Imogen 38: A Novel 38-kD Islet Mitochondrial Autoantigen Recognized by T Cells from a Newly Diagnosed Type 1 Diabetic Patient

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Abstract

Cell-mediated autoimmune attack directed against islet proteins of $\sim$ 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 NH_2-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 in-sulin (2) and carboxypeptidase H (3), which are localized to the secretory granule lumen; glutamate decarboxylase (GAD)[1] (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 diabetics 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.

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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-^{32}P]dCTP (Amersham International, Little Chalfont, UK) and used to screen a full-length cDNA library prepared from mouse Rip Tag 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 (31).

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 NH$_2$-terminal His$_6$-tagged construct in pRSET B (Invitrogen Corp., Abingdon, UK) and in COS 7 cells using a cytomegalovirus promoter construct in pCDNA3 (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 Extraphor apparatus (LKB, Stockholm, Sweden). Bacterial His$_6$-tagged proteins were purified by metal chelate chromatography (Ni^{2+} nitrilotriacetic acid agarose; QIAGEN Inc., Chatsworth, CA) under denaturing conditions (8 M urea, 50 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 K$^+$, 2 mM EGTA, and 1 mM MgSO$_4$, 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 MgSO$_4$, and 10 mM MES K$^+$, pH 6.5, and centrifuged for 45 min at 25,000 g in a rotor (Sorvall 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 media 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 carboxypeptidase H (38) or recombinant 38 kD (1C2).

Tissue culture cell lines were grown to subconfluency in DME containing 10% newborn calf serum, rinsed extensively in PBS, scraped off the tissue culture dish in 1 ml ice-cold 10-mM Tris-HCl, 1 mM EDTA, pH 8, sonicated for 20 s (MSE Soniprep, Crawley, UK), and then centrifuged at 20,000 g for 30 min in a refrigerated microcentrifuge to obtain a supernatant (soluble) and particulate fraction. Mouse tissues were transferred immediately into liquid nitrogen and pulverized while frozen with a mortar and pestle, and 100 mg wet wt samples were then sonicated and centrifuged as above.

Total RNA was prepared by homogenization of tissue in 6 M guanidinium HCl, 8% (wt/vol) mercaptoethanol, and 2% (vol/vol) laurylsarkosinate, followed by CsCl step-gradient centrifugation (39). Poly A$^+$ mRNA was then prepared by oligo dT cellulose chromatography, and samples were analyzed by Northern blotting after electrophoresis on denaturing formaldehyde gels. Blots were hybridized for 16 h at 42°C in 50% (vol/vol) formamide, 5× sodium chloride sodium phosphate EDTA buffer (SSPE), 5× Denhardt's reagent, and 50 μg/ml salmon testis DNA and finally washed in 0.1 × SSPE, 0.1% SDS at 55°C. ^{32}P-radiolabeled randomly primed probes were generated from the 955-bp EcoRI fragment in the pSVSPORT clone 1C2 and from mouse actin. Gel loadings were adjusted on the basis of actin probe hybridization to the samples subject to RNA dot blot analysis. Visualization and quantitation of blots was performed by phosphorimaging (BAS 2000; Fuji, Sheffield, UK).

In vitro translation analyses were performed with SP6 RNA polymerase transcripts of clone 1C2 and 9B2 (0.1 μg/25 μl incubation) in a reticulocyte lysate/dog pancreatic microsome system (Promega, Madison, WI). The efficiency of translocation of the products into the lumen of the endoplasmic reticulum was assessed by incubation for 30 min at 4°C with 10 μg/ml proteinase K. [^{35}S]Methionine-labeled products were analyzed by SDS-PAGE on 12.5% (wt/vol) acrylamide 0.1% bis-N,N′-bisacrylamide gels and prepared for fluorography (38) or analyzed by phosphorimaging after drying.

