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Immunology

The 37/40-kilodalton autoantigen in insulin-dependent diabetes mellitus is the putative tyrosine phosphatase IA-2

(islet cell antibodies/autoantibodies/expression cloning/prediction of type I diabetes/antigens)

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ABSTRACT **Major targets for autoantibodies associated with the development of insulin-dependent diabetes mellitus (IDDM) include tryptic fragments with a molecular mass of 37 kDa and/or 40 kDa of a pancreatic islet cell antigen of unknown identity. The assay identifying autoantibodies against the 37/40-kDa antigen in human sera is based on the immunoprecipitation of ^{35}S-labeled rat insulinoma cell proteins with sera from IDDM patients, followed by limited trypsin digestion of the immunoprecipitated material. To identify cDNA clones coding for the 37/40-kDa antigen, we have screened a cDNA expression library from rat insulinoma cells with a serum from an IDDM patient that precipitated the 37/40-kDa antigen in our assay. Among the cDNA products that reacted with the IDDM serum, we identified one cDNA clone whose open reading frame encodes a protein with a predicted mass of 105 kDa that we termed "ICA105" for 105-kDa islet cell antibody. The deduced amino acid sequence has high homology to a recently cloned putative tyrosine phosphatase IA-2 from human and mouse cDNA libraries. Translation of the cDNA *in vitro* results in a polypeptide with the expected molecular mass of 105 kDa. The evidence that ICA105 is indeed the precursor of the 37/40-kDa tryptic fragments is based on the following three results: (*i*) Sera from IDDM patients containing autoantibodies to the 37/40-kDa antigen precipitate the *in vitro* translated polypeptide, whereas sera from healthy subjects as well as sera from IDDM patients not reactive with the 37/40-kDa antigen do not precipitate the cDNA product. (*ii*) Immunoprecipitation of the *in vitro* translated protein with sera containing autoantibodies to the 37/40-kDa antigen followed by limited trypsin digestion of the precipitated proteins results in a 40-kDa polypeptide. (*iii*) The protein derived from our cDNA but not from an unrelated control cDNA clone can block immunoprecipitation of the 37/40-kDa antigen from a labeled rat insulinoma cell extract. The availability of the cloned 37/40-kDa antigen should facilitate the identification of individuals at risk of IDDM with increased accuracy. Furthermore, the identification of the 37/40-kDa antigen as the putative tyrosine phosphatase IA-2 is of relevance in elucidating the role of this antigen in the development of IDDM.**

Insulin-dependent diabetes mellitus (IDDM) is a chronic autoimmune disease characterized by progressive destruction of insulin-secreting pancreatic β cells leading to an impairment of glucose metabolism. Progression to IDDM is determined a long time before clinical onset of diabetes, and serological markers found at clinical onset are also detectable in the preclinical period.

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Antibodies reacting with islets on frozen sections of human pancreas (islet cell antibodies, ICAs) are the most commonly used marker for diagnosis and prediction of IDDM (1, 2). However, the precise molecular targets of islet cell antibodies are still not known (3).

Immunoprecipitation of detergent lysates of metabolically labeled islet cells with sera from IDDM patients resulted in the identification of a 64-kDa autoantigen (4). The 64-kDa antigen has been identified as glutamic acid decarboxylase (GAD) (5). Anti-GAD antibodies are present in >70% of newly diagnosed IDDM patients and have been detected up to 7 years before clinical onset of IDDM. However, anti-GAD autoantibodies are also present in nondiabetic patients with autoimmunity to other endocrine organs and thus are alone not strictly specific for diagnosis and prediction of IDDM (6). Analysis of antibody reactivity by immunoprecipitation of trypsin-digested islet homogenates results in three major fragments with molecular masses of 50, 40, and 37 kDa. Antibodies reacting with the 50-kDa fragment also precipitate GAD; in contrast, antibodies to the 37/40-kDa fragments do not correlate with anti-GAD antibodies (7). It has been subsequently suggested that the 37/40-kDa tryptic fragments (also called "37 K antigen") originate from an islet antigen with a molecular mass of 64 kDa that is distinct from GAD. Autoantibodies recognizing the 37/40-kDa tryptic fragments also react with the intact 64-kDa putative precursor, since limited trypsin digestion of the immunoprecipitated material results in the diagnostic 40-kDa band or in the 37-kDa and 40-kDa bands (8). In a study of identical twins, antibodies to 37/40-kDa fragments have been demonstrated to be both specific and sensitive to predicting IDDM (9). Furthermore, antibodies to the 37/40-kDa antigen identify rapid progressors to IDDM and a subgroup of patients with ICA in the absence of anti-GAD antibodies (6, 10, 11). Autoantibodies against 37/40-kDa tryptic fragments have been detected in 54% of newly diagnosed IDDM patients (10).

