Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 2307–2311, March 1996
Medical Sciences

Identification of a second transmembrane protein tyrosine phosphatase, IA-2β, as an autoantigen in insulin-dependent diabetes mellitus: Precursor of the 37-kDa tryptic fragment

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Communicated by Stanley B. Prusiner, University of California, San Francisco, CA, December 8, 1995 (received for review October 3, 1995)

ABSTRACT **A novel cDNA, IA-2β, was isolated from a mouse neonatal brain library. The predicted protein sequence revealed an extracellular domain, a transmembrane region, and an intracellular domain. The intracellular domain is 376 amino acids long and 74% identical to the intracellular domain of IA-2, a major autoantigen in insulin-dependent diabetes mellitus (IDDM). A partial sequence of the extracellular domain of IA-2β indicates that it differs substantially (only 26% identical) from that of IA-2. Both molecules are expressed in islets and brain tissue. Forty-six percent (23 of 50) of the IDDM sera but none of the sera from normal controls (0 of 50) immunoprecipitated the intracellular domain of IA-2β. Competitive inhibition experiments showed that IDDM sera have autoantibodies that recognize both common and distinct determinants on IA-2 and IA-2β. Many IDDM sera are known to immunoprecipitate 37-kDa and 40-kDa tryptic fragments from islet cells, but the identity of the precursor protein(s) has remained elusive. The current study shows that treatment of recombinant IA-2β and IA-2 with trypsin yields a 37-kDa fragment and a 40-kDa fragment, respectively, and that these fragments can be immunoprecipitated with diabetic sera. Absorption of diabetic sera with unlabeled recombinant IA-2 or IA-2β, prior to incubation with radiolabeled 37-kDa and 40-kDa tryptic fragments derived from insulinoma or glucagonoma cells, blocks the immunoprecipitation of both of these radiolabeled tryptic fragments. We conclude that IA-2β and IA-2 are the precursors of the 37-kDa and 40-kDa islet cell autoantigens, respectively, and that both IA-2 and IA-2β are major autoantigens in IDDM.**

IA-2 is a transmembrane protein with a molecular mass of 106 kDa and was recently cloned, sequenced, and expressed in a reticulocyte transcription/translation system (1, 2). By using recombinant IA-2 as a source of antigen, close to 70% of patients with insulin-dependent diabetes mellitus (IDDM) were found to have autoantibodies to this protein (M.S.L., C. Wasserfall, N.K.M., and A.L.N., unpublished results) and the presence of these autoantibodies was shown to be a good predictive marker for identifying individuals at high risk of developing IDDM (M. I. Hawa, R.E. Rowe, M.S.L., A.L.N., P. Pozzilli, M.R. Christie, and R.D.G. Leslie, unpublished results). Of particular interest was the finding that many patients who had autoantibodies to islet-cell antigens as measured by immunofluorescence (3) but who did not have antibodies to GAD_{65}, another important autoantigen in IDDM (4), were positive for autoantibodies to IA-2. Absorption of diabetic sera with recombinant GAD_{65} and/or IA-2 removed much, but not all, of the immunofluorescent reactivity of the diabetic serum with islet cells, suggesting that other still unidentified autoantigens were involved in the islet cell autoantibody response.

Recently, 21 members of the protein tyrosine phosphatase (PTP) family were identified from short nucleotide sequences isolated from a polymerase chain reaction (PCR)-amplified cDNA library that was constructed with cDNAs reverse-transcribed from pancreatic β cells and a pair of degenerative primers derived from known PTP nucleotide sequences (J.L., Q.L., G. Donadel, A.L.N. and M.S.L., unpublished results). Three of these 21 PTPs were previously unknown. One of them showed considerable sequence similarity to IA-2. In this report, we describe the molecular cloning of the intracellular domain of this molecule, designated IA-2β, characterize it as an autoantigen in IDDM, and show its relationship to a 37-kDa tryptic fragment from insulinoma cells to which IDDM patients are known to have autoantibodies (5).