T cell proliferation assays were performed using 1–2 × 10^4 T cells and 5–10 × 10^4 irradiated HLA-DR–matched PBMCs (antigen-presenting cells) in flat-bottomed 96-well plates in complete Iscove's modified Dulbecco's medium (18). PHA (250 ng/ml) and IL-2 (recombinant, 10% Lymphocult; Biotest, Dreieich, Germany) were used as positive controls along with 2–10 μg/ml crude ISG (18). Tissue samples and subcellular fractions prepared as above were irradiated to ensure sterility and analyzed at a range of concentrations (0.1–50 μg/ml).

Subconfluent cultures of COS 7 cells (3.5-cm dishes) were transfected with 1 μg 1C2 cDNA in pCDNA3 using the reagent Lipofectamine (GIBCO BRL, Paisley, UK) according to the manufacturer's recommended procedure. Cells were grown for a further 24–48 h and then extracted in ice-cold 10-mM Tris-HCl, 1 mM EDTA, pH 8, for the T cell proliferation assay or Western blotting as above, or were fixed in situ with 4% (wt/vol) paraformaldehyde in PBS for indirect immunofluorescence microscopy. In the latter case, cells were permeabilized with 0.1% Triton X-100 in PBS and then incubated with affinity-purified rabbit antibody (equivalent to 1:50 diluted serum) for 1 h at room temperature in 0.1 M glycine, 0.5% nonimmune horse serum in PBS, followed by fluorescein-conjugated pig anti–rabbit IgG (1:500; DAKO, Copenhagen, Denmark) for 1 h. Double-immunofluorescence labeling was performed by the simultaneous addition of mouse mAb (1:100) to a 65-kD human mitochondrial protein clone 2D1/82 (Biogenesis Ltd, Poole, UK) followed by Texas Red–conjugated sheep anti–mouse IgG (1:100; Amersham International) for 1 h.

Antibodies to the GST–1C2 fusion protein were preabsorbed with bacterial homogenate prepared from cells expressing wild-type GST, and then affinity-purified on the full-length His-tagged antigen transferred to nitrocellulose membranes (40). Western blotting was performed as previously described (38) using horseradish peroxidase–conjugated second antibody (DAKO) and enhanced chemiluminescence (Amersham International) as the detection system. Protein was determined with bicinchoninic acid reagent (Pierce, Rockford, IL) using BSA as standard. Samples were incubated at 60°C for 10 min in 10 μl 1% (wt/vol) SDS to solubilize membrane proteins or inclusion bodies before assay. Computational analyses were performed with the Genetics Computer Group (WI) package via the Human Genome Mapping Project (Sanger Centre, Hinxton, UK).

Results

Cloning and expression of the 38-kD antigen. 13 independent clones (7 between 1,231–1,267 bp) were obtained from screen-

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                                            †1C2
-45                     CGCTTGTGCTTCCGGCTGTGCTCTGGTTTGAGTTTGATTTACACG ATG CTC CAC AGA ATC
  1                                                                 Met Leu His Arg Ile


 16    CCG GCA TTT CTA CGC CCT CGC CCT TTC TCG GGC CTC CCT TTG TCC TGT GGA AAC CGG GAC
  6    Pro Ala Phe Leu Arg Pro Arg Pro Phe Ser Gly Leu Pro Leu Ser Cys Gly Asn Arg Asp
                                                                                      Î


 76    GTC TCG GTG GCT GTG CTA CCC GCG GCT CAG TCC GGA GCT GTG AGG ACA GAA AAT AAT ATC
 26    Val Ser Val Ala Val Leu Pro Ala Ala Gln Ser Gly Ala Val Arg Thr Glu Asn Asn Ile
                                                                         Î


136    CAA AGA CAT TTT TGC ACT AGC AGA TCA ATC TGT AGC AAG AAA GTT GAC CAG TCT GTT CCA
 46    Gln Arg His Phe Cys Thr Ser Arg Ser Ile Cys Ser Lys Lys Val Asp Gln Ser Val Pro
                   Î               Î

                                            †9B2
196    GCC AAT GAA ATT TCC CAG AAG GCA GCA GAG AGC CAA GGC AGG GGA AAG GAG ACT TTG AAA
 66    Ala Asn Glu Ile Ser Gln Lys Ala Ala Glu Ser Gln Gly Arg Gly Lys Glu Thr Leu Lys


