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### Research Article

We have identified a novel 69-kD peptide autoantigen (ICA69) associated with insulin-dependent diabetes mellitus (IDDM) by screening a human islet lambda gt11 cDNA expression library with cytoplasmic islet cell antibody positive sera from relatives of IDDM patients who progressed to the overt disease. The deduced open reading frame of the ICA69 cDNA predicts a 483-amino acid protein. ICA69 shows no nucleotide or amino acid sequence relation to any known sequence in GenBank, except for two short regions of similarity with BSA. The ICA69 cDNA probe hybridizes with a 2-kb mRNA in poly(A<sup>+</sup>) RNA from human pancreas, brain, heart, thyroid, and kidney, but not with skeletal muscle, placenta, spleen, or ovary. Expression of ICA69 was also detected in beta cells and cell lines, as well as in tumoral tissue of islet cell origin. The native ICA69 molecule migrates to 69 kD in SDS-PAGE as detected with specific antibodies. Serum samples from relatives of IDDM patients specifically reacted with affinity-purified recombinant ICA69 on Western blotting. The structural gene for ICA69 was designated ICA1. A homologue in the mouse, designated Ica-1 was mapped to the proximal end of chromosome 6 (within 6 cM of the Met protooncogene). ICA69 adds a novel autoantigen to the family of identified islet target molecules, and by the manner of its identification and characterization large amounts of antigen [...]

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# Islet Cell Autoantigen 69 kD (ICA69)

## Molecular Cloning and Characterization of a Novel Diabetes-associated Autoantigen

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### Abstract

We have identified a novel 69-kD peptide autoantigen (ICA69) associated with insulin-dependent diabetes mellitus (IDDM) by screening a human islet  $\lambda$ gt11 cDNA expression library with cytoplasmic islet cell antibody positive sera from relatives of IDDM patients who progressed to the overt disease. The deduced open reading frame of the ICA69 cDNA predicts a 483-amino acid protein. ICA69 shows no nucleotide or amino acid sequence relation to any known sequence in GenBank, except for two short regions of similarity with BSA. The ICA69 cDNA probe hybridizes with a 2-kb mRNA in poly(A<sup>+</sup>) RNA from human pancreas, brain, heart, thyroid, and kidney, but not with skeletal muscle, placenta, spleen, or ovary. Expression of ICA69 was also detected in  $\beta$  cells and cell lines, as well as in tumoral tissue of islet cell origin. The native ICA69 molecule migrates to 69 kD in SDS-PAGE as detected with specific antibodies. Serum samples from relatives of IDDM patients specifically reacted with affinity-purified recombinant ICA69 on Western blotting. The structural gene for ICA69 was designated *ICA1*. A homologue in the mouse, designated *Ica-1* was mapped to the proximal end of chromosome 6 (within 6 cM of the Met protooncogene). ICA69 adds a novel autoantigen to the family of identified islet target molecules, and by the manner of its identification and characterization large amounts of antigen are available for development of quantitative, convenient predictive assays for autoantibodies and analysis of the role of this molecule in diabetes autoimmunity, as well as its physiologic function. (*J. Clin. Invest.* 1993. 92:359–371.) **Key words:** autoantigens • preclinical diabetes • molecular cloning • autoimmunity • ICA69

### Introduction

There is evidence that insulin-dependent diabetes mellitus (IDDM)<sup>1</sup> is a chronic autoimmune disease in which the pres-

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1. *Abbreviations used in this paper:* APAAP, alkaline phosphatase-antialkaline phosphatase; GAD, glutamic acid decarboxylase; ICA, islet cell antibodies; IDDM, insulin-dependent diabetes mellitus; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; LB, Luria-Bertani; PAP, peroxidase antiperoxidase; pfu, plaque-forming units; RFLV, restriction length fragment variation.

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ence of autoantibodies, such as cytoplasmic islet cell antibodies or insulin autoantibodies, can be present years before the clinical onset of the disease (1). A common feature of type I diabetes and other autoimmune diseases is a humoral immune response characterized by the appearance of autoantibodies against cellular proteins, including islet peptides (2–4). Although all the target antigens in type I diabetes have not been identified, several autoantigens associated with the disease have been molecularly characterized using different experimental approaches, namely insulin (5), glutamic acid decarboxylase (GAD) (6), carboxypeptidase H (7), as well as the glycolipids GT3 (8) and GM2-1 (9). Recently, cDNA encoding for a fragment of carboxypeptidase H (7), a granule-associated enzyme, has been reported to react with sera from prediabetic patients and another peptide expressed in a  $\lambda$ gt11 phage from a human islet library appears to be recognized by IDDM sera (10). Cellular proteins of unknown sequence whose molecular masses are 38 (11), 52 (12), and 69 kD (13), have also been reported to be recognized by a humoral and/or a cellular immune response. It is of interest that almost all patients with type I diabetes have elevated levels of IgG anti-BSA antibodies related to a 69,000- $M_r$  islet peptide, which may represent a target antigen for cow milk-induced islet autoimmunity (14, 15).

The present study was undertaken to isolate clones that code for some of these or other unidentified autoantigens and characterize their molecular structure. Isolation of cDNA clones expressing antigenic determinants has been extensively used to identify clones coding for autoantigens in different autoimmune diseases (16). This approach offers the possibility of identifying and characterizing novel autoantigens that may be of restricted cellular distribution as well as low cellular expression (17). Such proteins may not be detected by routine screening tests such as immunofluorescence or immunoprecipitation.

We have used this approach to immunoscreen a human islet  $\lambda$ gt11 expression library with a pool of sera from prediabetic relatives of IDDM patients, identify, sequence the clones, and characterize the expressed proteins. In this report, we describe the cloning of a cDNA that encodes a novel islet autoantigen, whose apparent migration is 69 kD on SDS-polyacrylamide gel chromatography.

### Methods

**Serum samples.** Sera were obtained from first degree relatives of patients with type I diabetes. All of them were at high risk of developing IDDM, and some have already progressed to the overt disease on prospective follow-up. Clinical studies were performed with informed consent, as well as approval from the Joslin Clinic and University of Colorado institutional review boards. All the sera used for the screening of the human islet  $\lambda$ gt11 library expressed high titer of islet cell antibodies (> 80 Juvenile Diabetes Foundation units). The sera were repeatedly absorbed with a protein lysate of a wild  $\lambda$ gt11 phage-infected *Esche-*

*richia coli* strain Y1090 (18) to remove anti-*E. coli* antibodies. Absorbed antibodies were stored at  $-20^{\circ}\text{C}$  in the presence of 0.05% sodium azide until used for immunological screening. Originally, a pool of three sera was used to identify positive clones, and subsequently sera of three other relatives were studied for reactivity with the positive clone. 10 sera of normal individuals were also tested for reactivity with the positive clone. Sera from additional prediabetic relatives (subjects followed to diabetes onset) ( $n = 23$ ), autoantibody positive but currently nondiabetic relatives ( $n = 31$ ), and normal controls ( $n = 70$ ) were tested for reactivity to the expressed molecule on Western blots.

***λgt11 expression libraries.*** Two *λgt11* libraries were used, a human islet library provided by Dr. Alan Permutt (Washington University, St. Louis, MO) and a human insulinoma library generated by Alvin C. Powers (Vanderbilt University, Nashville, TN). A human *λgt11* islet library was constructed from human islet poly(A<sup>+</sup>) mRNA by Clontech (Palo Alto, CA), with  $\sim 1 \times 10^9$  plaque-forming units (pfu)/ml and 85% being recombinants. A human insulinoma library was generated from insulinoma poly(A<sup>+</sup>) mRNA and then cDNA was produced and packaged into the *λgt11* phage (19), with  $\sim 1.3 \times 10^9$  pfu/ml and a recombinant rate of more than 80%.

***Screening of λgt11 expression libraries with antibody and cDNA probes.*** A phage human islet *λgt11* expression library was screened with a pool of sera from preclinical IDDM relatives (20). Isolated recombinant phages were plated on Luria-Bertani (LB) agar plates (150 mm diameter) with *E. coli* strain Y1090 at  $\sim 0.5\text{--}1 \times 10^4$  pfu/plate. After a 3-h incubation at  $42^{\circ}\text{C}$ , a nitrocellulose filter (Schleicher & Schuell, Keene, NH) saturated with 10 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) (BRL, Grand Island, NY) was overlaid on the agar overnight at  $37^{\circ}\text{C}$  to induce the expression of  $\beta$ -galactosidase fusion proteins. After that, the filters were blocked with 1% BSA (Sigma Immunochemicals, St. Louis, MO) in Tris-buffered saline (TBS), incubated containing 0.05% Tween, incubated for 2 h at room temperature, and then incubated with 1/500 diluted sera overnight at  $4^{\circ}\text{C}$ . After several washes with TBS, the bound antibodies were detected by incubation with anti-human IgG alkaline phosphatase (Cappel Laboratories, Durham, NC) diluted 1/100 (2 h at room temperature). A phage human islet *λgt11* expression library was initially screened with pooled sera from three prediabetics. The original positive plaque was replated and rescreened sequentially until all progeny of plaques were recognized by the sera. To determine whether prediabetic sera or controls reacted with the product of the clone and to reduce the possibilities of false positivity, plaque-purified recombinant bacteriophage was mixed  $\sim 1:1$  with a wild-type *λgt11* and plated with *E. coli* Y1090 as for screening. Pieces of nitrocellulose carrying plaque proteins were then incubated with individual sera. Reactions were considered positive if significant staining of  $\sim 50\%$  of the plaques was observed. Intensity of staining was estimated to score reactivity of individual sera on a 0 (negative) to 4+ (strongest) scale. The cDNA insert of the original positive clone, termed PM1/1 (Fig. 1 B), was used as probe to further screen the human islet library and a human insulinoma library by plaque hybridization (21) to obtain several longer and overlapping cDNA clones. The probe was labeled with [ $\alpha^{32}\text{P}$ ]dCTP by random priming (21, 22) using Klenow fragment (Amersham Corp., Arlington Heights, IL) and used to rescreen the libraries.