MATERIALS AND METHODS

Cloning and Sequencing of Mouse IA-2β cDNA Clones. A 300-bp fragment of IA-2β isolated from a PCR-based PTP library was used as a probe to screen a mouse neonatal brain λ ZAPII cDNA library (Stratagene). Two clones extending ≈2 kb upstream from the poly(A) tail were isolated by screening 400,000 plaques. The insert of the longer clone was used to rescreen the same library. Seven additional cDNA clones were obtained that contained sequences overlapping with original clones. Nucleotide sequences were determined from both directions by the chain-termination method using the Sequenase Version 2.0 sequencing kit (United States Biochemical).

Northern Blot Analysis. Total RNAs were isolated from normal mouse tissues and tumor cell lines αTC-1 and βTC-1 by the acid guanidinium thiocyanate/phenol/chloroform extraction method (6). RNA samples (each at 20 μg) were electrophoresed in a 1% agarose/5.4% formaldehyde gel, transferred to Nytran membrane (Schleicher & Schuell) via capillary blotting. Hybridization was performed as described (1). IA-2β cDNA (aa 3–470) void of PTP domain or full-length IA-2 cDNA were used as the probe for Northern blot analysis. rRNAs were used to verify the quality of RNAs.

Human Subjects. Fifty newly onset IDDM patients who had been diagnosed within a week of their blood sampling and 50 age-matched controls with no history of autoimmune disease were studied. The median age of the new-onset patients was 8.8 years (range, 1–17 years), and the median age of the control subjects was 9.8 years (range, 6–16 years). Equal numbers of male and female patients were studied. Blood samples were collected under informed consent as approved by the University of Florida Institutional Review Board.

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; PTP, protein tyrosine phosphatase.

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Preparation of Rabbit Hyperimmune Serum and Mouse Monoclonal Antibody. Rabbit polyclonal antisera were prepared against the intracellular PTP domain of the IA-2 molecule by immunizing two male New Zealand White rabbits with multiple subcutaneous injections on the back of 125 μg of bacterial-expressed glutathione *S*-transferase–IA-2 fusion protein emulsified in incomplete Freund's adjuvant. Injections were performed every 2 weeks. Serum was collected and tested for immunoreactivity with bacterial-expressed fusion protein. Mouse monoclonal antibody IA-2/161 was prepared against the PTP domain of the IA-2 molecule by conventional hybridoma technology.

Radioimmunoprecipitation of *in Vitro*-Translated Intracellular Domain of IA-2β Autoantigen with IDDM Sera and Competitive Immunoprecipitation. The intracellular domain of mouse IA-2β cDNA (aa 351–723) was PCR-amplified with a perfect Kozak translational start sequence (GCCGCCACCATGG) that was engineered at the 5′ end of the sequence (7) and subcloned into a pCRII cloning vector (Invitrogen). PCR was performed with 10 ng of IA-2β cDNA as template. The PCR conditions were as follow: 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C for 35 cycles. A similar strategy, with a perfect Kozak sequence, was employed to clone the intracellular domain of mIA-2 (aa 604–979). The *in vitro* transcription/translation product was prepared with 1 μg of plasmid DNA in a TNT coupled rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine (Amersham) at 30°C for 2 h. Radiolabeled protein was determined by 10% (wt/vol) trichloroacetic acid precipitation. Immunoprecipitation was performed as described below. Briefly, translated reticulocyte lysate (50,000–75,000 cpm) and 5 μl of tested serum were mixed in 100 μl of immunoprecipitation buffer (20 mM Tris·HCl, pH 7.4/150 mM NaCl/1% Triton X-100). The reaction mixture was incubated overnight at 4°C and 50 μl of 50% (vol/vol) protein A-agarose (Life Technologies) was added to the solution at 4°C for 1 h. The immunoprecipitation mixture was washed four times with immunoprecipitation buffer, boiled in 1× SDS sample buffer, and applied to a 10% SDS/PAGE gel. The intensity of the IA-2β band (≈41 kDa) was scored from 1+ to 4+ by two investigators. The intensity of these bands corresponds very closely to counts in the immunoprecipitate as determined by liquid scintillation (unpublished data).