256    AAA GAC CTG CTG GAC ATT ATT AAG GAC ATG AAA GTT GAC CTG AGC ACA GCA AAT GTG AAA
 86    Lys Asp Leu Leu Asp Ile Ile Lys Asp Met Lys Val Asp Leu Ser Thr Ala Asn Val Lys


316    ACA CCA AAG CCA CGT GGC AGA AAA CCT TCT GCC AGT CTG GAG GCT ACC GTT GAC AGG CTT
106    Thr Pro Lys Pro Arg Gly Arg Lys Pro Ser Ala Ser Leu Glu Ala Thr Val Asp Arg Leu


376    CAG AAA GCT CCG GAA GAC CCT CCA AAG AAG AGG AAT GAG TTC CTG AGT CCT GAG TTG GTG
126    Gln Lys Ala Pro Glu Asp Pro Pro Lys Lys Arg Asn Glu Phe Leu Ser Pro Glu Leu Val


436    GCT GCA GCG TCG GCT GTT GCA GAT TCC CTA CCT TTT GAC AAG CAG ACA ACC AAG TCA GAG
146    Ala Ala Ala Ser Ala Val Ala Asp Ser Leu Pro Phe Asp Lys Gln Thr Thr Lys Ser Glu


496    CTG CTC AGG CAG CTG CAG CAA CAC GAG GAG GAG TTG AGG GCT CAG AAG GAC AGA GAG AAG
166    Leu Leu Arg Gln Leu Gln Gln His Glu Glu Glu Leu Arg Ala Gln Lys Asp Arg Glu Lys


556    CGC AGG ATC AGC TTC ACT CAC ATA ATA TCA AAT ATG AAA ATT GCC AAG TCT CCC TCC GGG
186    Arg Arg Ile Ser Phe Thr His Ile Ile Ser Asn Met Lys Ile Ala Lys Ser Pro Ser Gly

                    |31G start
616    AGA GCT AGT ACA AGG CCA CAG CAC CAG ATT CAG TTT GAC GAA GAC ATG GAC AGT TCT CTT
206    Arg Ala Ser Thr Arg Pro Gln His Gln Ile Gln Phe Asp Glu Asp Met Asp Ser Ser Leu


676    AAG CAG GAG AAG CCA ACT GAT TTC AGA AAA AGG AAA TAT TTA TTC AAG GGG AAA AGA CTT
226    Lys Gln Glu Lys Pro Thr Asp Phe Arg Lys Arg Lys Tyr Leu Phe Lys Gly Lys Arg Leu


736    TCA ATT TTT GCT GAT AAG GCG TTT GCT GAT GAA CCA CCT GAA CCA GAA GCA TCA CCT TCT
246    Ser Ile Phe Ala Asp Lys Ala Phe Ala Asp Glu Pro Pro Glu Pro Glu Ala Ser Pro Ser

                                             31G end|
796    CTC TGG GAG ATA GAG TTT GCT AAG CAG TTA GCC TCG GTA GCT GAC CAG CCC TTT GAG AAT
266    Leu Trp Glu Ile Glu Phe Ala Lys Gln Leu Ala Ser Val Ala Asp Gln Pro Phe Glu Asn


856    GGA TTT GAG GAG ATG ATA CAG TGG ACC AAG GAG GGG AAG CTG TGG GAG TTC CCA GTC AAC
286    Gly Phe Glu Glu Met Ile Gln Trp Thr Lys Glu Gly Lys Leu Trp Glu Phe Pro Val Asn


916    AAT GAA GCA GGT TTA GAT GAC GAT GGC TCC GAA TTC CAT GAG CAT ATA TTT TTG GAT AAG
306    Asn Glu Ala Gly Leu Asp Asp Asp Gly Ser Glu Phe His Glu His Ile Phe Leu Asp Lys


976    TAC CTG GAG GAT TTC CCC AAG CAA GGA CCA ATC CGT CTG TTC ATG GAG CTG GTG ACC TGT
326    Tyr Leu Glu Asp Phe Pro Lys Gln Gly Pro Ile Arg Leu Phe Met Glu Leu Val Thr Cys


