together with cells transfected with an unrelated protein (furin), a nontranslated truncated cDNA clone 9B2, and the full-length clone 1C2. Results are the mean cpm±SD of triplicate analyses using 5 μg/ml of antigen protein in the proliferation assay. A Western blot analysis of the same samples is shown in Fig. 6. CMF, crude membrane fraction from rat insulinoma.

Indirect immunofluorescence microscopy of the 1C2-transfected COS 7 cells revealed a distinctive pattern of antibody reactivity in positively transfected cells associated with large intracellular structures that ranged in morphology from discrete ovoid or doughnut-shaped organelles to long tubular elements with occasional bifurcation (Fig. 7). Such structures are characteristic of mitochondria in COS 7 cells, and it was shown that the antibody to 38 kD colocalized with an mAb to a human 65-kD mitochondrial protein (2D1/82; Biogenesis Ltd.). There was little or no anti–38-kD antibody reactivity on any of the control transfectants (data not shown), consistent with the low reactivity of the antisera on Western blots (Fig. 6) and the negative response in the 1C6 T cell proliferation assay (Table I). The specific pattern of anti–38-kD immunofluorescence was not observed with nonimmune serum or preabsorbed antibody.
Tissue distribution of the 38-kD antigen. Northern blot analyses of various mouse tissues (Fig. 8) revealed a single 1.5-kb mRNA that was widely distributed but variable in its level of expression. A second minor component of 2.4 kb was evident in kidney and liver tissue. Probes spanning the epitope-encoding region gave similar results to those shown with the major segment of the open reading frame, indicating that the mRNA that hybridized was related to the specific antigen in question. Phosphorimager quantitation showed that the signal generated with the 1C2 probe ranged from 1 to 4% of that with an actin probe of similar specific radioactivity at the same level of stringency of hybridization and washing (data not shown). Analysis of a series of mouse cell lines showed a higher abundance of the mRNA in βTC3 insulinoma cells relative to αTC2 glucagonoma cells, consistent with the results of the subtractive hybridization procedure used to isolate the cDNA fragment initially (26). The mRNA was also demonstrated in the pituitary corticotroph cell line AtT20.

The distribution of the protein antigen was initially investigated in a series of tissue culture cell lines (Fig. 9, Table II) using sonication to disrupt the tissue and centrifugation to prepare a soluble and particulate fraction. As in the case of insulinoma tissue, 1C6 T cell reactivity was associated with a particulate fraction. The growth hormone pituitary cell line GH4 and the pituitary corticotroph line AtT20 induced a marked 1C6 proliferative response, as did the insulinoma cell line βTC3. The glucagonoma cell αTC2 and the liver cell line BRL showed lower levels of expression. Western blot analyses, performed in parallel, showed a predominant 38-kD band that was associated mainly with the particulate fraction. There was a good correlation between the intensity of the reaction on the Western blot and the magnitude of the proliferative response evoked by these fractions.

Analysis of different mouse tissues using the 1C6 T cell proliferation assay (Table III) showed the presence of the antigen in a particulate fraction from testes, kidney, and liver but not in heart and skeletal muscle. Intermediate responses were demonstrated in lung, spleen, brain, and gastric fundus. When tested at a lower concentration (1 μg/ml), only the testes sample produced a response (data not shown), suggesting that most responses were close to the limit of detection. Western blot analyses performed on the same samples showed the pres-
ence of a 38-kD protein in all tissues (Fig. 10). This was lowest in the case of skeletal muscle and heart, which might account for the low T cell reactivity to these tissues. Immunoreactive proteins other than the 38-kD band were evident, notably a 33-kD protein in liver, kidney, and muscle and a 60-kD component in muscle, but were not investigated further.

Discussion

The cDNA cloned in these experiments appears to encode a normal mouse protein that represents a precursor form of a native 38-kD antigenic target of the human diabetic CD4+ T cell clone 1C6. Database searching at either the nucleotide or amino acid level and analysis for conserved protein motifs failed to reveal the identity of the protein or to provide clues to its biochemical properties or function. The previously reported homology of the 70-aa probe sequence with the bacterial superantigens *Streptococcus pyogenes* exotoxin A and *Staphylococcus* enterotoxin B (~25% identity over 60 aa) (26) did not extend beyond the immediate region originally identified. Likewise, homologies of the 11-aa minimal epitope with the prolactin receptor and lens β-crystallin were not borne out by the longer sequence. Attempts to define common linear epitopes by alignment of the amino sequence with GAD, carboxypeptidase H, heat shock protein 60, mycobacterial heat shock protein 65, and peripherin proved unsuccessful. It is concluded that the protein has not been previously identified at the structural level. In the absence of any functional information, it is given the name imogen 38 (islet mitochondrial antigen of 38 kD).