***In Vitro* Labeling of Islet Cells.** A cloned mouse insulinoma cell line, βTC-1 (insulin-secreting β cell), and a cloned mouse glucagonoma cell line, αTC-1 (glucagon-secreting α cell), were obtained from E. H. Leiter (The Jackson Laboratory) and maintained at low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum. Before labeling, a 70% confluent culture was incubated in methionine-free medium supplemented with 10% dialyzed fetal calf serum for 1 h to deplete the intracellular methionine pool. Subsequently [^{35}S]methionine (100 μCi/ml; 1 Ci = 37 GBq) was added to the culture for 5 h. The labeled cells were harvested and the membrane fraction was prepared as described (8).

Immunoprecipitation and Blocking of 37/40-kDa Tryptic Fragments from IA-2β/IA-2 Autoantigens. Radiolabeled cell lysates (1×10^7 cpm) were precipitated with diabetic serum overnight and coprecipitated with protein A-agarose beads. After being washed three times with precipitation buffer, the beads were washed with water once and incubated with trypsin (50 or 100 μg/ml) on ice for 20 min. The precipitate was washed once again with water, boiled in 1× SDS sample buffer, and separated on a 10% SDS/PAGE gel. The blocking reaction was performed by preincubation of the serum with a reticulocyte lysate containing the unlabeled intracellular domain of IA-2 or IA-2β for 2 h. Radiolabeled cell lysate was added, precipitated, and trypsinized as described above. Labeled reticulocyte lysates of full-length IA-2 and the intracellular domains of IA-2 and IA-2β were also directly treated with trypsin (50 μg/ml) on ice for 20 min before loading on a 10% SDS/PAGE gel.

RESULTS

Molecular Cloning and Sequence Analysis of IA-2β. A 300-bp fragment of IA-2β, obtained from a PCR-based mouse insulinoma PTP fragment library, was used to screen a mouse neonatal brain cDNA library. Nine cDNA clones were isolated and their nucleotide sequences were determined by double-stranded sequencing. The insert sizes ranged from 0.6 to 2.5 kb and the total overlapped sequence was ≈3.5 kb. Comparison of the nucleotide sequence of the different clones revealed that