1036   GGC CTT TCC AAA AAC CCA TAT CTG AGT GTT AAA CAG AAG GTC GAG CAC ATA GAG TGG TTT
346    Gly Leu Ser Lys Asn Pro Tyr Leu Ser Val Lys Gln Lys Val Glu His Ile Glu Trp Phe


1096   AGA AAT TAT TTT AAT GAA AAA AGA GAT ATT CTC AAA GAA AAT AAT ATA GCG TTC ACT TAA
366    Arg Asn Tyr Phe Asn Glu Lys Arg Asp Ile Leu Lys Glu Asn Asn Ile Ala Phe Thr End


1156   CATCACAAAGAAAAAAATTTTATTTAAACTTGAAGATGTATATTAAAGCTAAATAGAAATTTTATCA
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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 (Î). 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.

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.

The first 25 aa of the NH$_2$ terminus of the deduced 384-aa translation product contained five positively charged amino

Mr (kD)

66-

43-

31-

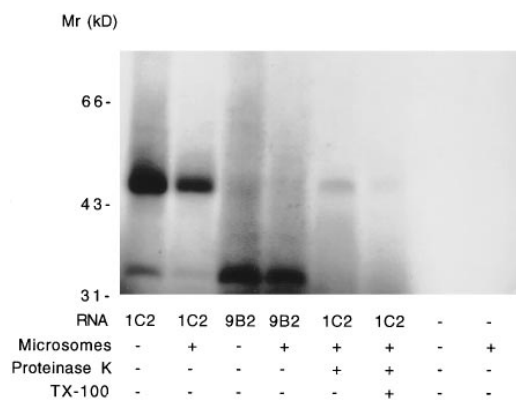| RNA	1C2	1C2	9B2	9B2	1C2	1C2	-	-
Microsomes	-	+	-	+	+	+	-	+
Proteinase K	-	-	-	-	+	+	-	-
TX-100	-	-	-	-	-	+	-	-

Figure 2. In vitro translation of the cloned cDNA. RNA transcripts of clones 1C2 and 9B2 were performed in the presence or absence of dog pancreatic microsomes and subsequently treated with proteinase K in the presence or absence of detergents to determine the extent of protection afforded by translocation into the lumen of the endoplasmic reticulum.

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_{24} Asp_{25}) 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 Thr_{41}, Arg His_{48}, or Arg Ser_{54}.

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 RsaI and AluI sites consistent with the original subtraction cloning procedure (42), which used PCR amplification of doubly digested fragments of βTC3 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 M_r (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 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 $CD4^+$ 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 His-tagged 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-
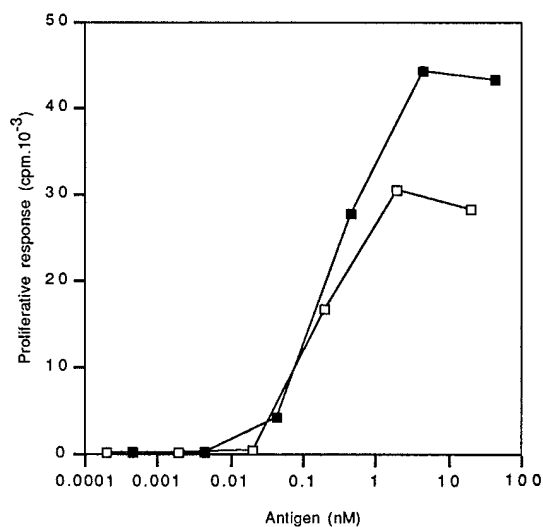