In this report, we describe the cloning of a cDNA that codes for the precursor of the 37/40-kDa antigen. This antigen is highly homologous to a recently described tyrosine phosphatase.

MATERIALS AND METHODS

Subjects. Preclinical IDDM subjects (one female, three males, mean age of 20.4 years, age range of 12–27 years) defined operationally as asymptomatic were first-degree relatives of patients with IDDM who were found to have ICAs at a level ≥ 20 Juvenile Diabetes Foundation (JDF) units.

Abbreviations: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; GAD, glutamic acid decarboxylase; ICA, islet cell antibody; IDDM, insulin-dependent diabetes mellitus; NP-40, Nonidet P-40; RIN, rat insulinoma.

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Recent-onset IDDM subjects (15 females, 20 males, mean age of 28.7 years, age range of 12–55 years) were insulin-dependent patients, having presented with typical clinical features of IDDM in the previous 2 weeks. Control subjects were normal volunteers (six females, nine males, mean age of 31.8 years, age range of 25–45 years).

Preparation of Cell Extracts. Rat insulinoma (RIN) cells were cultured in Hepes-buffered RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum; simian virus 40 (SV40)-transformed mouse β-islet cells (βTC) (12) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 0.45% glucose supplemented with 15% (vol/vol) horse serum and 2.5% fetal calf serum. For metabolic labeling 2×10^8 cells were washed twice with labeling medium (methionine-free RPMI 1640 medium) and incubated for 5 hr with labeling medium supplemented with 7.15 mCi (1 Ci = 37 GBq) of ^{35}S-labeled methionine/cysteine *in vitro* cell labeling mix (Amersham). Cells were scraped into the medium and washed twice with Hepes buffer (10 mM Hepes·NaOH, pH 7.4/0.15 M NaCl). Cell extracts were prepared essentially as described (8). All steps were performed at 4°C. Briefly, 10^8 cells were resuspended in 1 ml of 10 mM Hepes·NaOH, pH 7.4/0.25 M sucrose/1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and homogenized by 30 passes in a motor-driven homogenizer (Wheaton Scientific). The homogenate was centrifuged for 30 min at 12,000 × *g*. The pellet was resuspended in 0.5 ml of Hepes buffer and centrifuged for 20 min at 12,000 × *g*. The pellet was resuspended in 1 ml of extraction buffer [10 mM Hepes·NaOH, pH 7.4/0.15 M NaCl/1% Nonidet P-40 (NP-40)/0.5% sodium deoxycholate/1 mM AEBSF] and incubated for 1 hr on a rocking platform. The lysate was centrifuged for 20 min at 12,000 × *g*, and the supernatant (detergent extract) was frozen in aliquots at −80°C.

Immunoprecipitation Assay for the 37/40-kDa Antigen. An amount of detergent extract equivalent to 10^7 cpm was incubated for 4 hr with 5 μl of human serum at 4°C on a shaker. Immunocomplexes were collected on 50 μl of protein A-Sepharose (Pharmacia) and washed three times with NP-40-buffer (20 mM Tris·HCl, pH 8.0/1 M NaCl/1% NP-40). After washing once with H_2O, the immunobeads were incubated for 20 min with 0.1 ml of 50 μg of trypsin per ml in Hepes buffer followed by a final wash with H_2O. The beads were resuspended in SDS sample buffer and boiled, and the reaction products were separated by SDS/10% PAGE and were visualized by fluorography.