***Amplification of λgt11 cDNA insert and cloning.*** The *λgt11* cDNA insert from the positive clones was amplified by PCR (23, 24) using *λgt11* primers complementary to the  $\beta$ -galactosidase portion of the *λgt11* template (primer 1218: 5'-GGTGGCGACGACTCTGGAGC-CCG-3'; primer 1222: 5'-TTGACACCAGACCAACTGGTAATG-3', New England Biolabs, Beverly, MA). Reaction mixtures for PCR (0.1 ml) contained cDNA template, 100 pmol each of the primers, and 2.5 U of *Taq* I DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT) in 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub> containing dNTPs at 0.2 mM each and 0.01% gelatin. Reactions were carried out in a thermal cycler (Perkin-Elmer Cetus) for 30 cycles of denaturation ( $92^{\circ}\text{C}$ , 1 min), annealing ( $60^{\circ}\text{C}$ , 1.5 min), and elongation ( $72^{\circ}\text{C}$ , 1 min). After *Eco* RI digestion and fractionation on 1% agarose gel stained with ethidium bromide to visualize the PCR products, the product of interest was excised, purified, and subcloned into

the *Eco* RI site of pBluescript II vector. This vector was used to transform *E. coli* strain XL1 Blue, and to sequence the PCR products across its polylinker arms (Stratagene, La Jolla, CA). cDNA samples for PCR were obtained from phage suspension.

***DNA sequencing and computer analysis of nucleic acid and protein sequences.*** Nucleotide sequences were determined by using the dideoxynucleotide chain termination method of Sanger et al. (25), using T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH). To avoid compression in G + C-rich sequences, additional sequencing reactions were performed with dTTP alternating with dGTP (26).

Sequences were aligned and analyzed using the EUGENE, SAM, PIMA.SH, and PROSITE programs. The GenBank (DNA and Amino Acid Databank) was searched for similarities, and the PLSEARCH program analyzed for protein sequence patterns derived from the sequences of homologous protein families (Molecular Biology Computing Research Resource, Dana Farber Cancer Institute, and Harvard School of Public Health, Cambridge, MA). Hydropathy plots from the deduced amino acid sequence were prepared as described by Kyte and Doolittle (27, 28) and Klein et al. (29).

***Cell lines.*** Cells were used at late log phase, when almost all were viable. RIN 1046-38, derived from a rat insulinoma (kindly provided by Christopher Newgard, Southwestern Medical Center, University of Texas, Dallas, TX), were cultured in DME supplemented with 10% FBS, and 5.6 mM glucose in a humidified atmosphere of 10% CO<sub>2</sub>/90% air at  $37^{\circ}\text{C}$  (30).  $\beta\text{TC-1}$  and  $\alpha\text{TC-1}$  were derived from progeny of transgenic mice expressing SV40 large T-antigen under control of the rat insulin II 5'-flanking region or rat preproglucagon 5'-flanking region respectively (31-33). The  $\beta\text{TC-1}$  and  $\alpha\text{TC-6}$  cell lines were maintained in DME supplemented to a final concentration of 16.5 mM glucose and supplemented with Eagle's MEM, nonessential amino acids component, 44 mM sodium bicarbonate, 15 mM HEPES, 50  $\mu\text{g/l}$  gentamicin sulphate, and 10% heat-inactivated FBS in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. HIT cells, derived from a hamster insulin producing cell line (34), were grown in 5% CO<sub>2</sub>/95% air in RPMI 1640 medium containing 10% FCS and 11.1 mM glucose. HeLa cells (ATCC CCL 2.2; American Type Culture Collection, Rockville, MD) (35), JEG cells (human choriocarcinoma; ATCC HTB 36) (36), and HepG2 cells (human hepatoma; ATCC HB 8065) (37) were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine and 5  $\mu\text{g/ml}$  gentamicin sulfate in 10% CO<sub>2</sub>/90% air incubator. A human islet carcinoid cell line designated BON-1 (provided by Dr. Cortney Townsend, Department of Surgery, University of Texas Medical Branch, Galveston, TX) was maintained in DME with 10% heat-inactivated FCS and 5.6 mM glucose in a humidified atmosphere of 10% CO<sub>2</sub>/90% air.

***RNA isolation and Northern analysis.*** Total RNAs and poly(A<sup>+</sup>) RNAs from various tissues and cell lines were prepared by the guanidinium isothiocyanate method, enriched for the polyadenylated (poly-A) fraction with an oligo(dT)-cellulose column and analyzed on Northern blots according to standard procedures (38). The hybridization was carried out for 18 h at  $42^{\circ}\text{C}$  in the prehybridization buffer (50% formamide, 5 $\times$  SSPE [1 $\times$  SSPE consists of 150 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA, pH 7.4]), 5 $\times$  Denhardt's solution, 100  $\mu\text{g/ml}$  denatured salmon sperm DNA, and 0.1% SDS) (18) containing [ $\alpha^{32}\text{P}$ ]dCTP labeled cDNA purified probe. The probes consisted of either a 0.95-kb fragment from the original PM1/1 positive clone identified, or a 1.78-kb *gt11* insert from an overlapping clone; 100 ng of each probe was labeled by the random priming method. 100 ng of a 2-kb human  $\beta$ -actin cDNA was used as control probe (39). The fresh hybridization solution contained the denatured radiolabeled DNA probes at a concentration of  $2\text{--}4 \times 10^6$  cpm/ml with a specific activity  $\geq 5 \times 10^8$  cpm/ $\mu\text{g}$  (18, 40). The nitrocellulose filters were washed in three changes of 2 $\times$  SSC and 0.05% SDS at room temperature each time. The final three washes were carried out in 0.1 $\times$  SSC and 0.1% SDS from room temperature to  $65^{\circ}\text{C}$  depending upon the stringency conditions required for each experiment. Filters were exposed to Kodak film at  $-80^{\circ}\text{C}$  with intensifying screens. Ribosomal bands were used as size markers (41, 42).

*Preparation of anti-ICA69 antibodies from synthetic peptides and from the purified molecule.* Rabbit antibodies were produced using synthetic peptides from the deduced amino acid sequence as well as the ICA69 recombinant expressed molecule. Rabbits were immunized in order to generate antibodies against specific domains (28, 43). Two regions of the molecule, one corresponding to the COOH terminus, residues 471–483: GKTDKEHELLNA, and one to an internal polypeptide close to the COOH terminus, residues 458–470: ADLDPLSNPDAV, and the serum generated against the whole molecule, were used and found to yield antisera which reacted with the native ICA69 molecule on Western blots (44). The synthetic polypeptides were coupled to a carrier protein, keyhole limpet hemocyanin linked to bromoacetyl bromide. Five female New Zealand white rabbits were immunized with 1 mg of the keyhole limpet hemocyanin-peptide conjugate suspended in 1 ml of complete Freund's adjuvant. Rabbits were boosted three times with 1 mg of the specific polypeptide in incomplete Freund's adjuvant at 30-d intervals and serum samples were collected and stored in aliquots at  $-20^{\circ}\text{C}$ . An ELISA was used to detect specific anti-peptide antibodies.

*Indirect ELISA.* Indirect ELISA was performed for the detection of specific antibodies generated in rabbits against ICA69 polypeptides (45). 1  $\mu\text{g}$  of specific polypeptide was used to coat each well of a microtiter plate (Immulon; Dymatech Laboratories, Inc., Chantilly, VA) (46), and after blocking residual binding of the plate with a PBS solution containing 1% BSA for 2 h, appropriate dilutions of rabbit pre- and postimmune sera were added to each well (1:100–1:32,000) and incubated overnight. All dilutions were tested in triplicate. After washing away unbound antibodies, a solution containing anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma Immunochemicals) as developing reagent was added to the wells. After 2 h incubation, unbound conjugate was washed away and a substrate solution (*o*-phenylenediamine dihydrochloride) (Sigma Immunochemicals), was added. A specific hyperimmune serum raised to another polypeptide (PEP-80, of the IRS-1 molecule kindly provided by Dr. M. White, Joslin Diabetes Center) (47) and preimmune sera from normal rabbits were used in each assay as positive and negative controls respectively. The optical density of the solutions in the wells was measured with a spectrophotometer through a 405-nm filter.

*SDS-PAGE and immunoblotting.* Cell line extracts and total homogenates of rat brain tissues were prepared as described by Laemmli (48). Cell line extracts and total-homogenate proteins were separated by SDS-PAGE using a constant voltage of 180 V for 4 h through stacking and the resolving gel. Bromophenol blue was included in the sample buffer to visualize buffer front. A mixture of individually colored and purified proteins were used as protein standards (Rainbow<sup>®</sup> Protein Molecular Weight Markers, Amersham Corp.): myosin, mol wt 200,000, blue; phosphorylase b, mol wt 97,400, brown; BSA, mol wt 69,000, red; ovalbumin, mol wt 46,000, yellow; carbonic anhydrase, mol wt 30,000, orange; trypsin inhibitor, mol wt 21,000, green; and lysozyme, mol wt 14,300, magenta. Homogenate protein concentrations were determined by Lowry's method (Pierce Chemical Co., Rockford, IL) and 4–50  $\mu\text{g}$  of proteins per lane (depending on the size of the PAGE) were run on a 10% SDS-PAGE under reducing conditions. Proteins were then transferred onto nitrocellulose according to Towbin et al. (49) in transfer buffer (12.5 mM Tris, 96 mM glycine, 20% methanol) for 1 h on a semi-dry electrophoretic transfer cell at 15 V. The nitrocellulose was cut into strips, and incubated for 2 h at  $37^{\circ}\text{C}$  in 5% (wt/vol) nonfat dried milk diluted in PBS (Blotto buffer) to block the nonspecific binding sites. The nitrocellulose strips were then incubated with a 1:100 dilution of a rabbit anti-ICA69 antiserum and then washed in 5% (wt/vol) nonfat dried milk diluted in PBS adding Tween 20 to a final concentration of 0.01%. After incubation of the filters at room temperature for 2 h with  $^{125}\text{I}$ -Protein A (Amersham Corp.) to detect the rabbit anti-ICA69 antibodies, unbound  $^{125}\text{I}$ -Protein A was removed by washing as described above. Blots were exposed to Kodak film at  $-80^{\circ}\text{C}$  with intensifying screens for 12–24 h.