Figure 3. T cell clone 1C6 proliferative response to recombinant antigens. Results are shown for the GST fusion protein of aa 1–315 (□) and a synthetic 20-aa peptide (aa 259–278) (■) bearing the epitope.
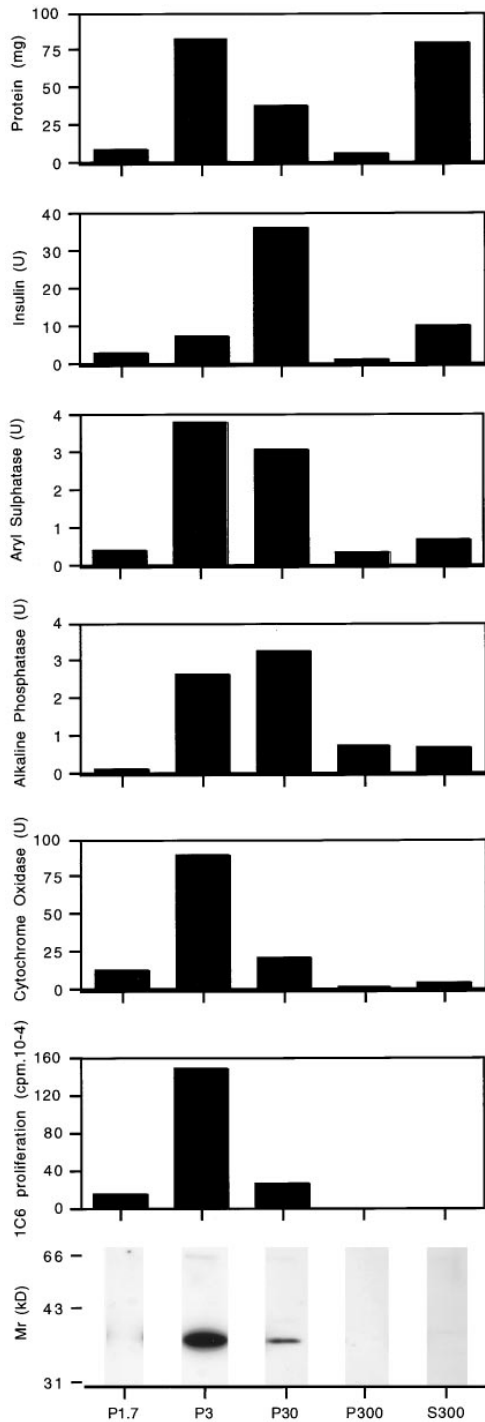
Figure 4. Differential centrifugation analysis of the subcellular distribution of the 38-kD antigen in insulinoma tissue. A postnuclear supernatant from a rat insulinoma homogenate in isotonic medium was subjected to successive centrifugation steps to yield a 1,700-*g* × 10 min pellet (P1.7), a 3,000-*g* × 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 a 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.
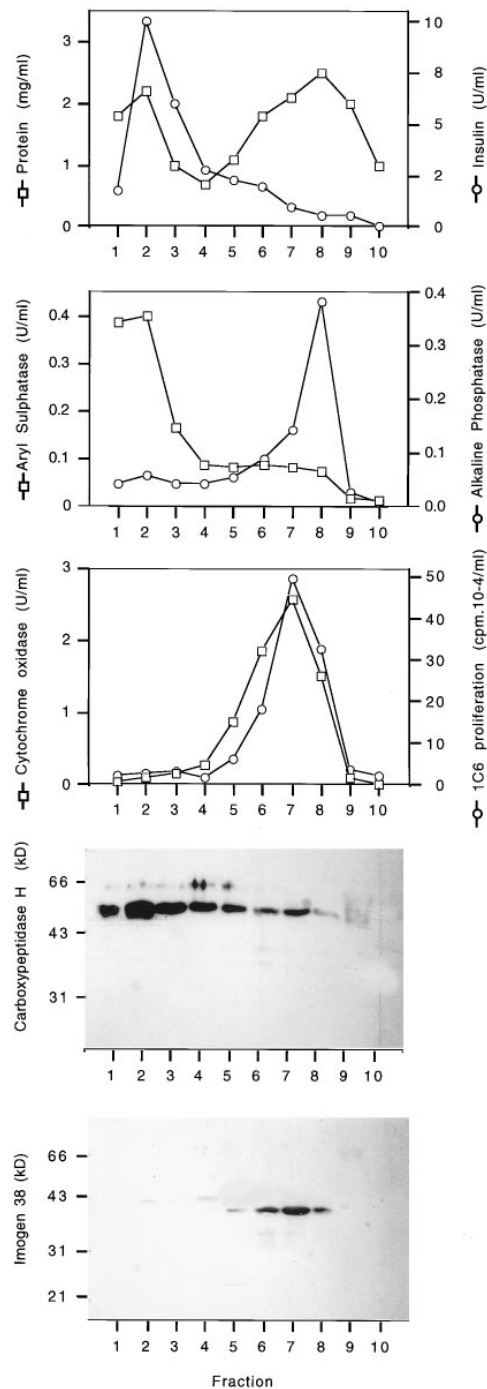