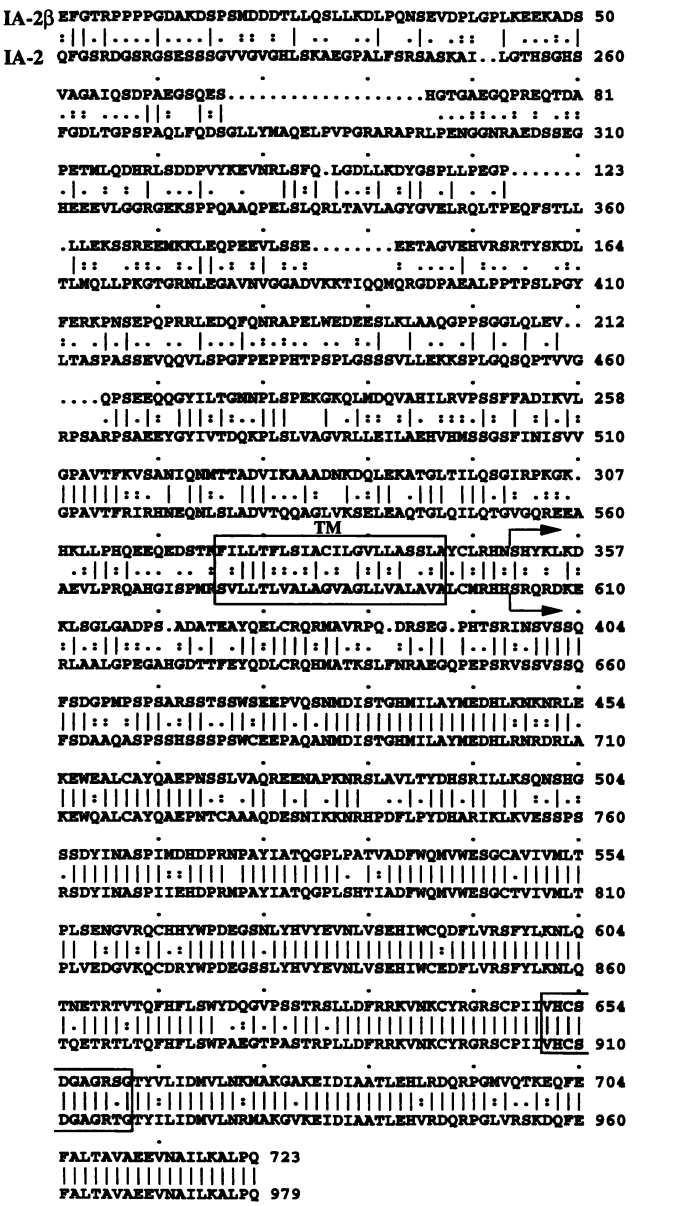

FIG. 1. Amino acid sequences of mouse IA-2β and IA-2. Protein sequences were lined up with a Genetics Computer Group BESTFIT program. Identical amino acid residues are shown by a vertical bar, highly similar amino acids are shown by a colon, and similar amino acids are shown by a period. The putative transmembrane segment (TM) is boxed and the PTP core sequence is boxed and shaded. Residue number represents mouse IA-2 (2). The N-terminal residue of the partial mouse IA-2β protein sequence was arbitrarily designated residue 1. Arrows indicate the start of the intracellular sequences constructed in the pCRII vector for the *in vitro* translation study.

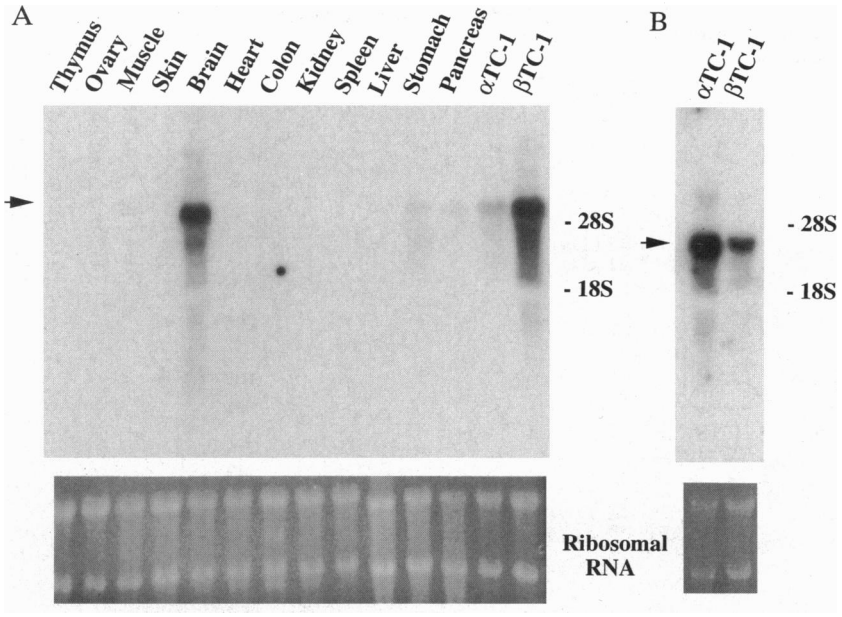

FIG. 2. Northern blot analysis. Total RNA (20 μg) from normal mouse tissues and cell lines were separated on a 1% agarose/formaldehyde gel, transferred to a Nytran membrane, and hybridized with ^{32}P-labeled IA-2β cDNA probe (*A*) or IA-2 cDNA probe (*B*). Message sizes of IA-2 [3.8 kb (arrow)] and IA-2β [5.5 kb (arrow)] were estimated by rRNAs. rRNAs are indicated.

the 3′ untranslated region of some of the clones lacked a stretch of 306 bp, perhaps the result of alternative splicing. A compressed GA-rich stretch at the 3′ end of the untranslated region further hampered resolution of the exact sequence. Nonetheless, all nine cDNA clones possessed the same open reading frame that translated into a protein of 723 aa with intracellular, transmembrane, and extracellular domains (Fig. 1). Sequence analysis using the GenBank data base showed that IA-2β is a member of the transmembrane PTP family. Its intracellular segment contains a single PTP domain that shows 74% identity to IA-2. The PTP core sequence of IA-2β (VHCSDGAGRS/TG) differs from that of IA-2 by only 1 aa. In contrast, the partial extracellular sequence of IA-2β shows only 26% identity with the extracellular domain of IA-2.