Library Construction and Screening. Total RNA was isolated from RIN cells with Ultraspec (Biotecx Laboratories, Houston). Poly(A)$^+$ RNA was enriched by two cycles of oligo(dT) affinity chromatography (13). A directional, size-selected (in the range of 1–9 kb) cDNA library was constructed and cloned into Lambda ZAP Express by following a protocol provided by the manufacturer (Stratagene). Immunoscreening was performed essentially as described (14). An aliquot of the library (4×10^5 clones) was screened with a 1:40 dilution of a serum from an IDDM patient that precipitated the 37/40-kDa antigen in our standard immunoprecipitation assay. Reacting phages were purified by secondary and tertiary screening and converted into plasmid DNA by *in vivo* excision of the pBK-CMV phagemid from the Lambda ZAP Express vector by following a protocol provided by the manufacturer (Stratagene).

Analysis of cDNA Clones. The polypeptides encoded by the cDNA clones were synthesized in the presence of [^{35}S]methionine by coupled *in vitro* transcription–translation using a coupled reticulocyte lysate system (TNT, Promega). Coupled transcription–translation reactions (50 μl) were performed with 1.5 μg of pBK-CMV plasmid DNA containing the cDNA of interest. Unincorporated [^{35}S]methionine was separated from the completed translation reactions by size-exclusion chromatography on Nick columns (Pharmacia) equilibrated with NP-40-buffer. Immunoprecipitations with *in vitro* translated cDNA products (IVT extract) were performed essentially as above; however, 5×10^4 cpm of IVT extract was used per reaction, and limited trypsin digestion was omitted where indicated. For blocking experiments, a coupled transcription–translation reaction (100-μl volume) was performed with 3 μg of cDNA in pSPUTK (Stratagene) in the absence of radiolabeled amino acids. Unincorporated amino acids were separated as above, and the IVT extract was preincubated for 2 hr with 5 μl of IDDM serum, followed by the addition of 10^7 cpm of detergent extract. After a 5-hr incubation on a rocking platform, 50 μl of protein A-Sepharose (Pharmacia) was added, and immunoprecipitation reactions were processed as described above.

The DNA sequence of the longest cDNA clone encoding the precursor of the 37/40-kDa antigen was determined by using an automated ABI 373 A DNA sequencer. Sequence analysis was performed by using the GCG package (Genetics Computer Group, Madison, WI).

RESULTS

Detection of 37/40-kDa Tryptic Fragments in Insulinoma Cell Extracts. We have established an assay for the 37/40-kDa antigen based on the immunoprecipitation of ^{35}S-labeled insulinoma cell proteins with sera from IDDM patients. This assay identifies polypeptides with molecular masses of both 37 and 40 kDa in some cases or of 40 kDa only in others after trypsin treatment of the immunobeads (8). The relative amount of radioactivity present in the 40-kDa and 37-kDa bands depends mainly on the serum used for the immunoprecipitation (8). We detected the 37/40-kDa antigen in a RIN cell line and in a cell line derived from simian virus 40-transformed mouse islet β cells (Fig. 1).

Molecular Cloning of the 37/40-kDa Antigen. We constructed a directional cDNA expression library from a RIN cell line. Double-stranded cDNA was size-selected in the range from 1 to 9 kb before cloning into Lambda ZAP Express. The library had a complexity of 3.5×10^6 independent clones. We identified 11 positive clones by immunoscreening of 4×10^5 plaques with a 1:40 dilution of an IDDM serum containing autoantibodies to the 37/40-kDa antigen. The reacting plaques were purified by two rounds of rescreening, and the phages were converted into plasmid DNA by *in vivo* excision of the pBK-CMV phagemid containing the cDNA.

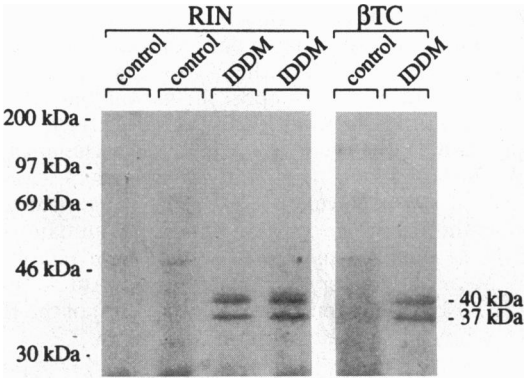

FIG. 1. Immunoprecipitation of the 37/40-kDa antigen from insulinoma cell extracts. RIN and mouse βTC cells were biosynthetically labeled with [^{35}S]methionine and cysteine. A detergent extract was prepared after separating cytoplasmic proteins. Immunoprecipitations were performed with sera from IDDM patients and healthy control individuals and with detergent extract (10^7 cpm per lane). Immunocomplexes were collected on protein-A beads, washed, partially digested with trypsin, and solubilized in sample buffer. The products were separated by SDS/10% PAGE and were visualized by fluorography.