*Expression of the recombinant ICA69.* PM1/3 clone cDNA was amplified by PCR. The PCR product was generated using a primer spanning the PM1/3 start codon and encoding the first eight amino

acids (5'-TCAGGACACAAATGCAGTTATCCC-3'), and a primer containing the codon sequence for the last seven amino acids, a translational stop codon and a HindIII restriction site (5'-TTTAAGCTTCA-TGCATTGAGCAATTCGTGTTCC-3'). The pMAL-c vector (50, 51), which encodes for maltose binding protein as product of the *maltE* gene, was cut with StuI and HindIII restriction enzymes and ligated with the PM1/3 PCR product. The constructs were then transfected into the appropriate *E. coli*, strain TB1 (52). Ampicillin-resistant colonies were grown overnight in 3 ml LB medium containing 100  $\mu\text{g}/\text{ml}$  ampicillin. 100  $\mu\text{l}$  of TB1 pMAL-c-PM1/3 transformants were diluted in 1 ml LB/ampicillin medium and grown 1 h at  $37^{\circ}\text{C}$  followed by induction with 1 mM IPTG for 2 h. Lysates were prepared by centrifugation of 200  $\mu\text{l}$  bacterial cultures for 1 min and boiling the cell pellet with 50  $\mu\text{l}$  SDS sample buffer with 5% mercaptoethanol. After SDS-PAGE of lysates (10  $\mu\text{l}$ ) with and without IPTG induction, gels were stained with Coomassie blue. One colony expressing a protein whose molecular mass migrated  $\sim 105$  kD was identified, and the correct size of the insert was confirmed by restriction analysis.

Another vector system was used to express the purified recombinant ICA69 protein without maltose binding protein. The coding region of the PM1/3 cDNA clone, was amplified by PCR using the primers 5'-GAAGGATCCATGTCAGGACACAAATGCAG-3' and 5'-GGTCTCGAGTCATGCATTGAGCAATTCGTG-3' and cloned into the BamHI and XhoI sites of the expression vector pTrc99A (His<sub>6</sub>). This vector was constructed by insertion of a synthetic DNA fragment encoding six histidines ([CAC]<sub>6</sub>) into the polylinker of pTrc 99A (53). Recombinant proteins were tagged with six histidine residues at the NH<sub>2</sub> terminus. The plasmid construct was transformed into *E. coli*-Tg1 and protein expression was induced by the addition of IPTG to the culture medium. After 2 h at  $37^{\circ}\text{C}$ , bacteria were lysed in 100 mM Tris pH 8.0, 6 M GuHCl, and 10 mM DTT, and insoluble material was removed by centrifugation at 40,000 *g* for 30 min. Recombinant (His)<sub>6</sub>-ICA69 was purified using Ni-NTA-agarose (Qiagen, Chatsworth, CA) in the presence of 6 M GuHCl, 1 mM DTT buffer. The correct size of the ICA69 cDNA in the vector was confirmed by sequencing.

Lysates containing ICA69 and maltose binding protein fusion protein, as well as the purified recombinant ICA69, have been used as source for performing Western blots with control sera such as rabbit anti-ICA69 sera (pre- and postimmune), human control sera, and prediabetic sera at a dilution 1:100. Optical density of the bands corresponding to the ICA69 fusion protein and the affinity-purified ICA69 has been evaluated to quantitate the reactivity of the serum samples to ICA69 using a video densitometer (Bio Rad Laboratories, Hercules, CA), and the results were expressed as relative densitometric units.

*Immunohistochemistry.* Immunohistochemistry has been performed in formalin fixed rat pancreas paraffin embedded sections (4  $\mu\text{m}$  thickness). A double immunoenzymatic labeling of rat islet cellular constituents has been performed (54, 55) using as detection system horseradish peroxidase antiperoxidase (PAP) and alkaline phosphatase-antialkaline phosphatase (APAAP). The PAP immune complex served for the identification of ICA69, whereas the APAAP complex for the identification of insulin, glucagon and somatostatin. After removal of paraffin and rehydration of tissue, the pancreas sections were first treated with an hydrogen peroxidase solution to suppress possible endogenous peroxidase activity. This was followed by an incubation with normal serum to quench nonspecific protein binding to certain tissue elements, and then the sections were incubated with a primary antibody mixture (rabbit anti-ICA69 antibody generated to the whole molecule, and a mouse mAb generated insulin [HPI-005] to glucagon [GLU 001], or anti-ICA69 and a mouse mAb generated to somatostatin [SOM 018] [Novo Nordisk, Denmark]) for 30 min at room temperature. Unbound antibodies were washed with TBS. Antibody to target antigens (primary antibody), antibody to the primary antibody (link antibody: swine anti-rabbit for ICA69, or goat anti-mouse for antiinsulin, antiglucagon or antisomatostatin mAbs), APAAP and PAP reagents (Dako Corp., Santa Barbara, CA) were applied sequentially for simultaneous double staining. The color development was stopped by washing the slides thoroughly in deionized water.

**a**

CGGGCGGGGATACCCAGGAGATGGGGTTCGAGGAGAGACCCCGGGGAGTAGAGAGAGAGAACTCACTCCCGAGTCCCGACCCCTCCCAAG -178  
CAAGGTTAATAATACTTATCTCTCATGCTTTTTCCTGCCCTTCTCCCAAATCATCAACAATAGAAGAAGAAGAAAAC ATG TCA GGA CAC 12  
Met Ser Gly His 4

AAA TGC AGT TAT CCC TGG GAC TTA CAG GAT CGA TAT GCT CAA GAT AAG TCA GTT GTA AAT AAG ATG CAA CAG AGA 87  
Lys Cys Ser Tyr Pro Trp Asp Leu Gln Asp Arg Tyr Ala Gln Asp Lys Ser Val Val Asn Lys Met Gln Gln Arg 29  
AMD  
TAT TGG GAG ACG AAG CAG GCC TTT ATT AAA GCC ACA GGG AAG AAG GAA GAT GAA CAT GTT GTT GCC TCT GAC GCG 162  
Tyr Trp Glu Thr Lys Gln Ala Phe Ile Lys Ala Thr Gly Lys Lys Glu Asp Glu His Val Val Ala Ser Asp Ala 54  
GAC CTG GAT GCC AAG CTA GAG CTG TTT CAT TCA ATT CAG AGA ACC TGT CTG GAC TTA TCG AAA GCA ATT GTA CTC 237  
Asp Leu Asp Ala Lys Leu Glu Leu Phe His Ser Ile Gln Arg Thr Cys Leu Asp Leu Ser Lys Ala Ile Val Leu 79  
CK2  
TAT CAA AAG AGG ATA TGT TTC TTG TCT CAA GAA GAA AAC GAA CTG GGA AAA TTT CTT CGA TCC CAA GGT TTC CAA 312  
Tyr Gln Lys Arg Ile Cys Phe Leu Ser Gln Glu Glu Asn Glu Leu Gly Lys Phe Leu Arg Ser Gln Gly Phe Gln 104  
PKC  
GAT AAA ACC AGA GCA GGA AAG ATG ATG CAA GCG ACA GGA AAG GCC CTC TGC TTT TCT TCC CAG CAA AGG TTG GCC 387  
Asp Lys Thr Arg Ala Gly Lys Met Met Gln Ala Thr Gly Lys Ala Leu Cys Phe Ser Ser Gln Gln Arg Leu Ala 129  
PKC  
TTA CGA AAT CCT TTG TGT CGA TTT CAC CAA GAA GTG GAG ACT TTT CGG CAT CGG GCC ATC TCA GAT ACT TGG CTG 462  
Leu Arg Asn Pro Leu Cys Arg Phe His Gln Glu Val Glu Thr Phe Arg His Arg Ala Ile Ser Asp Thr Trp Leu 154  
ACG GTG AAC CGC ATG GAA CAG TGC AGG ACG GAA TAT AGA GGA GCA CTA TTA TGG ATG AAG GAC GTG TCT CAG GAG 537  
Thr Val Asn Arg Met Glu Gln Cys Arg Thr Glu Tyr Arg Gly Ala Leu Leu Trp Met Lys Asp Val Ser Gln Glu 179  
CTT GAT CCA GAC CTC TAC AAG CAA ATG GAG AAG TTC AGG AAG GTG CAA ACA CAA GTG CGC CTT GCA AAA AAA AAC 612  
Leu Asp Pro Asp Leu Tyr Lys Gln Met Glu Lys Phe Arg Lys Val Gln Thr Gln Val Arg Leu Ala Lys Lys Asn 204

TTT GAC AAA TTG AAG ATG GAT GTG TGT CAA AAA GTG GAT CTT CTT GGA GCG AGC AGA TGC AAT CTC TTG TCT CAC 687  
Phe Asp Lys Leu Lys Met Asp Val Cys Gln Lys Val Asp Leu Leu Gly Ala Ser Arg Cys Asn Leu Leu Ser His 229

ATG CTA GCA ACA TAC CAG ACC ACT CTG CTT CAT TTT TGG GAG AAA ACT TCT CAC ACT ATG GCA GCC ATC CAT GAG 762  
Met Leu Ala Thr Tyr Gln Thr Thr Leu Leu His Phe Trp Glu Lys Thr Ser His Thr Met Ala Ala Ile His Glu 254  
PKC  
AGT TTC AAA GGT TAT CAA CCA TAT GAA TTT ACT ACT TTA AAG AGC TTA CAA GAC CCT ATG AAA AAA TTA GTT GAG 837  
Ser Phe Lys Gly Tyr Gln Pro Tyr Glu Phe Thr Thr Leu Lys Ser Leu Gln Asp Pro Met Lys Lys Leu Val Glu 279  
CK2  
AAA GAA GAG AAG AAG AAA ATC AAC CAG CAG GAA AGT ACA GAT GCA GCC GTG CAG GAG CCG AGC CAA TTA ATT TCA 912  
Lys Glu Glu Lys Lys Lys Ile Asn Gln Gln Glu Ser Thr Asp Ala Ala Val Gln Glu Pro Ser Gln Leu Ile Ser 304  
AMP PKC  
TTA GAG GAA GAA AAC CAG CGC AAG GAA TCC TCT AGT TTT AAG ACT GAA GAT GGA AAA AGT ATT TTA TCT GCC TTA 987  
Leu Glu Glu Glu Asn Gln Arg Lys Glu Ser Ser Ser Phe Lys Thr Glu Asp Gly Lys Ser Ile Leu Ser Ala Leu 329

GAC AAA GGC TCT ACA CAT ACT GCA TGC TCA GGA CCC ATA GAT GAA CTA TTA GAC ATG AAA TCT GAG GAA GGT GCT 1062  
Asp Lys Gly Ser Thr His Thr Ala Cys Ser Gly Pro Ile Asp Glu Leu Leu Asp Met Lys Ser Glu Glu Gly Ala 354

TGC CTG GGA CCA GTG GCA GGG ACC CCG GAA CCT GAA GGT GCT GAC AAA GAT GAC CTG CTG CTG TTG AGT GAG ATC 1137  
Cys Leu Gly Pro Val Ala Gly Thr Pro Glu Pro Glu Gly Ala Asp Lys Asp Asp Leu Leu Leu Ser Glu Ile 379  
CK2