Figure 5. Percoll density gradient centrifugation analysis of the 38-kD antigen in insulinoma tissue. A particulate fraction prepared from insulinoma homogenate at 30,000 *g* for 20 min was subjected to isopycnic density gradient centrifugation and recovered in 10 fractions of decreasing density. Each 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.

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

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

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.

tion prepared at 3,000 *g* for 20 min. Western blot analysis showed the presence of a major immunoreactive 38-kD protein and a minor 60-kD component whose distributions paralleled that of 1C6 reactivity. Marker protein analyses showed that, whereas the distribution of the antigen overlapped with secretory granules, it appeared to be concentrated in organelles of larger size and/or greater density. Its sedimentation behavior was similar to that of cytochrome oxidase, a mitochondrial marker, and differed from that of alkaline phosphatase, a 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 immunoassay) or carboxypeptidase H (determined by Western blotting). The distribution of the antigen overlapped with that of the plasma membrane marker, alkaline phosphatase, though the latter was mostly concentrated in fractions of a lower buoyant density.

Transient transfection of COS 7 cells was achieved using the clone 1C2 inserted under a cytomegalovirus promoter in the vector pCDNA3. The shorter cDNA clone, 9B2 (Fig. 1), which could be translated in vitro to a protein of lower M_r (33 kD) (Fig. 2), was also analyzed, along with mock-transfected cells and cells transfected with an unrelated protein (furin). Nontransfected, mock-transfected, and 9B2-transfected cells showed background reactivity in the 1C6 T cell proliferation assay, whereas 1C2 transfectants produced a response comparable to particulate fractions of rat insulinoma tissue (Table I). Western blot analysis showed the presence of immunoreactive protein in 1C2-transfected cells (Fig. 6), which was indistinguishable in size from the native rat insulinoma protein (38 kD) and clearly smaller in size than the product of translation of the corresponding cRNA in vitro (44 kD) (Fig. 3). Control cells showed much smaller amounts of a 38-kD immunoreactive protein, which, in any case, appeared in the soluble fraction. The low reactivity in controls appears to relate to the antigen content rather than to a lack of species cross-reactivity, since the 1C6 T cell clone is responsive to the antigen in the human islets (Roep, B. O., and J. C. Hutton, unpublished findings).

The appearance of Western blot immunoreactivity in the soluble fraction of 1C2-transfected cells in the absence of significant 1C6 T cell reactivity was an unexpected observation. A mixture of the particulate fraction from 1C2-transfected

cells with the soluble fraction of nontransfected cells (10 μg each) induced the same proliferative response as the particulate fraction alone. Mixture of the soluble fraction from the 1C2-transfected cells with the particulate fraction of nontransfected cells failed to produce a proliferative response. Thus, it appeared unlikely that the soluble fraction contained an inhibitor of the T cell proliferation assay or that the presence of membranes per se enhanced the presentation or T cell reactivity to the antigen.