The standard assay for the 37/40-kDa antigen is based on the immunoprecipitation of labeled islet cell proteins with sera from IDDM patients or controls followed by limited trypsin digestion of the precipitated material (8). We tested the products of our cDNA clones in a similar assay. We generated [^{35}S]methionine-labeled polypeptides derived from our cDNA clones by coupled *in vitro* transcription/translation in a reticulocyte lysate system. These polypeptides were immunoprecipitated with sera from IDDM patients and healthy controls. IDDM sera, but not control sera, precipitate the polypeptides derived from three of our cDNA clones. The insert size of these clones are 2.4, 2.8, and 3.6 kb. Restriction analysis suggests that these cDNA clones originate from the same transcript but differ in their extension at the 5′ end. Polypeptides derived from these clones have apparent molecular masses of 64, 73, and 105 kDa, respectively (Fig. 2 *Upper*). To test whether these cDNAs encode the precursor of the 37/40-kDa antigen, we subjected the immunoprecipitated material to limited trypsin digestion, which resulted in all cases in polypeptides with a molecular mass of 40 kDa (Fig. 2 *Upper*). These polypeptides comigrated with the 40-kDa band observed in our standard assay for the 37/40-kDa tryptic fragments performed with labeled RIN cell proteins. Further evidence that the isolated cDNA clones are related to the 37/40-kDa antigen resulted from the analysis of immunoreactivity of the *in vitro* translated cDNA products with IDDM and control sera that we had tested previously in our standard assay for the 37/40-kDa antigen. We performed this experiment with the 105-kDa protein generated from the 3.6-kb-long cDNA clone. None of the control sera showed reactivity with the cDNA product. Five sera from IDDM patients containing autoantibodies to the 37/40-kDa antigen precipitated the *in vitro* translated polypeptide, whereas two sera from IDDM patients not reactive with the 37/40-kDa antigen also did not precipitate the cDNA product (data not shown). We termed the 105-kDa protein derived from the 3.6-kb cDNA clone "ICA105" (for 105-kDa ICA).

We finally investigated whether recombinant ICA105 can block immunoprecipitation of the 37/40-kDa antigen from a labeled RIN cell extract. Coupled *in vitro* transcription/translation reactions were performed with a control clone and the cDNA clone encoding ICA105 without incorporating radiolabeled amino acids. IDDM sera containing autoantibodies to the 37/40-kDa antigen were incubated for 2 hr with the *in vitro* translated cDNA products prior to being tested in the standard immunoprecipitation assay with labeled RIN cell extract. Fig. 2 *Lower* shows that ICA105, but not a control protein synthesized in parallel under identical conditions, can block immunoprecipitation of the 37/40-kDa antigen. Taken together, our data strongly support the notion that ICA105 is indeed the precursor of the 37/40-kDa autoantigen.

Autoantibodies to Recombinant ICA105 in Human IDDM Sera. We tested 35 sera from IDDM patients, 4 sera from individuals at risk to develop IDDM, and 15 control sera to determine the relevance of ICA105 as an autoantigen in IDDM. None of the control sera reacted with radiolabeled ICA105 in our immunoprecipitation assay. Autoantibodies against ICA105 were detected in 54% (19 of 35) of the IDDM sera. Recombinant ICA105 was precipitated by 75% (3 of 4) sera from subjects at risk to develop IDDM. All sera positive for anti-37/40-kDa antibodies in the assay with labeled RIN cell extracts precipitated the *in vitro* translated ICA105 polypeptide.