Tissue Expression of IA-2β. Normal mouse tissues were examined for the expression of IA-2β message. Since the intracellular domain of IA-2β shows high similarity to the intracellular domain of IA-2, Northern blot analysis was performed using as the probe a ^{32}P-labeled IA-2β sequence void of the PTP domain. Fig. 2*A* shows that a 5.5-kb mRNA band was prominent in the insulinoma cell line βTC-1 and brain, less prominent in the glucagonoma cell line αTC-1, pancreas, and stomach, and barely detectable in colon. The other tissues tested including thymus, ovary, muscle, skin, heart, kidney, spleen, and liver were negative. For comparison, Northern blot analysis was performed on αTC-1 and βTC-1 cells with a ^{32}P-labeled IA-2 sequence as the probe. Fig. 2*B* shows that a 3.8-kb mRNA band was very prominent in αTC-1 cells but less prominent in βTC-1 cells. A weaker signal of larger message was detected in αTC-1 cells that may be the result of alternative splicing. Because of its sequence similarity with IA-2 and strong expression in βTC-1 cells, we refer to this new cDNA as IA-2β.

Immunoprecipitation of Intracellular Domain of IA-2β with IDDM Serum. The intracellular domain of IA-2β (aa 351–723) was translated into a 41-kDa product in a TNT-coupled rabbit reticulocyte system. Radiolabeled IA-2β then was immunoprecipitated with serum from IDDM patients and separated on a 10% SDS/PAGE gel. Fig. 3 *Inset* shows seven representative sera from IDDM patients that recognized the *in vitro*-translated product. The intensity of the bands ranged from 1+ to 4+.

Fifty coded sera from diabetic patients and 50 coded sera from controls then were tested by radioimmunoprecipitation for autoantibodies to the IA-2β intracellular domain. As seen in Fig. 3, 46% of the sera from IDDM patients, but none of the sera from controls, reacted with IA-2β.

Identification by Competitive Immunoprecipitation of Cross-Reactive Autoantibodies to the Intracellular Domains of IA-2 and IA-2β. By using the rabbit reticulocytes transcription/translation system, the intracellular domains of IA-2 (aa 604–979) and IA-2β (aa 351–723) translated into a 42-kDa product and a 41-kDa product, respectively. As seen in Fig. 4*A*, hyperimmune rabbit sera and mouse monoclonal antibody to the intracellular domain of IA-2 reacted not only with IA-2 but

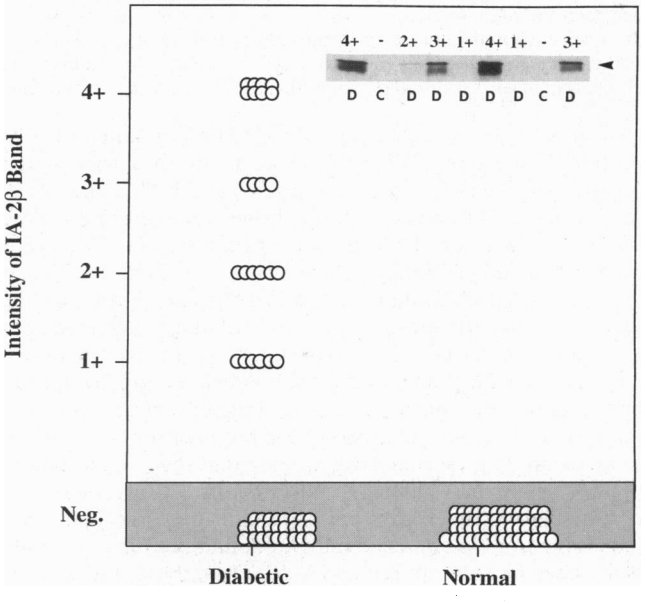

FIG. 3. Sera from 50 patients with clinically documented IDDM and 50 normal controls were tested for autoantibodies to the intracellular domain of IA-2β by radioimmunoprecipitation. The intensity of bands with a molecular mass of 41 kDa was scored on a 1+ to 4+ scale. (*Inset*) Representative sera with different band intensities. Lanes: D, diabetic sera; C, control sera.