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.
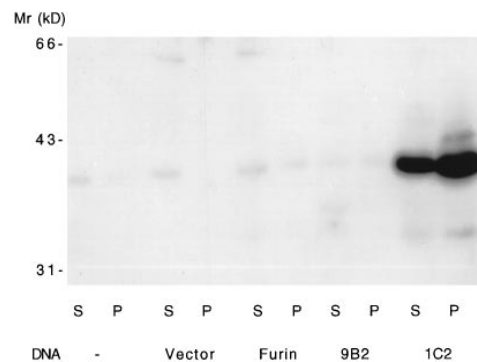

Figure 6. Expression of 38-kD cDNA in transiently transfected COS 7 cells. Transfected cells were cultured for 24–26 h, sonicated in a low-salt buffer, and separated into a soluble (*S*) and particulate (*P*) fraction by centrifugation. Results with untransfected and mock-transfected cells are shown together with cells transfected with an unrelated protein (furin), a truncated cDNA clone 9B2, and the full-length clone 1C2. Samples were separated on 12.5% SDS-PAGE gels for blotting (20 μg protein) and assayed in parallel for 1C6 T cell reactivity (Table I).
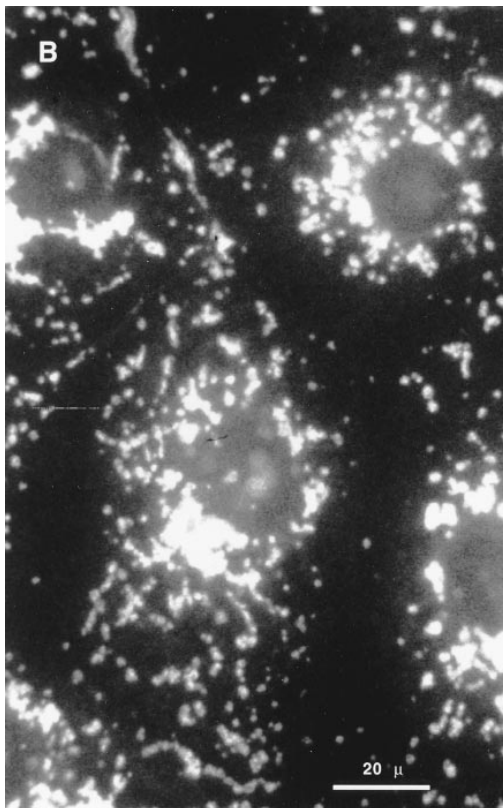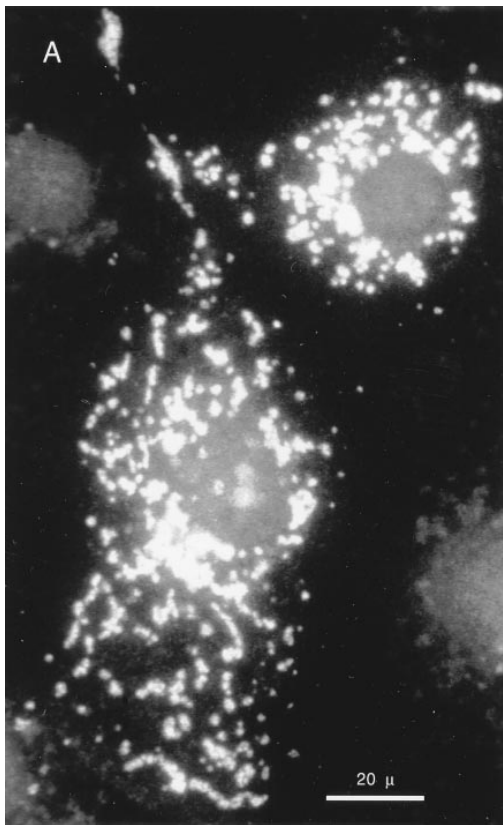
Figure 7. Immunohistochemical localization of the 38-kD antigen. COS 7 cells were transiently transfected with clone 1C2 and developed with rabbit anti–38-kD antibody (*A*) and a mouse mAb to a human mitochondrial protein (*B*). The pattern of immunoreactivity in the positively transfected cells coincides with the mitochondrial marker.