TTC AAT GCT TCC TCC TTG GAA GAG GGC GAG TTC AGC AAA GAG TGG GCC GCT GTG TTT GGA GAC GGC CAA GTG AAG 1212  
Phe Asn Ala Ser Ser Leu Glu Glu Gly Glu Phe Ser Lys Glu Trp Ala Ala Val Phe Gly Asp Gly Gln Val Lys 404

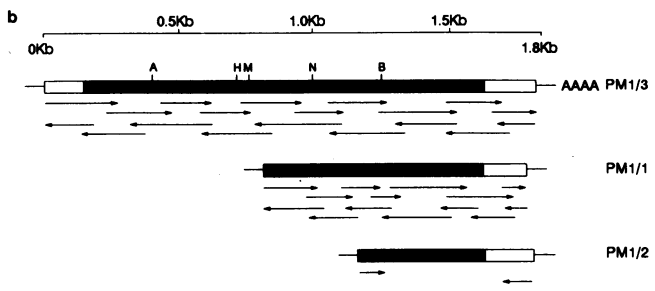
GAG CCA GTG CCC ACT ATG GCC CTG GGA GAG CCA GAC CCC AAG GCC CAG ACA GGC TCA GGT TTC CTT CCT TCG CAG 1287  
Glu Pro Val Pro Thr Met Ala Leu Gly Glu Pro Asp Pro Lys Ala Gln Thr Gly Ser Gly Phe Leu Pro Ser Gln 429  
CK2

CTT TTA GAC CAA AAT ATG AAA GAC TTA CAG GCC TCG CTA CAA GAA CCT GCT AAG GCT GCC TCA GAC CTG ACT GCC 1362  
Leu Leu Asp Gln Asn Met Lys Asp Leu Gln Ala Ser Leu Gln Glu Pro Ala Lys Ala Ala Ser Asp Leu Thr Ala 454  
CK2

TGG TTC AGC CTC TTC GCT GAC CTC GAC CCA CTC TCA AAT CCT GAT GCT GTT GGG AAA ACC GAT AAA GAA CAC GAA 1437  
Trp Phe Ser Leu Phe Ala Asp Leu Asp Pro Leu Ser Asn Pro Asp Ala Val Gly Lys Thr Asp Lys Glu His Glu 479

TTG CTC AAT GCA TGA ATCTGTACCCTTCGGAGGGCACTCACATGCCGCCAGCAGCTCCCTGGGGCTAGCAGAAGTATAAAGTGATCAGT 1531  
Leu Leu Asn Ala END 483

ATGCTGTTTTAATAATTATGTGCCATTTTAAATAAATGAAAGGGTCAACGGCCCTGTTAAAAAATAAAAAAAAAAAAAAAAAA 1607



**Figure 1.** (a) Complete nucleotide and deduced amino acid sequence of the ICA69 molecule. Underlined are the first upstream in frame stop codon (TAA) at nucleotide -75 and the polyadenylation signal 29 bp upstream of the poly(A) tail. Homologous subunits with BSA are in boxes. A potential N-linked glycosylation site is indicated by an asterisk. Potential phosphorylation sites are as follows: PKC (protein kinase C), CK2 (casein kinase II), and AMP (cAMP/cGMP-dependent kinase). A potential amidation site is indicated as AMD. (b) Strategy for sequencing cDNA encoding ICA69 polypeptide and partial restriction map. Sequencing was performed with synthetic oligonucleotide primers, and the direction of sequencing is indicated by arrows. Restriction sites are: A, AccII; B, BglII; H, HgiAI; M, MaeII; and N, NdeI. The poly(A) tail (AAAA) was found in the PM1/3 clone. The coding region is indicated by solid bars and 5' and 3' untranslated regions are represented by empty bars. These sequence data are available from GenBank under accession number L01100.

The sections were then counterstained with Mayer's hematoxylin. Coverslips were mounted with an aqueous mounting medium without alcohol (Glicergel; Dako Corp.).

**Mapping of the mouse homologue of *Ica-1*.** Presence of a homologous locus in the mouse genome (*Ica-1*) was established by analysis of Southern blots of kidney DNA from the NOD (nonobese diabetic) mouse digested with a variety of restriction endonucleases and probed with the PM1/1 cDNA insert according to the procedure described in detail elsewhere (56). An XbaI restriction fragment length variation (RFLV) distinguishing the *Ica-1* locus in the diabetes-susceptible NOD/Lt (~ 8 kb fragment) from the related, but diabetes-resistant NON/Lt strain (~ 11 kb fragment), was used both to assign a chromosomal location and to assess whether a gene conferring susceptibility to IDDM was closely linked to the NOD/Lt allele of *Ica-1*. Segregation of this *Ica-1* RFLV was studied in a panel of kidney DNAs prepared from 19 first backcross diabetic mice from an NOD/Lt × NON/Lt outcross previously typed for other DNA markers (57), plus kidney DNAs from 40 diabetic F2 mice produced in an outcross between NOD/Lt and a diabetes-resistant NON/Lt stock congenic for the diabetogenic *H-2<sup>g7</sup>* haplotype of NOD (NON.NOD-H-2<sup>g7</sup>) (58). A HindIII RFLV distinguished NOD/Lt (~ 8.5 kb fragment) from both NON/Lt and NON-NOD-H-2<sup>g7</sup> (~ 7.8 kb fragment). Comparison of the *Ica-1* segregation pattern with previously typed markers indicated that this locus was linked to the *Met* protooncogene on proximal chromosome 6. To confirm this putative linkage, segregation of *D6Rck2*, *D6Mit1*, and *D6Mit16* was assessed by PCR using the oligonucleotide primer sequences described by Dietrich et al. (59). The recombination frequencies reported represent a weighted average using the information function (60).

**Statistical analysis.** Differences between groups of relatives and controls were analyzed by Wilcoxon rank sum test.

## Results

**Isolation of cDNA clones encoding the ICA69 molecule.** A human islet  $\lambda$ gt11 expression library was immunoscreened with a pool of sera from three prediabetic relatives of IDDM patients, which contained a high titer of cytoplasmic islet cell antibodies. Approximately  $0.4 \times 10^6$  plaques were screened and a single, consistently positive 0.95 kb clone, designated PM1/1, was identified. Fusion protein from the purified clone induced by IPTG, reacted with three out of six ICA positive prediabetic subjects' sera (individually tested at 1:500 dilutions of the sera), whereas no reaction was obtained with 10 control individual sera. A labeled cDNA probe derived from the PM1/1 clone was used to screen both a human  $\lambda$ gt11 islet library and a human insulinoma  $\lambda$ gt11 library to obtain the full-length cDNA. Screening ~  $6.5 \times 10^4$  pfu, two additional hybridizing and overlapping clones were identified from the human islet  $\lambda$ gt11 expression library, both of which retained specificity after secondary and tertiary screening to 100% purity. DNA sequence analysis (see below) confirmed that the clones contained fragments of the same gene.

**DNA sequence.** After PCR amplification and subcloning into pBluescript II vector, partial sequence indicates that the smallest overlapping clone (PM1/2), whose size is 0.6 kb, reveals sequence totally contained within the original sequenced PM1/1 insert (Fig. 1 B). The results of sequencing both cDNA strands of the longest clone (PM1/3), whose size is 1.78 kb, indicates complete identity in the region of the molecule overlapping with the two clones and sequence not contained within the previous clones.

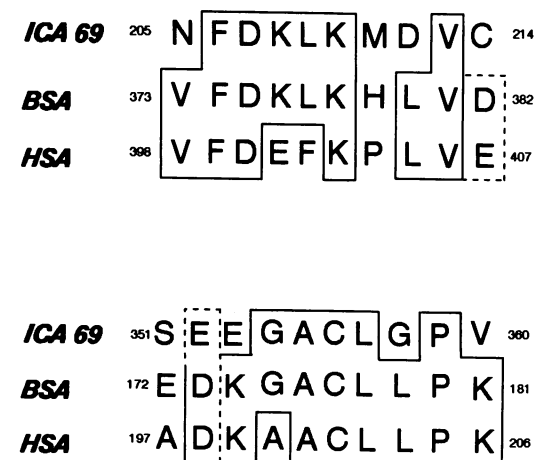
Analysis of the nucleotide sequence 1,785 bp cDNA reveals a 1,449-bp open reading frame coding for 483 amino acids and ending in a poly(A) tail 29 bases downstream of the canonical

polyadenylation signal (AATAAA). Translation of the ICA69 message putatively initiates from the first in frame ATG according to the criteria defined by Kozak (61). Upstream from the first ATG, there is an in frame stop codon (TAA) at -75 bp (Fig. 1 A). The predicted open reading frame from the deduced ATG start codon, codes for a protein with an estimated linear relative molecular mass of 54,600, which contains one potential N-linked glycosylation site.

Using computer programs to search databases of known nucleic acid or amino acid sequences, no significant similarities were found except for minimal homology with BSA, implying that our sequence was new and unique. Two short regions of bovine but not human serum albumin precursor appear to have similarities with the ICA69 molecule (Fig. 2).

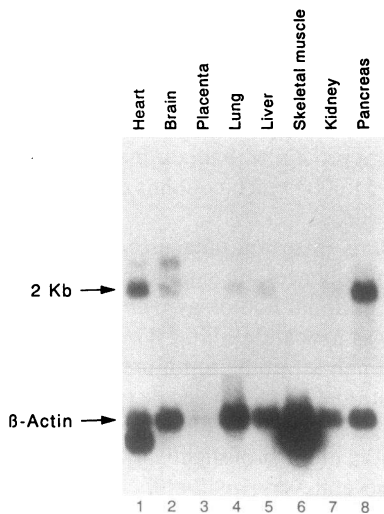
A hydrophobicity plot generated from the ICA69 inferred amino acid sequence reveals a number of slightly hydrophobic regions, alternating with several very hydrophilic segments, which suggests that the molecule does not contain any membrane spanning domain, according to the criteria defined by both Kyte and Doolittle (27, 28) and Klein et al. (29). The segments of hydrophobicity appear not to be long enough to be potential transmembrane-spanning regions. The molecule is predominantly hydrophilic, with 27% of its amino acid residues positively or negatively charged.