The 37/40-kDa Autoantigen Is the Putative Tyrosine Phosphatase IA-2. The nucleotide sequence of the 3.6-kb-long cDNA coding for ICA105 has been determined. Analysis of the DNA sequence reveals a 2949-bp open reading frame coding for 983 amino acids (Fig. 3). The predicted open reading frame codes for a protein with a molecular mass of 105 kDa. Analysis of the deduced amino acid sequence reveals that

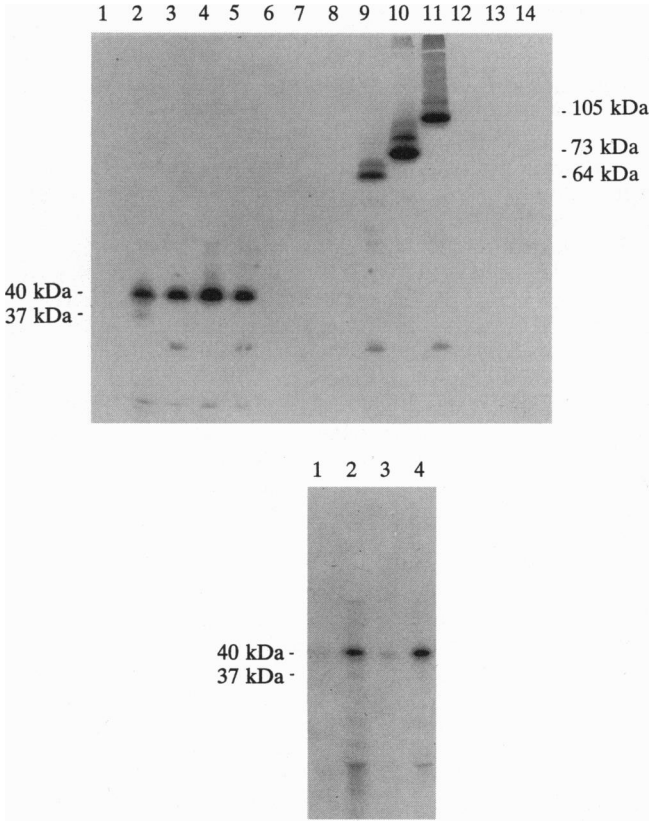

FIG. 2. Identification of ICA105 as the precursor of the 37/40-kDa antigen. (*Upper*) Immunoprecipitation of labeled RIN cell proteins (lanes 1 and 2) and radiolabeled polypeptides generated by coupled *in vitro* transcription and translation of three different cDNA clones isolated by immunoscreening (lanes 3–14). Immunoprecipitations were performed with a control serum (lanes 1, 6–8, and 12–14) and an IDDM serum containing anti-37-kDa antibodies (lanes 2, 3–5, and 9–11). The immunoprecipitated material was subjected to limited trypsin digestion (lanes 1–8) before resuspending in SDS sample buffer and separation by SDS/10% PAGE or partial trypsin digestion was omitted (lanes 9–14). Immunoprecipitation of the three *in vitro* translated cDNA products with IDDM sera without subsequent trypsin digestion treatment results in polypeptides of molecular mass 64 kDa (lane 9), 73 kDa (lane 10), and 105 kDa (lane 11). However, immunoprecipitation of these cDNA products with IDDM sera followed by limited trypsin digestion results in all cases in a 40-kDa polypeptide (lanes 3–5) that comigrates with the 40-kDa band observed in the standard assay performed with labeled RIN cell extract (lane 2). (*Lower*) Recombinant ICA105 can block immunoprecipitation of the 37/40-kDa antigen from RIN cell extracts. A coupled transcription/translation reaction was performed in a 100-μl volume with a control cDNA clone and the cDNA encoding ICA105 in the absence of radiolabeled amino acids. Lanes: 1 and 2, standard assay for the 37/40-kDa antigen with a control serum (lane 1) and an IDDM serum (lane 2); 3, an IDDM serum (5 μl) preincubated for 2 hr with *in vitro* translated unlabeled ICA105, followed by the addition of 10^7 cpm of detergent extract from labeled RIN cells (after a 5-hr incubation on a rocking platform, 50 μl of protein A-Sepharose was added, and immunoprecipitation reactions were processed as described above); 4, same reaction as in lane 3, but preincubation was performed with a control protein generated in parallel under identical conditions.

the putative start codon of the cDNA is followed by a hydrophobic signal peptide (positions 1–31). A second hydrophobic stretch was found between positions 581 and 602 and constitutes most likely a transmembrane domain. These findings suggest that ICA105 is a type I transmembrane protein.