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-
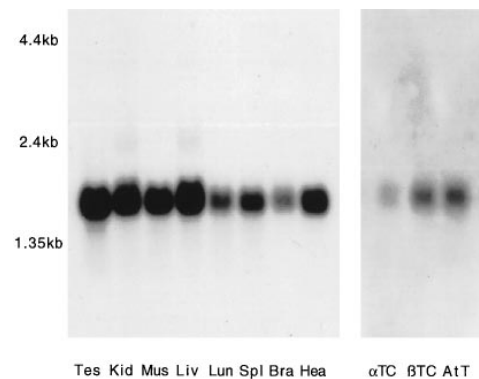

Figure 8. Northern blot analyses. Poly A⁺ mRNA samples (5 μg) prepared from the indicated mouse tissues and cell lines were hybridized to a probe prepared from a 955-bp fragment from clone 1C2. Sample sizes were adjusted in each case to give an equivalent signal to an actin probe.
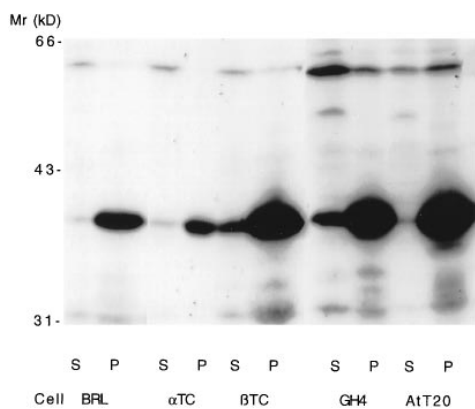
38-kD Diabetic Autoantigen **557**

Figure 9. Western blot analysis of the 38-kD antigen in mouse tissue culture cell lines. Cells were harvested when semiconfluent, sonicated in a low-salt buffer, and separated into a soluble (*S*) and particulate (*P*) fraction by centrifugation. Samples were separated on 12.5% SDS-PAGE gels for blotting (20-μg protein) and assayed in parallel for 1C6 T cell reactivity (Table II).

Figure 10. Western blot analysis of the 38-kD antigen in different mouse tissues. Tissues were pulverized in liquid nitrogen and then sonicated in a low-salt buffer, and a particulate fraction was recovered by centrifugation. Samples were separated on 12.5% SDS-PAGE gels for blotting (20-μg protein) and assayed in parallel for 1C6 T cell reactivity (Table III).

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
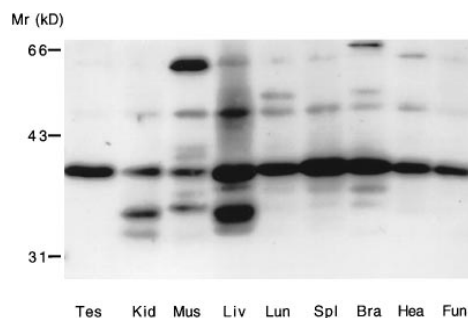
homology of the 70-aa probe sequence with the bacterial superantigens *Streptococcus pyogenes* exotoxin A and *Staphylococcus* entertoxin B ($\sim$ 25% identity over 60 aa) (26) did not extend beyond the immediate region originally identified. Likewise, homologies of the 11-aa minimal epitope with the 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).

Imogen 38 could generally be distinguished from other 38-kD diabetic autoantigens previously described. Its subcellular localization and sequence differ from those of the nuclear transcription factor Jun B, which was cloned from an islet cell expression library by autoantibody screening (24). The observa-

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

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

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

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

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 autoantisera (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$_{25}$ would produce a protein of 41 kD with an estimated pI of 8.0 and expose an NH$_2$-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 IMP1 or the octapeptidyl peptidase–like enzyme (43) to a soluble protein of 39.5 kD and pI of 7.6. Alternatively, the precursor could be processed by the matrix-processing peptidase complex after the Arg Thr$_{41}$ (39.5 kD, pI 7.6), Arg His$_{48}$ (38.6 kD, pI 7.5), or Arg Ser$_{54}$ (38.0 kD, pI 7.1). The last product fits best with the migration of the rat, mouse, or human antigen on SDS-PAGE and with the pI 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 is 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 that they possess potential or ancestral "signal peptide"–like structures within their mitochondrial targeting sequences, suggests a possible molecular mechanism by which a sequestered organellar 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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