**Analysis of ICA69 transcripts in normal and tumor cells.** The cDNA derived from two clones (PM1/1, PM1/3) was used to probe for transcripts in human and animal tissues and in several cell lines by Northern analysis (Figs. 3 and 4). Probes consisting of 0.95 kb (PM1/1) and 1.78 kb (PM1/3) hybridized with a 2-kb mRNA band in islet-derived cell lines, and in some tissues, with a 2.7-kb band. Fig. 3 shows a Northern blot of ICA69 transcripts in human tissues. A 2-kb poly(A<sup>+</sup>) RNA band was detected in abundant amounts in both human pancreas and heart, and then in brain, and in small amounts in lung, liver and kidney, but not in placenta and skeletal muscle. In brain and heart, an additional 2.7-kb band is visible. A 2-kb poly(A<sup>+</sup>) RNA band was also detected in human thyroid, but not in ovary and spleen (not shown). The  $\beta$ -actin control probe hybridized with a 2-kb band in all tissues with different intensity, and with a smaller band (1.6 kb) in both heart and



**Figure 2.** Regions of similarities between ICA69 molecule, BSA, and human serum albumin precursor (HSA). Solid line encloses identical amino acid residues. Dashed line encloses amino acid residues with similar charge. Numbers correspond to the amino acid residue numbers.

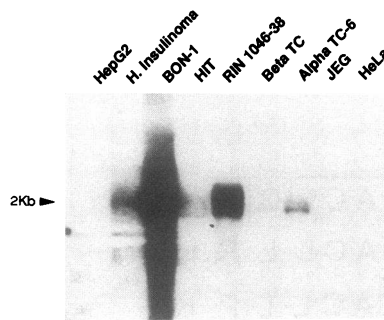




**Figure 3.** Northern blot analysis of poly(A<sup>+</sup>) RNA from different human tissues hybridizing with the 0.95-kb cDNA insert of the PM 1/1 clone. The poly(A<sup>+</sup>) RNA was obtained from Clontech. 2 μg of pure poly(A<sup>+</sup>) RNA from each tissue was applied and resolved on denaturing gel as described under Methods. Lane 1, human heart; lane 2, human brain; lane 3, human placenta; lane 4, human liver; lane 5, human lung; lane 6, human skeletal muscle; lane 7, human kidney; and lane 8, human pancreas. The same blot was rehybridized with a human β-actin cDNA control probe to show that the mRNA was not degraded. Hybridization and washing were performed with identical conditions in the two different experiments. The blot was exposed for 4 h (β-actin cDNA probe) and 24 h (PM1/1 cDNA probe) at -80°C with intensifying screen.

lane 7, human kidney; and lane 8, human pancreas. The same blot was rehybridized with a human β-actin cDNA control probe to show that the mRNA was not degraded. Hybridization and washing were performed with identical conditions in the two different experiments. The blot was exposed for 4 h (β-actin cDNA probe) and 24 h (PM1/1 cDNA probe) at -80°C with intensifying screen.

skeletal muscle. This is because β-actin is not expressed in equal amount in all tissues and because there are two forms of β-actin mRNA in both heart and skeletal muscle (2 and 1.6 kb) (40). The labeled cDNA PM1/1 insert hybridizes with a 2-kb mRNA band in total RNA from rat pancreas, brain, and cerebellum and kidney (in the last three tissues, also with a 5-kb band). In contrast, no ICA69 transcripts were detectable in rat spleen, thymus, bowel, lymph nodes, and salivary gland (not shown). The heterogeneity of mRNA size among tissues may be caused by an alternative splicing of the *ICA1* gene. As shown in Fig. 4, the 2-kb *ICA1* transcript was detected in total RNA from human insulinoma and from a variety of endocrine cell lines, such as a human islet carcinoid cell line (BON-1), a hamster insulin-producing cell line (HIT), and three rodent islet cell lines, namely the rat RIN 1046-38 insulinoma cell line, the mouse βTC-1 (producing primarily insulin, but also some glucagon, and which shows a transcript after longer exposure than shown in Fig. 4), and the mouse αTC-6 (glucagon-



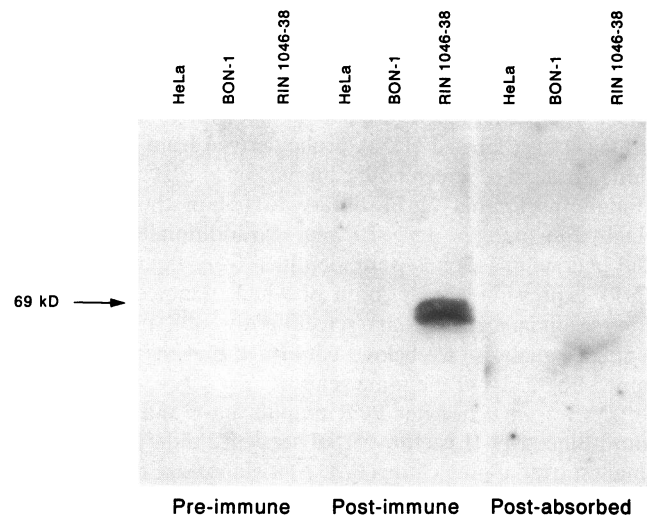
**Figure 4.** Northern blot analysis of ICA69 expression in human insulinoma, and rat, hamster, and mouse transformed cell lines. A 2-kb mRNA is detected in total RNA from human (H) insulinoma, a human islet carcinoid cell line (BON-1), a hamster insulin-producing cell line (HIT), RIN

1046-38, and the mouse islet lines βTC-1 (which is visible after longer exposure), and αTC-6 (a clonal line producing glucagon). No detectable mRNA was found in total RNA from three human nonislet cell lines: HepG2-hepatoma, HeLa-fibroblast, and JEG-choriocarcinoma. Autoradiograph exposure time was 2-7 d.

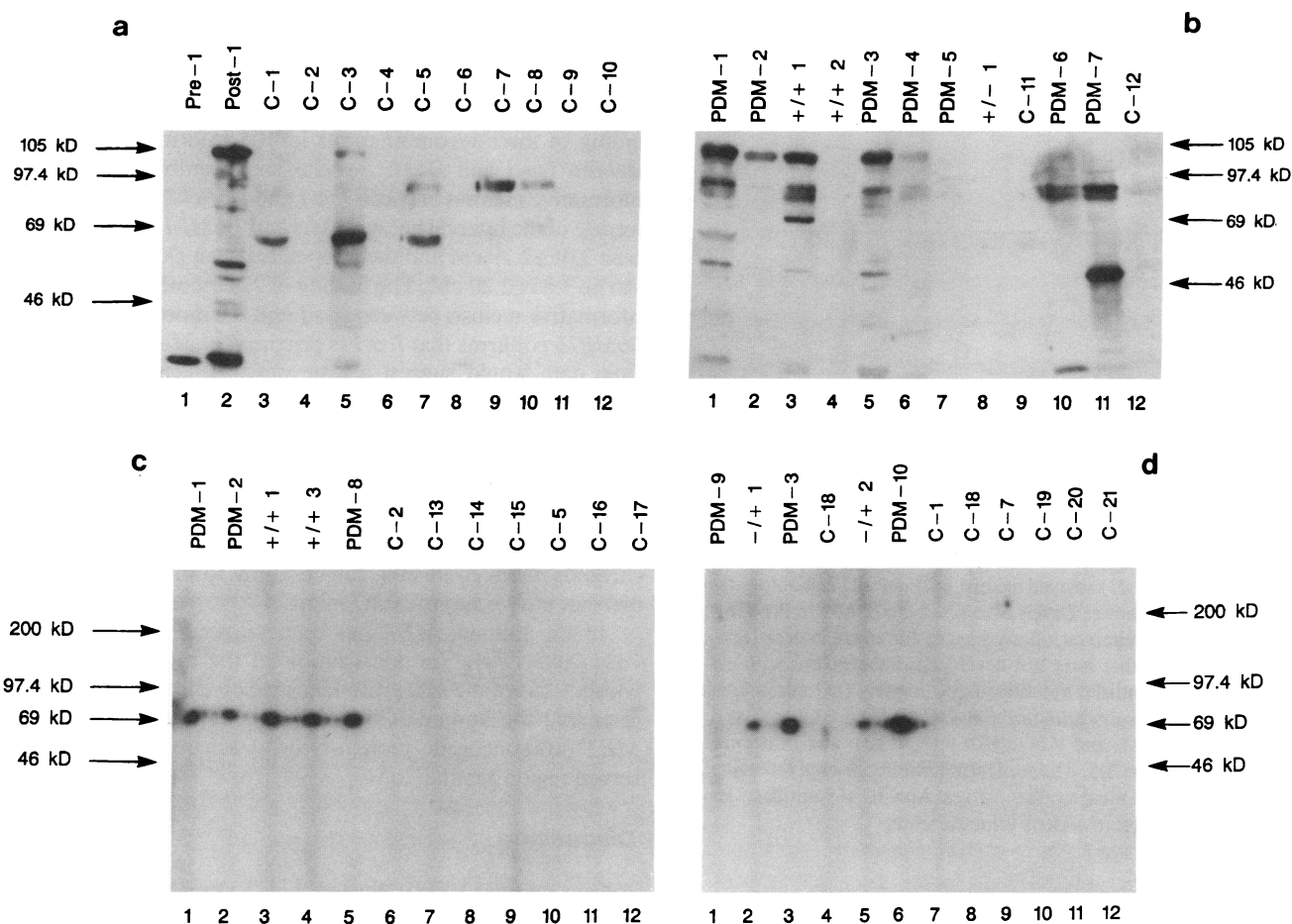
producing clonal line). No *ICA1* transcripts were detected in total RNA from three human nonislet cell lines, namely HepG2-hepatoma, HeLa-cells, and JEG-choriocarcinoma (Fig. 4).

**Immunoblotting.** Western blots of cell line extracts (RIN 1046-38, BON-1) and brain tissue homogenate revealed a specific band of 69 kD after incubation with rabbit antibodies raised to the COOH terminus of ICA69 and an internal polypeptide. Fig. 5 illustrates that rabbit antiserum (rabbit number 1) raised against the COOH terminus of the molecule specifically reacted with a protein of 69 kD in RIN 1046-38 and BON-1 (visible after longer exposure than shown in Fig. 5) total cell homogenate, but not with HeLa cell line homogenate. The specific 69-kD band disappears after absorption with the polypeptide against which specific antibodies were produced. The control serum from the same rabbit before the polypeptide immunization does not show any 69-kD reactivity. The same specific 69-kD reactivity was also detectable in rat brain total homogenate (not shown), and also using hyperimmune sera generated to an internal polypeptide as well as antiserum produced against the whole ICA69 molecule (see Methods). Since the deduced amino acid sequence of ICA69 is 483 residues with an estimated relative molecular mass of 54,600, the difference between the Western blot size of the protein fractionated in the SDS-PAGE and the estimated size based on the deduced amino acid sequence is likely caused by an aberrant migration of the RIN and the brain tissue proteins in SDS-PAGE as a result of detergent solubilization, as previously observed for a brain protein of approximately the same sequence deduced molecular mass and a similarly discrepant migration on SDS-PAGE (62, 63).