The deduced amino acid sequence is 86% and 93% identical to a putative tyrosine phosphatase IA-2 with a predicted molecular mass of 105 kDa recently cloned from human and mouse cDNA libraries, respectively (15, 16). Sequence align-

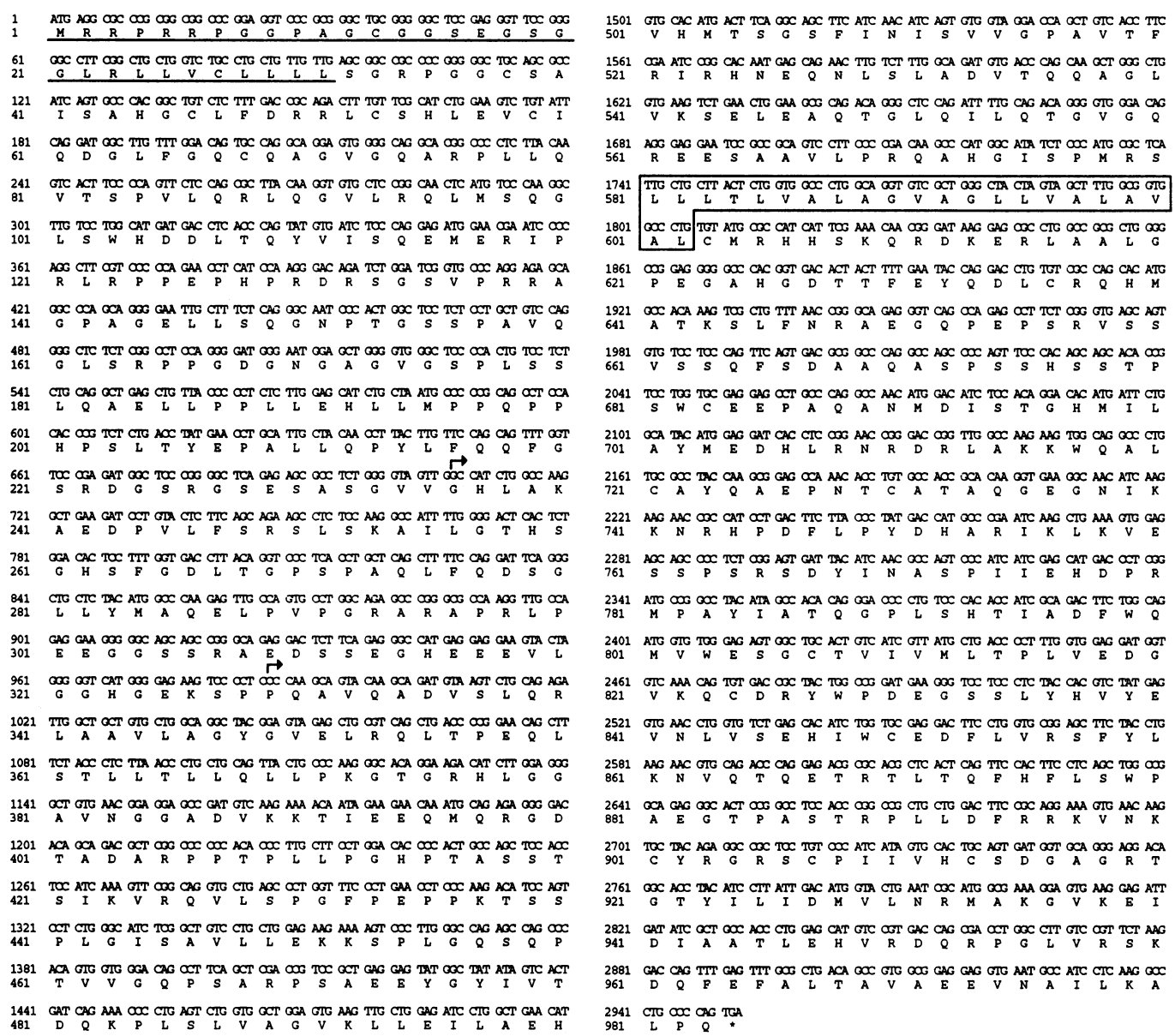

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the rat ICA105-encoding cDNA. The putative signal peptide is underlined and the predicted membrane spanning region is boxed. The 5′ ends of two additional cDNA clones encoding shorter versions of ICA105 that were isolated by immunoscreening are indicated with arrows.

ment demonstrates that the region of homology extends over the whole predicted amino acid sequence (data not shown). Additional homologies are found to a cloned human islet cell antigen 512 (ICA512) with a predicted molecular mass of 60 kDa (17). Amino acid sequence homology is found between positions 400 and 918 of the rat ICA105 sequence. Considering the degree of homology between rat ICA105, human IA-2, and human ICA512, it can be assumed that the latter is a partial cDNA clone encoding IA-2. We conclude that ICA105 is the rat homologue of the human and mouse putative tyrosine phosphatase IA-2.