Table II. 1C6 T Cell Proliferative Response to Mouse Tissue Culture Cell Lines

<table>
<thead>
<tr>
<th></th>
<th>BRL</th>
<th>αTC2</th>
<th>βTC3</th>
<th>GH4</th>
<th>AtT20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td>33±12</td>
<td>226±219</td>
<td>48±3</td>
<td>171±14</td>
<td>217±165</td>
</tr>
<tr>
<td>Particulate</td>
<td>2,653±261</td>
<td>5,325±2,290</td>
<td>7,851±1,453</td>
<td>24,798±3,533</td>
<td>17,986±5,560</td>
</tr>
<tr>
<td>Controls</td>
<td>PHA 43,973±11,371</td>
<td>IL-2 17,808±2,926</td>
<td>Medium 58±28</td>
<td>CMF 29,770±3,031</td>
<td></td>
</tr>
</tbody>
</table>

Cells were harvested when semiconfluent, sonicated in a low-salt buffer, and separated into a soluble and particulate fraction by centrifugation. Results are the mean cpm±SD of triplicate analyses using 5 μg/ml of antigen protein in the proliferation assay. A Western blot analysis of the same samples is shown in Fig. 9. CMF, crude membrane fraction from rat insulinoma.

Table III. 1C6 T Cell Proliferative Response to Membrane Preparations of Different Mouse Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Testis</th>
<th>Kidney</th>
<th>Skeletal muscle</th>
<th>Liver</th>
<th>Lung</th>
<th>Spleen</th>
<th>Brain</th>
<th>Cardiac muscle</th>
<th>Gastric fundus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5,860±276</td>
<td>3,910±2,892</td>
<td>114±58</td>
<td>2,859±410</td>
<td>994±220</td>
<td>4,722±924</td>
<td>1,326±715</td>
<td>110±18</td>
<td>2,294±430</td>
</tr>
<tr>
<td>Controls</td>
<td>PHA 12,596±2,848</td>
<td>IL-2 5,240±3,240</td>
<td>Medium 152±140</td>
<td>CMF 2,596±290</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

Results are the mean cpm±SD of triplicate analyses using 5 μg/ml of antigen protein in the proliferation assay. A Western blot analysis of the same samples is shown in Fig. 10 and related Northern blot analyses in Fig. 8. CMF, crude membrane fraction from rat insulinoma.
tion that imogen 38 is derived from a precursor of 44 kD and the fact that it has no N-glycosylation consensus sequences distinguish it from the 37- and 40-kD antigens described by Christie and colleagues (7), which are both derived from glycoproteins of 64 kD. The lack of N-glycosylation and different solubility characteristics also differentiate between imogen 38 and the amphipathic 38-kD molecule identified in islet immunoprecipitation studies using diabetic autoantibodies (22, 23). More subtle relationships, such as the possession of common conformational epitopes, cannot be presently excluded, and serological studies are being performed using the purified recombinant imogen 38 to evaluate the incidence, prevalence, and magnitude of immune responses to this antigen to clarify this issue.

The structural evidence that suggested mitochondrial localization of the encoded protein was strongly supported by immunofluorescence microscopy of transfected COS 7 cells using an antibody generated to the recombinant protein and by differential and Percoll density gradient centrifugation analyses of the native antigen in insulinoma tissue. Previous investigations based on analysis of discontinuous Nycodenz density gradients of insulinoma homogenates had indicated that 1C6 T cell reactivity was associated with ISGs (18). Although granule-enriched fractions from Nycodenz gradients contain only a small proportion of all the mitochondria of the cell, the specific activity of mitochondrial marker proteins within them is comparable to fractions containing the bulk of the mitochondria, since the latter also contain most of the endoplasmic reticulum proteins. This, combined with the difficulty of obtaining reliable estimates of the initial content and recoveries of the antigen because of the nonlinearity of the T cell proliferation assay, made it impossible to distinguish granule from mitochondrial localization under these circumstances. The present studies, which used a different density gradient system and were backed up by Western blotting analyses, were unequivocal and showed that the mitochondrion is the major site of imogen 38 localization in the normal pancreatic β cell.