**Reactivity of recombinant ICA69 with serum of first degree relatives of IDDM patients on Western blotting.** To determine whether sera from prediabetic subjects reacted with the recombinant ICA69 fusion protein, and then with the purified ICA69 recombinant molecule, further testing of Western blots was



**Figure 5.** Rabbit antiserum raised against a COOH-terminal ICA69 peptide sequence specifically reacted with a protein of 69 kD in RIN 1046-38 and BON-1 (visible after longer exposure) cell total homogenate but not with homogenate from HeLa cells. The specific 69-kD band disappears after absorption with the polypeptide against which specific antibodies were produced. The serum from the same rabbit (rabbit number 1) before the polypeptide immunization does not react with the 69-kD band.



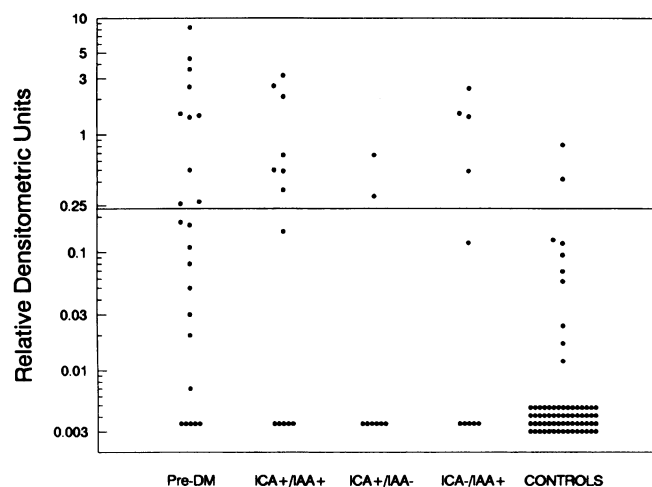
**Figure 6.** Anti-ICA69 sera from relatives of IDDM patients recognize a maltose binding protein-ICA69 fusion protein (*a* and *b*) and affinity-purified recombinant ICA69 without fusion protein (*c* and *d*) on Western blots. Fluorograms of 10% SDS-PAGE showing reactivity of normal control (C), prediabetic relatives' sera (PDM) from preclinical IDDM subjects (at dilution 1:100) and reactivity of serum samples from autoantibody positive relatives (cytoplasmic islet cell antibody/insulin autoantibody +/+, +/-) who have not yet developed diabetes. Bands at ~ 105 kD (arrows) represent the PM1/3 clone fusion protein as indicated by reactivity with rabbit-anti ICA69 peptide antibody. The ~ 105 kD bands correspond to the SDS-PAGE migration of the whole fusion protein. Lanes 1-2 (*a*), samples from the same rabbit (rabbit number 1) before (*Pre-1*) and after (*Post-1*) the immunization with the deduced COOH terminus polypeptide of ICA69 (see Methods). Lanes 3-12, serum samples from 10 healthy individuals. In *b*, sera from seven prediabetics (PDM-1 to 7), from two cytoplasmic islet cell antibody+/insulin autoantibody+ relatives who have not developed diabetes (+/+), from one cytoplasmic islet cell antibody+/insulin autoantibody negative (+/-) and two additional human control sera (C-11, C-12). Immunoblots showing reactivity of preclinical IDDM relatives cytoplasmic islet cell antibody-positive serum samples (PDM) with Ni-NTA-agarose purified recombinant (His)<sub>6</sub>-ICA69 (*c* and *d*). The purified ICA69 was separated by a 10% SDS-PAGE, and probed with sera from relatives of IDDM patients, and from controls (at 1:100 dilution). Lanes 1, 2, and 5 (*c*) and lanes 3 and 6 (*d*) show reactivity of sera from relatives of IDDM patients followed to the overt disease (PDM) with the 69-kD band. Cytoplasmic islet cell antibody/insulin autoantibodies (+/+ and +/-), serum samples from relatives of IDDM patients that have not developed the disease, show also reactivity with ICA69. Note the absence of detectable reactivity with control sera (lanes 6-12 in *c*; lanes 4 and 7-12 in *d*). All the sera from both prediabetics and controls used in the preliminary MBP/PM1/3 clone fusion protein Western blot assay gave consistent results using the affinity-purified recombinant ICA69 as source of antigen for performing Western blots (some of the same serum samples, used in the immunoblots in *a* and *b*, are indicated with the same label in the two sets of immunoblots [Fig. 6, *a*, *b*, *c* and *d*]). Rabbit hyperimmune sera produced against the whole ICA69 and the COOH terminus of the molecule specifically reacted also with the Ni-NTA-agarose purified (His)<sub>6</sub>-ICA69 (not shown). Positions of molecular mass markers ( $M_r \times 10^{-3}$ ), and ~ 105 kD bands are indicated at right and left edges. The gels were exposed for 6-12 h.

performed. We first assessed whether antiserum raised against the COOH terminus and the whole ICA69 molecule, as well as the three sera from prediabetics previously reacting with the  $\lambda$ gt11 IPTG-induced PM1/1 insert, could react on a Western blot with the PM1/3 clone expressed as a fusion protein with maltose binding protein. The rabbit hyperimmune serum and 6 out of 10 sera from relatives with IDDM specifically reacted with a ~ 105 kD band (Fig. 6, *a* and *b*) which corresponds to the SDS-PAGE migration of the whole fusion protein, whereas none of the 12 control sera reacted significantly (quantitating

the sera reactivity to the ~ 105 kD band by scanning densitometry).

Sera from first degree relatives from patients with insulin-dependent diabetes mellitus demonstrated specific binding to the affinity purified recombinant ICA69 on Western blotting (Fig. 6, *c* and *d*, and Fig. 7). Serum samples from 23 relatives of IDDM patients who were initially found to be ICA positive and then followed to the clinical onset of the disease (*Pre-DM*; Fig. 7), 70 healthy volunteers, serum samples from 13 ICA+/IAA+ (cytoplasmic islet cell antibody+/insulin autoanti-





**Figure 7.** Quantitative comparison of serum binding to recombinant affinity-purified ICA69. Results of Western blots for individual serum samples (dilution 1:100) assayed against purified islet cell autoantigen 69 kD (ICA69) are shown. Levels of antibody to ICA69 in Pre-DM (preclinical IDDM relatives followed up to the overt disease [ $n = 23$ ]); in relatives that have not developed diabetes, but with evidence of humoral antiislet autoimmunity, namely (cytoplasmic islet cell antibody/antiinsulin autoantibody) ICA+/IAA+ ( $n = 13$ ), ICA+/IAA- ( $n = 8$ ), and ICA-/IAA+ ( $n = 10$ ); and in normal control subjects ( $n = 70$ ). The horizontal line represents the value 2 SD above the pooled mean control values. Anti-ICA69 antibody levels are expressed as relative densitometric units.

body+), 8 ICA+/IAA-, and 10 ICA-/IAA+ who have not developed type I diabetes to date were assayed to determine their reactivity to highly purified ICA69. Most serum samples from the control group reacted minimally with the purified ICA69 in this assay format. When significant antigen binding was defined as an optical density of  $> 2$  SD above the mean values for the control group (Fig. 7), serum from 10 of the 23 Pre-DM group, exceeded 2 SD of normal binding to ICA69 (43%), as well as serum samples from 7 of the 13 ICA+/IAA+ (54%), from 2 of the 8 ICA+/IAA- (25%), and from 4 of the 10 ICA-/IAA+ (40%). It is of interest that in the ICA+/IAA- group all the serum samples from relatives of IDDM patients that are considered negative, detected by our Western blot assay format, are restricted ICA, who rarely progress to the overt IDDM.

**ICA69 islet immunohistochemistry.** Staining of formalin-fixed sections of rat pancreas with antibodies raised to the human recombinant ICA69 revealed selective  $\beta$  cell reactivity (Fig. 8). In islets double immuno-enzymatically labeled with a polyclonal antibody to ICA69 and with mAbs to glucagon or somatostatin, antibodies to ICA69 reacted with the  $\beta$  cell core (Fig. 8a) but not with either glucagon or somatostatin containing cells (Fig. 8, b and c).

**Chromosome localization of *Ica-1*, the mouse homologue, to chromosome 6.** Linkage data summarized in Fig. 9 indicate that *Ica-1* (*Ica-1* is the mouse gene symbol for the gene coding for ICA69; ICA1 is the human gene symbol for the gene coding for ICA69) is linked to the *Met* protooncogene (located 6 cM proximal on chromosome 6). Linkage to two other proximal *Met*-linked markers (*D6Mit1*, *D6Rck2*, 5 cM proximal) was demonstrated. The weighted average of the interval between *Met* and *Ica-1* was estimated to be  $6.23 \pm 2.5$  cM based on 1/17