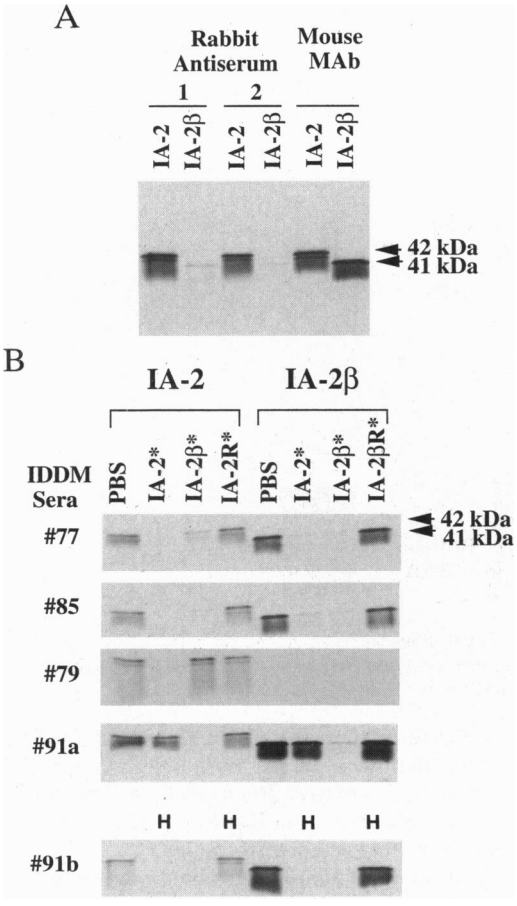

FIG. 4. Competitive radioimmunoprecipitation. (*A*) Radiolabeled recombinant intracellular domains of IA-2 and IA-2β were immunoprecipitated by two hyperimmune rabbit antisera or a mouse monoclonal antibody raised against the PTP domain of the IA-2 molecule. (*B*) *In vitro*-radiolabeled intracellular domains of IA-2 or IA-2β were precipitated with sera from IDDM patients. Sera were preincubated with PBS or unlabeled (*) *in vitro*-translated recombinant IA-2 or IA-2β (10-fold excess as compared to radiolabeled antigen) for 2 h, and then the radiolabeled antigen was added. IA-2R and IA-2βR are the respective antisense *in vitro* translation products. Lanes H represent higher concentration (50-fold excess compared to radiolabeled antigen) of *in vitro*-translated recombinant IA-2 or IA-2β for blocking.

also with IA-2β, precipitating a 42-kDa protein and a 41-kDa protein, respectively. The difference in the intensity of the reactivity of the rabbit hyperimmune sera with IA-2 and IA-2β may reflect a difference in the epitopes recognized on these two molecules. Sera from diabetic patients (sera 77 and 85) recognized and precipitated radiolabeled IA-2 and IA-2β and both unlabeled IA-2 and IA-2β blocked this precipitation (Fig. 4*B*). Some diabetic sera (e.g., serum 91a) that recognized both IA-2 and IA-2β could be blocked by a 10-fold excess of unlabeled IA-2β (compared to radiolabeled antigen) but not by the same concentration of IA-2. Higher concentrations of unlabeled IA-2 (50-fold excess) were required for blocking the same serum (e.g., serum 91b), arguing that the autoantibodies in this serum have a higher affinity for and/or recognize predominantly the IA-2β epitopes. Still other diabetic sera (e.g., serum 79) recognized only IA-2, and the reaction could be blocked by IA-2 but not by IA-2β. Thus, these studies show that autoantibodies to IA-2 and IA-2β have a high degree of cross-reactivity and that both common and distinct epitopes are present on these molecules.