DISCUSSION

ICA105 is the Precursor of the 37/40-kDa Autoantigen. We have cloned the precursor of the 37/40-kDa autoantigen in IDDM by immunoscreening of a RIN cDNA library. We have called this polypeptide ICA105. Immunoprecipitation of labeled RIN cell proteins with IDDM sera containing autoantibodies to the 37/40-kDa antigen followed by limited trypsin digest of the precipitated material results in two bands with molecular masses of 37 and 40 kDa or in one band with a mass of 40 kDa, depending on the IDDM serum used (Fig. 1 and ref. 8). Immunoprecipitation of *in vitro* translated ICA105 with IDDM sera reacting only with the 40-kDa band in the assay with labeled RIN cell proteins results, as expected, in a 40-kDa band after limited trypsin digestion of the precipitated material (Fig. 2 *Upper*). Performing the same experiment with IDDM sera that react with the 37-kDa and the 40-kDa bands in the standard assay with RIN cell extracts results only in a 40-kDa band after limited trypsin digestion of the immunobeads (data not shown). These findings raised the question of whether the 37-kDa and 40-kDa tryptic fragments originate from a single precursor. Therefore, we analyzed whether preincubation of IDDM sera with unlabeled *in vitro* translated ICA105 can block immunoprecipitation of the 40-kDa and 37-kDa tryptic fragments in the standard assay. As expected, preincubation of IDDM sera with recombinant ICA105 blocks immunoprecipitation of the 40-kDa fragment. However, we could also detect a significant reduction of the 37-kDa frag-

ment (Fig. 2 *Lower*). A possible explanation for these results is that the 37-kDa and 40-kDa tryptic fragments originate from the same polypeptide and that the 37-kDa tryptic fragment results from the posttranslational processing of ICA105 that occurs *in vivo* but not *in vitro*. Alternatively, the 37-kDa and 40-kDa tryptic fragments originate from distinct, but immunologically related polypeptides. This issue should be addressed once monoclonal antibodies directed against recombinant ICA105 become available.

ICA105 and Prediction of IDDM. Precise prediction of IDDM requires the knowledge of genetic and serum markers specific for IDDM (18). Previous studies have suggested that a combined analysis of autoantibodies can yield sufficient sensitivity and specificity to predict development of IDDM (11). Autoantibodies to 37/40-kDa tryptic fragments of islet cell antigens were shown to be specific and sensitive for the prediction of IDDM (6, 9, 19). We have demonstrated that we can detect anti-37-kDa autoantibodies in IDDM serum samples, using *in vitro* translated radiolabeled ICA105. Importantly, all the sera that were found to be positive for anti-37/40-kDa antibodies in the standard assay with labeled RIN cell extract reacted *in vitro* with the translated ICA105 and none of the control sera showed reactivity to recombinant ICA105. The availability of recombinant ICA105 should allow testing of large sample numbers, thus facilitating the diagnosis and the identification of individuals at risk for IDDM.

ICA105 As an Autoantigen. A GenBank data base search showed sequence homology between ICA105 with both the human and mouse putative tyrosine phosphatase IA-2. IA-2 was originally isolated from a subtractive cDNA library constructed from human insulinoma and glucagonoma tissue. IA-2 message was detected in insulin-secreting β-cells or in cell lines derived from insulinoma tissue and in brain tissue (15, 16). GAD has been recently indicated as a key autoantigen in IDDM. Interestingly, GAD is also found in both brain and islets (15). IDDM is a T-cell-mediated disease, and both $CD4^+$ and $CD8^+$ GAD-specific T cells are present in recent onset IDDM patients and individuals at risk to develop diabetes (20, 21). The availability of recombinant ICA105 in sufficient amount should allow us to determine T-cell reactivity to this antigen in IDDM patients. The molecular definition of this IDDM-related autoantigen could hold implications for novel immunotherapeutic strategies such as immunointervention based on administration of recombinant ICA105 in tolerogenic form.

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