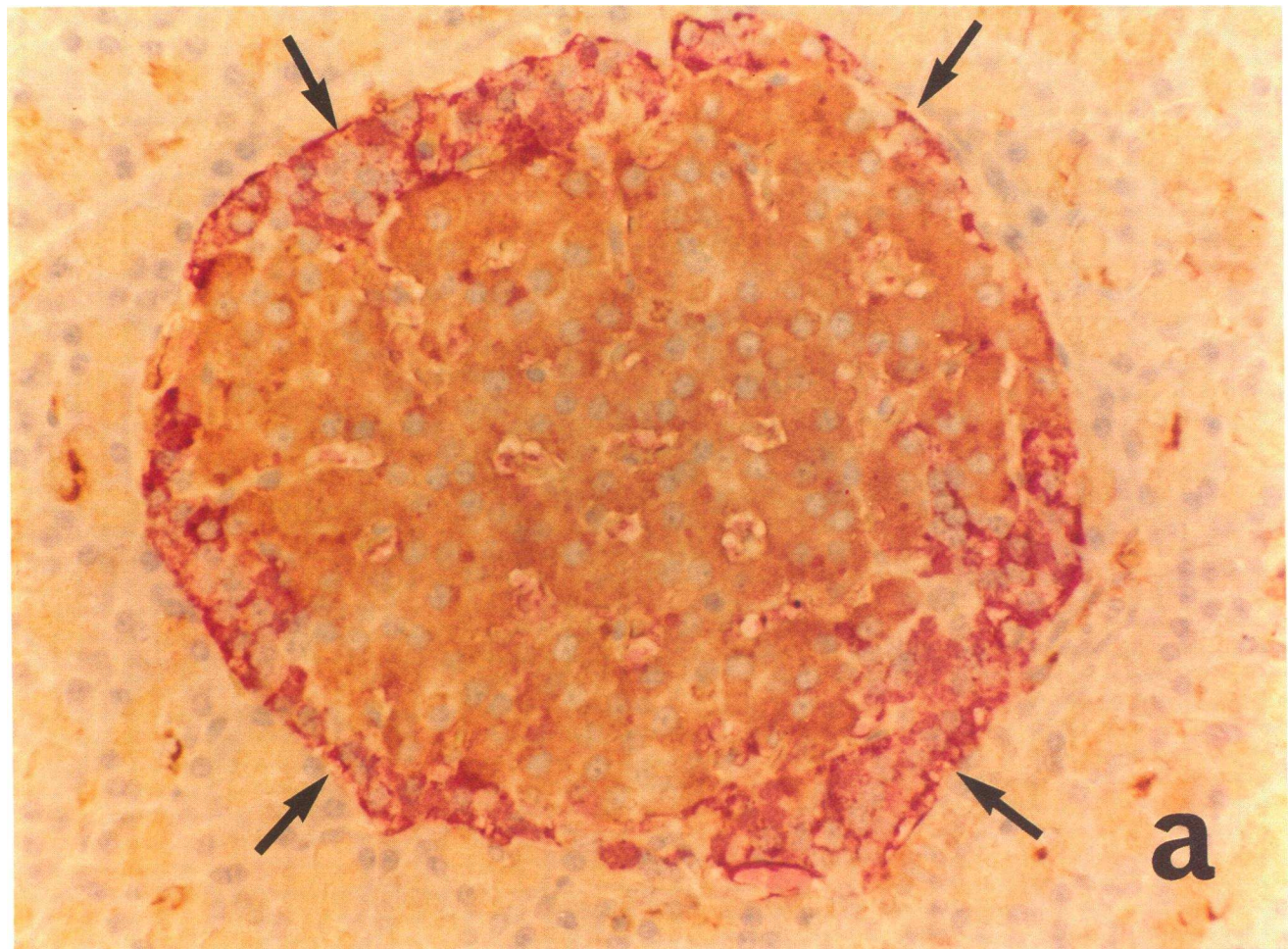
recombinant in the backcross panel and 5/40 obligate recombinant in the F2 panel (total of 97 informative meioses). The proximal *D6Mit1* and *D6Rck2* markers were separable by the finding of three recombinations in 101 informative meioses between *Ica-1* and *D6Mit1* versus six recombinations in 94 informative meioses between *Ica-1* and *D6Rck2*. The weighted average of the interval between *Met* and *D6Mit1* was estimated to be  $3.05 \pm 2.29$  cM and the interval between *D6Mit1* and *Ica-1* to be  $3.85 \pm 2.20$  cM. The finding of 22 recombinations in 74 informative meiosis between *Ica-1* and the more distal marker *D6Mit16* confirms that *Ica-1* is proximal to *Met*. Collectively, these data would suggest a gene order of centromere-*Ica-1*-*D6Mit1*-*D6Rck2*-*Met*-*D6Mit16* as indicated in Fig. 9. Though a polymorphism between NOD and NON exists for the *Ica-1* gene, and segregation of the NOD-derived *Ica-1* allele was not significantly associated with diabetes in either the backcross or the F2 generation ( $P > 0.05$  by chi square analysis), indicating that it was not closely linked to a locus at the proximal end of chromosome 6 conferring susceptibility to insulin dependent diabetes (*Idd* gene) of NOD mice.

In the human, *MET* has been mapped to chromosome 7q21-7q31 (65). The localization of the mouse *Ica-1* gene within 6 cM of the *Met* protooncogene on chromosome 6, suggests that the human *ICA1* gene may be found close to the *MET* protooncogene protein-tyrosine kinase locus in a conserved region around 7q31.

## Discussion

Recent findings have indicated that the range of autoantigens related to type I diabetes is more diverse than was originally thought. Sera from IDDM patients, as well as from stiff-man syndrome patients, appear to recognize one or both forms of the neuroendocrine-associated enzyme GAD (6, 66), namely GAD 65 (67), and GAD 67 (68), and one portion of the antibody response to islet cells, termed "restricted ICA," has been reported to recognize GAD (69, 70). In the present study, we have cloned, sequenced, and characterized a novel 69-kD diabetes-related autoantigen.

In an attempt to identify molecular targets for antiislet autoimmunity in IDDM, we have used sera from preclinical IDDM subjects as probe to isolate cDNA clones from a human islet  $\lambda$ gt11 expression library. We have obtained a cDNA of a novel protein that codes for a full-length amino acid sequence 54.6 kD with an apparent mobility of 69 kD on SDS-PAGE. The detection of mRNA in neural tissues studied such as brain, the presence of ICA69 transcripts in islet-derived cell lines, namely, RIN 1046-38, BON-1, HIT,  $\beta$ TC-1,  $\alpha$ TC-6, and in human insulinoma tissue, in contrast to nonneuroendocrine cell lines such as HeLa cells, JEG-choriocarcinoma, and HepG2-hepatoma likely reflect the sharing of many molecules between islets and neurons. A low level of ICA69 mRNA was also found in human lung, liver, and kidney. It is of interest that a high level of ICA69 mRNA is present in heart, whereas mRNA is undetectable in skeletal muscle and this could be related to the presence of selective cells expressing high levels of this molecule. Islets and neuronal cells both contain secretory granules and microvesicular bodies; for instance, GAD has been localized to microvesicular structures in both pancreatic  $\beta$  cells, as well as in synaptic nerve microvesicular structures (71). Many of the molecules of both of these shared structures appear to be prominent targets of the autoimmunity related to



**Figure 8.** Microphotographs of peroxidase and alkaline phosphatase double immunoenzymatic rat islets labeling using anti-ICA69, antiinsulin, anti-glucagon, and antisomatostatin antibodies. (a) The islet was double-labeled for ICA69 and glucagon. Anti-ICA69 antiserum reacted with the  $\beta$  cell core of the islet (brown), whereas the glucagon containing cells are stained with an anti-glucagon mAb (red, arrows) and are not reactive with ICA69 ( $\times 350$ ). (b) The islet was double-labeled for ICA69 and somatostatin. Anti-ICA69 antiserum (brown) reacted with the  $\beta$  cell core, whereas the somatostatin containing cells reacted with an anti-somatostatin mAb (red); additional peripheral cells (arrows), negative for both ICA69 and somatostatin, are apparently non- $\beta$  cells ( $\times 315$ ). (c) An islet was double-labeled with mAb anti-insulin (brown) and anti-glucagon (red, arrows), showing a pattern identical to that of (a) (anti-ICA69 and anti-glucagon) ( $\times 350$ ).

type I diabetes. The fact that ICA69 mRNA is detected mostly in endocrine cell lines of islet cell origin, and that ICA69 mRNA is also detected in brain, thyroid and heart, but not skeletal muscle, suggests that ICA69 may be in fact related to the neuroendocrine system. A 2-kb mRNA band is visible in total RNA a glucagon producing cell line, namely  $\alpha$ TC-6, whereas no staining is visible on normal rat glucagon containing cells detecting by immunohistochemistry.

The putative polypeptide encoded by the longest open reading frame of ICA69 clones has a molecular mass of 54,600 D. On Western blots, immunoreactive ICA69 has a molecular mass of 69 kD suggesting aberrant migration on SDS-PAGE. A molecular mass discrepancy between that calculated from the deduced amino acid sequence and that measured by SDS-PAGE, has been reported in the cloning of a number of proteins (62, 63, 72–74). These proteins have highly charged regions that appear to be related to retarded SDS-PAGE gel migration, resulting thus in an overestimation of the true molecular mass. Our protein has several strongly charged re-

gions, such as the segment of the molecule between residues 307 and 320. The fact that cDNA encoding the full length of the molecule produces a 69-kD protein when expressed in bacteria whose molecular mass is higher than the one inferred by the relative molecular mass of the deduced sequence (54.6 kD), supports aberrant gel migration as the likely cause of the relative molecular mass disparity.

Environmental factors are potential triggers of autoimmunity in type I diabetes (13). There is evidence in animal models of IDDM such as BB rats that the elimination of cow milk proteins from the diet significantly reduces the incidence of the clinical onset of diabetes in these animals (75). Sera from patients with type I diabetes as well as BB rats are reported to have high titer of IgG anti-BSA antibodies (but not of antibodies to other milk proteins) or IgG anti-ABBOS (a region of the BSA molecule extending from 152 to 158 amino acid residues) antibodies (13–15). It has been reported that antibodies raised to one short BSA unique peptide region (ABBOS) or serum from a newly diagnosed IDDM child react on a Western blot format