Trypsin Treatment Converts IA-2β and IA-2 into 37- and 40-kDa Fragments. The *in vitro*-translated products of full-length IA-2 (106 kDa) and intracellular IA-2 (42 kDa) were

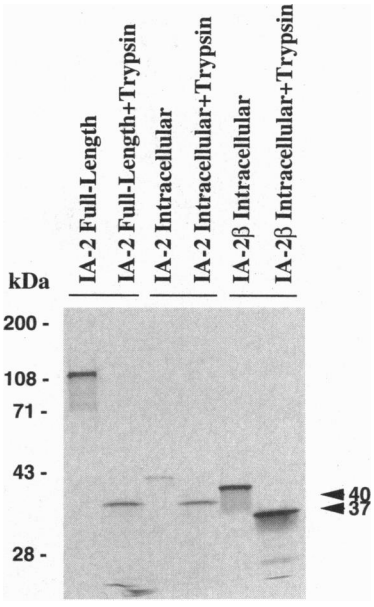

FIG. 5. Trypsin treatment converts IA-2 and IA-2β into 40- and 37-kDa fragments, respectively. *In vitro*-translated full-length IA-2 (lanes 1 and 2), intracellular domain of IA-2 (lanes 3 and 4), and IA-2β (lanes 5 and 6) were trypsinized (50 μg/ml) before electrophoresis on a 10% SDS/PAGE gel.

subjected to trypsin treatment. As seen in Fig. 5, the predominant tryptic fragment migrated as a 40-kDa band. In contrast, the tryptic fragment of intracellular domain of IA-2β (41 kDa) migrated as a 37-kDa band. Both tryptic fragments could be precipitated by diabetic sera (data not shown).

Blocking of 37/40-kDa Tryptic Fragments from Islet Cells by the Intracellular Domain of IA-2β and/or IA-2. To further show that the 37- and 40-kDa tryptic fragments were derived from IA-2β and IA-2, tryptic fragments from αTC-1 and βTC-1 cell lines were precipitated with diabetic sera. As seen in Fig. 6, diabetic sera precipitated only a 40-kDa band from αTC-1 cells and a 37/40-kDa doublet from βTC-1 cells. These findings are consistent with the relative abundance of IA-2 mRNA in αTC-1 and IA-2β mRNA in βTC-1 cells (Fig. 2). Blocking experiments (Fig. 6*A*) showed that both unlabeled recombinant IA-2 and IA-2β were capable of preventing the precipitation of radiolabeled IA-2 and IA-2β by diabetic sera from patient A. Similarly, unlabeled recombinant IA-2 completely blocked the precipitation of radiolabeled IA-2 by diabetic serum from patient B, but unlabeled recombinant IA-2β was somewhat less effective (e.g., αTC-1 cells) (Fig. 6*B*). This may be due to the different epitopes recognized on IA-2 and IA-2β molecules by sera from different diabetic patients.

DISCUSSION

IA-2 is a novel receptor-type PTP isolated from a human insulinoma subtraction library (1) and now known to be a major autoantigen in IDDM. Further studies led to the identification of 21 PTPs in pancreatic islets, three of which were novel. In the present study, the entire sequence of the intracellular and transmembrane domain and a partial sequence of the extracellular domain of one of these novel PTPs, IA-2β, were determined. Our data indicate that IA-2β is closely related but different than IA-2. The intracellular domain of IA-2β shows 74% identity to the intracellular domain of IA-2. The PTP core sequence differs by only 1 aa. A partial sequence (322 aa) of the extracellular domain of IA-2β, however, shows only 26% identity to the extracellular domain of IA-2. The functional role of IA-2β, or for that matter IA-2, remains unknown.