The presence of a leader sequence of largely hydrophobic character punctuated by basic amino acid residues indicates that imogen 38 is initially imported into the mitochondrion via a voltage-dependent and HSP70-dependent process (41). Cleavage by the matrix-processing peptidase complex at the first consensus sequence Arg Asp signifies that this occurs at a position where a leader sequence of largely hydrophobic stretch of 14 amino acids (residues 26–39) would produce a protein of 39.5 kD, and exposure of an NH₂-terminal hydrophobic stretch of 14 amino acids (residues 26–39). Within the paradigm of the endosymbiotic origin of mitochondria, this could constitute the equivalent of a bacterial signal sequence and direct the protein from the matrix through the inner mitochondrial membrane, where it could be processed further by IMP 1 or the octapeptidyl peptidase–like enzyme to a soluble protein of 39.5 kD and pl 7.6. Alternatively, the precursor could be processed by the matrix-processing peptidase complex after the Arg Thr or Arg His or Arg Ser residues. The last product fits best with the migration of the rat, mouse, or human antigen on SDS-PAGE and with the pl of the native rat insulinoma antigen (7.0 ± 0.1, n = 8) determined with the 1C6 T cell proliferation assay by isoelectric focusing in 8 M urea and 4% NP-40 (Arden, S. D., B. O. Roep, and J. C. Hutton, unpublished findings). The 55–384 sequence, however, contains no hydrophobic stretches longer than 9 aa, and thus the association of the antigen with membranes as detected previously in the 1C6 T cell proliferation assay (18) is likely to occur by a process other than membrane spanning. The finding that transient overexpression of the antigen in COS 7 cells results in an antigenic form that can be solubilized by sonication suggests that its interaction with membrane structure may depend on other anchored proteins or posttranslational modification of the protein. These experiments also revealed that the solubilized antigen is a poorer stimulus for 1C6 T cell proliferation than the particulate form, a phenomenon which biases interpretation of localization studies based on T cell proliferation data to favor the conclusion that the antigen is exclusively membrane associated. These questions are likely to be resolved by structural analysis of purified forms of the native antigen and studies of its biosynthesis and interaction with other cellular constituents. Such studies will be facilitated by the availability of antibodies that were developed in the current experimental series.

Although imogen 38 appeared to be more abundant in β cells than in α cells, its tissue distribution was broad and consistent with being a housekeeping gene or a structural component common to all oxidative tissues. Although the existence of tissue-specific isoforms of the antigen could not be ruled out, the majority would appear to possess the antigenic epitope recognized by the T cell clone 1C6. On these grounds, it is unlikely that imogen 38 is a primary target for cellular autoimmune attack in type 1 diabetes. This does not, however, preclude it from playing an important pathological role as a target for bystander autoimmune attack and a contributor to insulitis. Indeed, it may be argued that such a role is more consistent with a mitochondrial protein that is neither accessible nor easily presentable to the cellular or humoral arm of the immune system without tissue disruption or dysfunction. Nevertheless, mitochondrial antigens with a similarly broad tissue distribution are implicated in other tissue-specific autoimmune diseases, notably, the ADP/ATP transporter of the inner mitochondrial membrane in autoimmune myocarditis and dilated cardiomyopathy (44), the mitochondrial matrix chaperonin heat shock protein 60 (the mammalian homologue of mycobacterial HSP65) in rheumatoid arthritis, type-1 diabetes and myositis (45–47), and the inner mitochondrial membrane 2-oxoacid multienzyme complexes in primary biliary cirrhosis (48, 49). In the latter instance, immunohistological studies with autoantibodies and mAbs to the immunodominant E2 component (dihydrolipoyl acetyltransferase) reveal immunoreactive structures not only in mitochondria but also in the apical region of the biliary epithelium and the ductal lumen. Moreover, such reactivity is only evident in diseased tissue, which raises the possibility that the E2 subunit is actually mistargeted to the secretory pathway in response to inflammation. The fact that proteins like E2 and imogen 38 probably need to interact with cytosolic chaperonins of the HSP70 family to be directed to mitochondria (41), and they possess potential or ancestral “signal peptide”–like structures within their mitochondrial targeting sequences, suggests a possible molecular mechanism by which a sequestered organelar protein may be redirected to the secretory pathway and gain access to the cell surface or compartments involved in antigen presentation. Such a phenomenon probably occurs with a number of different mitochondrial proteins (e.g., 50–52) and could account for the apparent tissue specificity of an autoimmune attack directed at otherwise widely distributed tissue antigens. There is no direct evidence, at the moment, for such a mechanism either in the
case of E2 or imogen 38, which emphasizes the need to understand more of the cell biology of imogen 38 in both the normal and prediabetic islet and the mechanism by which it is presented to T cells in vivo. Such questions can be more easily addressed now that imogen 38 has been cloned and recombinant antigens and antibodies have been generated. These reagents will likewise facilitate investigations, both clinical and experimental, of the importance of this particular antigen to the pathogenesis of type 1 diabetes and examination of its potential as a diagnostic marker or therapeutic agent.

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