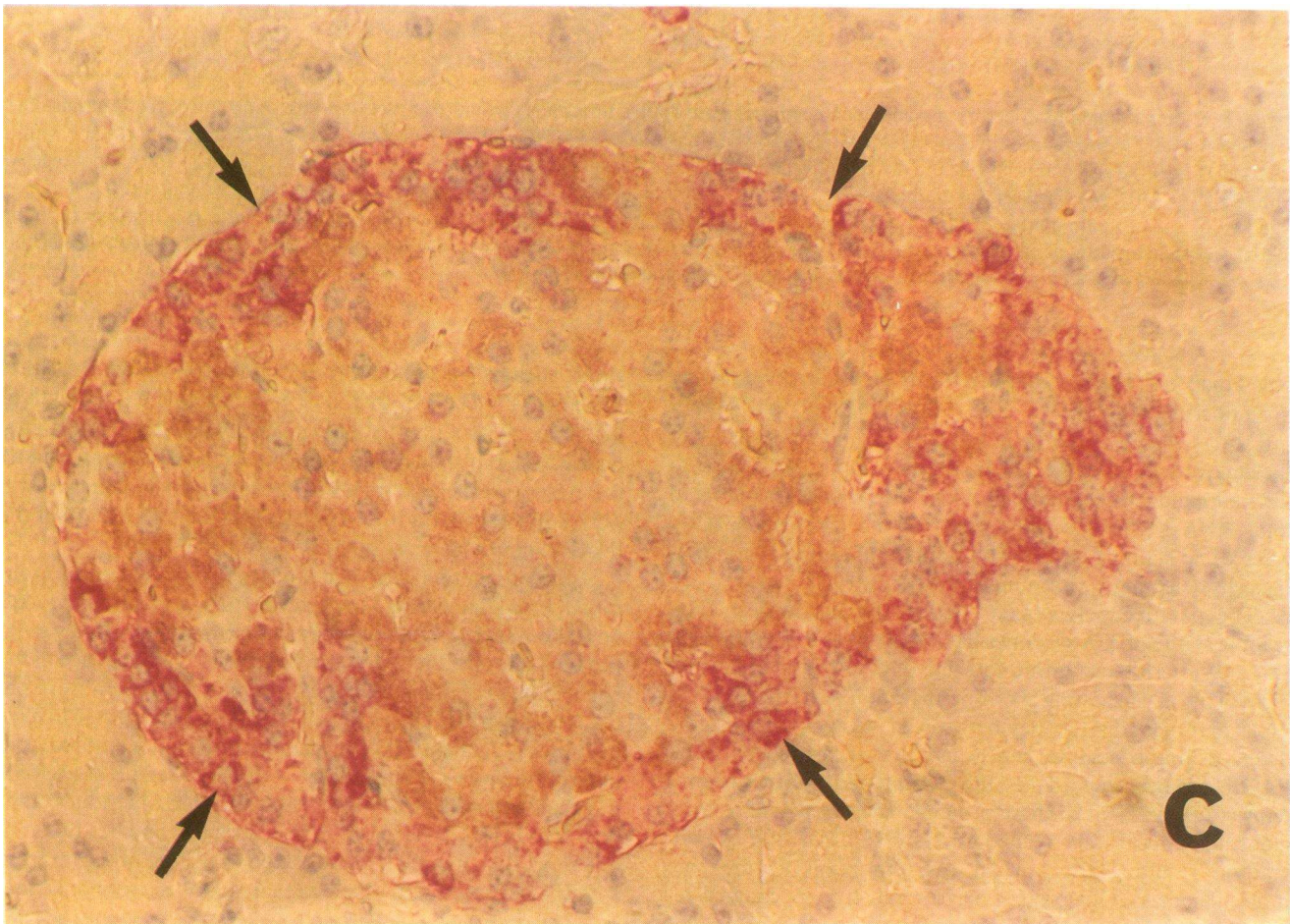
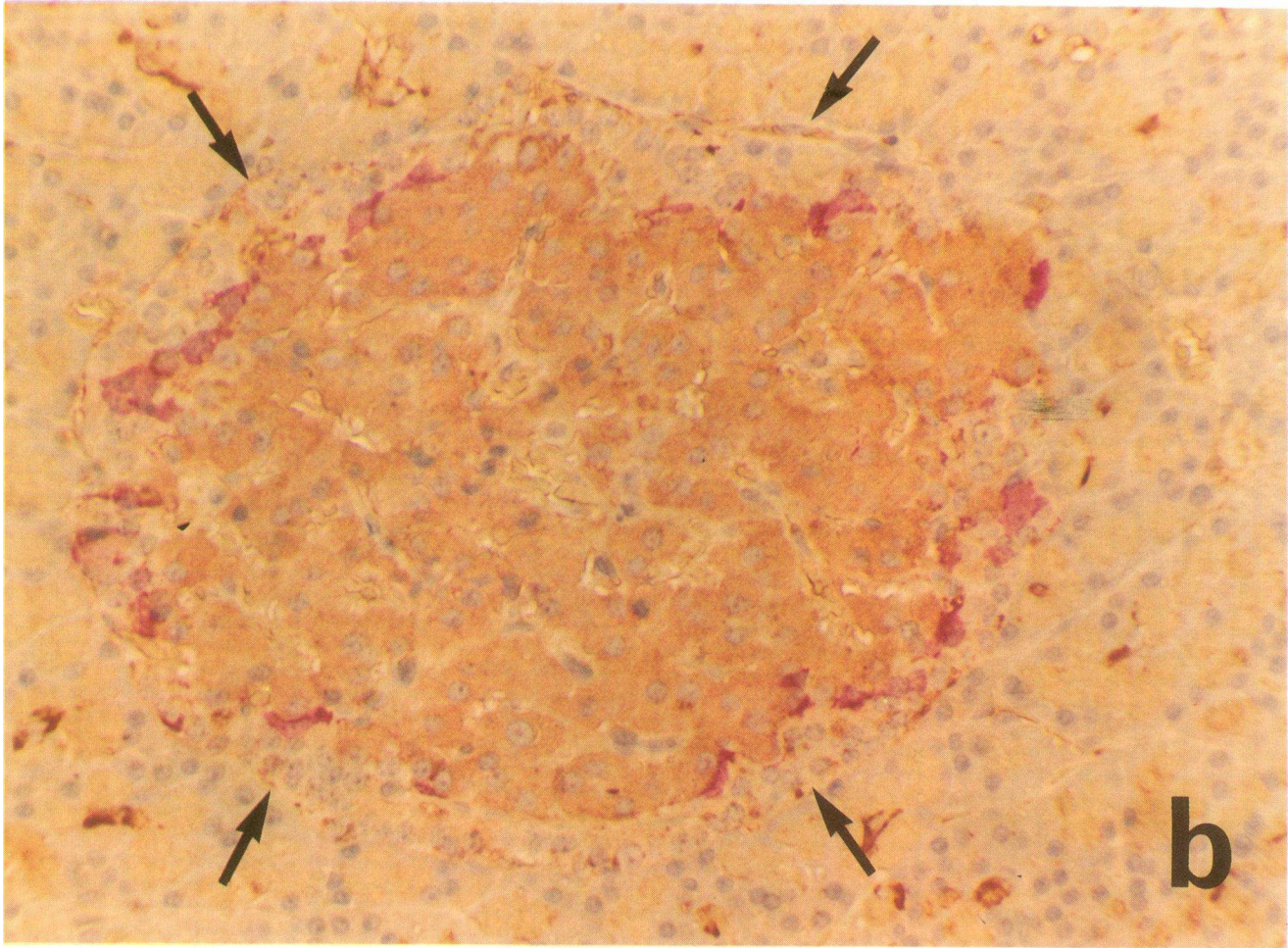
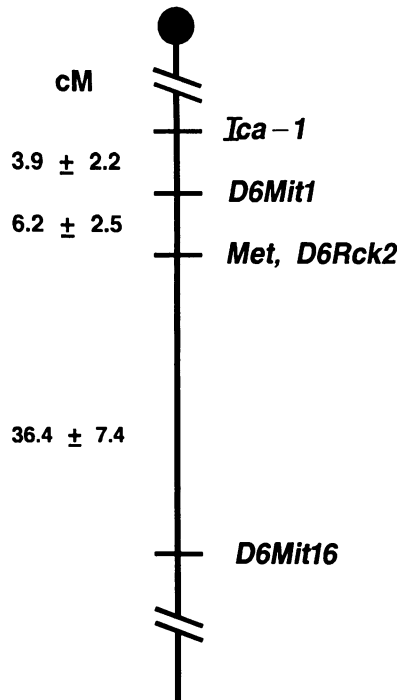


Figure 8 (Continued)





**Figure 9.** Localization of *Ica-1* on proximal chromosome 6 of the mouse. The recombination frequencies between linked loci are expressed in cM $\pm$ SEM (shown at the left) and were estimated using a weighted average for backcross and F2 data. Multipoint linkage map constructed using MAPMAKER 1.9 (64) gave maximum likelihood estimates for the best three gene orders as follows: order 1, *Ica-1-D6Mit1-D6Rck2-Met* log likelihood -79.10; order 2, *D6Rck2-Met-Ica-1-D6Mit14* log likelihood -82.85; and order 3, *D6Rck2-Met-D6Mit1-Ica-1* log likelihood -86.88. \*Recombination frequency estimated from F2 data only.

with  $\gamma$ -interferon-induced RIN 69-kD protein or with islet proteins with a similar mobility (60–70 kD), respectively (13).

It is of interest that our 69-kD molecule shares two short regions of similarity with bovine serum albumin (not with human serum albumin precursor). These two antigenic determinants perhaps could play a role in the induction of cow milk-induced autoimmunity based upon the hypothesis of pathogenesis related to molecular mimicry, or may be simply a cause of a cross-reactivity with anti-ICA69 antibodies and with anti-BSA antibodies. To prove one of these two hypotheses, further studies are required. We have just learned that a partial peptide sequence available from a p69 BSA-related molecule studied by Dosch and co-workers is identical to amino acids 262 to 325 of ICA69 (H.-M. Dosch, personal communication).

Our study shows that first degree relatives of patients with IDDM who developed the disease carry antibodies reacting with ICA69. Antibodies to ICA69 are detected also in relatives who have not yet developed the disease. Such antibodies are found in 43% of the serum samples from preclinical IDDM relatives assayed so far, and we believe that with the optimization of a more sensitive assay (such as a radioassay or an ELISA), the percentage of reactivity with ICA69 among IDDM relatives will increase.

Detecting by Western blotting, the percentage of anti-ICA69 reactivity among sera from relatives of IDDM patients that developed the disease and in from ICA+/IAA+ group appears to be higher than the ICA+/IAA- group (25%) (Fig. 7), seemingly because all the anti-ICA69 negative sera from ICA+/IAA- relatives assayed thus far, show a  $\beta$  cell-selective ICA pattern (termed "restricted"), that includes recognition of GAD, which is associated with the lack of progression to overt diabetes (69, 70). Therefore, the low percentage of anti-ICA69 positive sera in the ICA+/IAA- group as compared to the

other groups appears to be related to the fact that most of the ICA+/IAA- IDDM serum samples from IDDM relatives assayed so far are restricted ICA and rarely progress to the overt IDDM. In light of these observations, we believe that with the combination of different assays for the detection of autoantibodies in IDDM, such as anti-IAA, anti-GAD, anti-ICA69, and in the future against other islet autoantigens, combinatorial algorithms for improving the prediction of type I diabetes will be designed.

The identification, the characterization of the molecular structure and the availability of the ICA69 molecule will help to add this autoantigen to a battery of markers for the prediction of diabetes risk, and will enhance our understanding of islet biochemistry. A family of islet antigen specific therapies are considered for the suppression of insulin-dependent diabetes; e.g., oral tolerance to insulin (76). ICA69 with its  $\beta$  cell expression and autoantigenicity becomes an additional candidate molecule for manipulation of  $\beta$  cell autoimmunity.

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