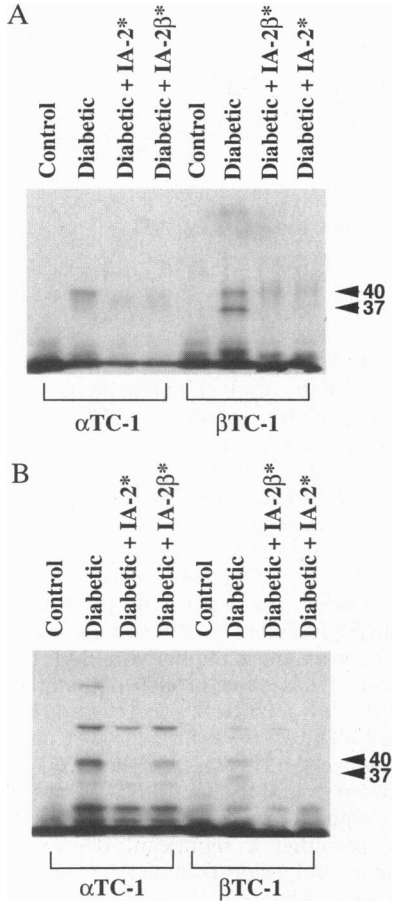

FIG. 6. Blocking of 37/40-kDa tryptic fragments by unlabeled (*) recombinant intracellular domains of IA-2β and/or IA-2. *In vitro*-radiolabeled preparations of αTC-1 and βTC-1 lysates (1×10^7 cpm) were immunoprecipitated with control or diabetic sera from two patients (*A* and *B*). Precipitates were treated with trypsin (100 μg/ml) before loading onto a 10% SDS/PAGE gel. Blocking reactions were performed by incubating IDDM sera for 2 h with the unlabeled recombinant intracellular domain of IA-2 or IA-2β before adding the radiolabeled lysate from αTC-1 and βTC-1 cells.

The tissue distributions of IA-2β and IA-2 show similarities as well as differences. IA-2β and IA-2 are expressed primarily in pancreatic islets and brain. Of particular interest is the fact that IA-2β is preferrentially expressed in our β-cell line (βTC-1), whereas IA-2 is preferrentially expressed in our α-cell line (αTC-1). Whether this differential expression is found in normal pancreatic α and β cells remains to be determined.

The intracellular domain of IA-2β, expressed in a reticulocyte transcription/translation system, was used as antigen to search for autoantibodies in the sera of diabetic patients. Our studies showed that close to 50% of sera from diabetic patients reacted with IA-2β. Studies are now underway to determine the percentage of newly diagnosed vs. long-term diabetic patients with antibody to IA-2β and the predictive value of antibodies to IA-2β for development of IDDM. It is already clear, however, that many sera that react with IA-2β also react with IA-2 because of common antigenic determinants. Screening also indicates that there are unique epitopes on both IA-2 and IA-2β and that certain diabetic sera preferrentially recognized one or the other of these autoantigens. Examination of a large number of diabetic sera are needed to evaluate the clinical importance of these unique epitopes. Although the intracellular domain of mouse IA-2β has yielded considerable information, even more data may be obtained when the entire sequence of IA-2β is available, especially that of the human counterpart. Efforts are now underway to isolate human IA-2β by using mouse IA-2β cDNA as the probe.

Autoantibodies to islet-cell antigens have been the subject of a number of reports (9–13). In 1990, Christie *et al.* (8) reported that a radiolabeled lysate of insulinoma cells incubated with diabetic sera and then treated with trypsin resulted in the precipitation of 37-kDa and 40-kDa fragments. Autoantibodies to these fragments were found in a high percentage of diabetic patients and their presence in nondiabetic subjects was highly predictive of the subsequent development of IDDM (14–16). Since autoantibodies to the 37-kDa and 40-kDa tryptic fragments showed a strong positive correlation, it was suggested that these antibodies bound to epitopes common to both fragments (5). However, the identity of the precursor protein(s) from which these tryptic fragments were derived remained unclear. Recently, Christie and coworkers (17) reported, based on blocking experiments with the intracellular domain of IA-2, that IA-2 appears to be the precursor of the 40-kDa, but not the 37-kDa, fragment. Our data, in fact, showed that recombinant IA-2 and IA-2β yielded tryptic fragments of 40 kDa and 37 kDa, respectively. Moreover, both unlabeled recombinant IA-2 and IA-2β blocked the binding of diabetic sera to the 40-kDa and 37-kDa tryptic fragments prepared from insulinoma cells. Therefore, we conclude that IA-2 is the precursor of the 40-kDa tryptic fragment and IA-2β is the precursor of 37-kDa tryptic fragment.

The cloning and sequencing of IA-2 and IA-2β and the identification of these molecules as major autoantigens are first steps in elucidating their roles in the pathogenesis of IDDM. The development of a panel of autoantibody assays using recombinant IA-2 and/or IA-2β together with recombinant GAD_{65} should provide a powerful tool for screening large populations and accessing their relative predictive values in identifying individuals at high risk for IDDM.

We acknowledge Janice Solomon for excellent editorial assistance.

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