Autoantibodies to IA-2 in IDDM

Location of Major Antigenic Determinants

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Thirty-three IDDM sera that immunoprecipitated full-length IA-2 were tested for reactivity with different fragments of the IA-2 molecule. The fragments were prepared by PCR amplification of IA-2 cDNA and by expression in a rabbit reticulocyte transcription/translation system. Whereas all 33 sera reacted with the intracellular domain (amino acid 604 to 979), none of the sera reacted with the extracellular domain of IA-2 (amino acid 31 to 577). Analysis of the reactivity of IDDM sera with the different regions of the intracellular domain showed that 94% (31 of the 33) reacted with the COOH-terminus (amino acid 771 to 979), 40% reacted with the NH_2-terminus (amino acid 604 to 776), and 40% reacted with the middle portion (amino acid 692 to 875). Of the 31 sera that reacted with the COOH-terminus, 14 of these reacted only with the COOH-terminus and with no other region. Of the 13 sera that reacted with the NH_2-terminus, only one reacted exclusively with the NH_2-terminus. Treatment of the different domains of IA-2 with trypsin showed that only the COOH-terminus was resistant to trypsin, arguing that it is from this region of the IA-2 molecule that the 40-kDa tryptic fragment from insulinoma cells is derived. From these experiments, it is concluded that the major antigenic determinant of IA-2 is located at the COOH-terminus and that minor antigenic determinants are located at the NH_2-terminus and middle portion of the intracellular domain. *Diabetes* 46:40–43, 1997

IA-2 is a new member of the protein tyrosine phosphatase (PTP) family (1,2) and is a major autoantigen in IDDM (3–7). Up to 70% of patients with IDDM have autoantibodies to IA-2, and the presence of autoantibodies in nondiabetic subjects strongly indicates that the individual is at a high risk of eventually developing clinically apparent IDDM. IA-2 has a signal peptide, an extracellular domain, a transmembrane region, an intracellular domain, and a molecular mass of 106 kDa. Treatment of the full-length molecule or the intracellular domain with trypsin results in a fragment of 40 kDa to which IDDM patients have autoantibodies (5–8). Absorption of IDDM sera containing islet cell autoantibodies (ICA) with recombinant IA-2 protein results in a decrease of the immunofluorescent reactivity of the sera with pancreatic islet cells (3,7). These and other experiments argue that IA-2, together with glutamic acid decarboxylase (GAD_{65}), represent major autoantigens in IDDM (9–12). However, the location of the amino acid sequence within the IA-2 molecule to which autoantibodies react is not known. The present experiments, using radiolabeled in vitro translated segments of the IA-2 molecule, were initiated to locate the major antigenic determinants.

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PTP, protein tyrosine phosphatase; PCR, polymerase chain reaction; ICA, islet cell autoantibodies; ID, intracellular domain; ED, extracellular domain; ID-N, intracellular domain NH_2-terminus; ID-M, intracellular domain middle portion; ID-C, intracellular domain COOH-terminus.

RESEARCH DESIGN AND METHODS

Sera. Thirty-three IDDM sera were obtained from clinically diagnosed IDDM patients 3–45 years old. These sera, kindly provided by Dr. Noel Maclaren, have been used extensively in the study of IA-2 (3). In the examination of the antigenicity of the wild type IA-2, using a conventional immunoprecipitation procedure followed by electrophoresis and autoradiography, all of these sera were shown to contain antibodies against full-length IA-2. Twenty nondiabetic sera were obtained from healthy individuals who had no history of IDDM among their first-degree relatives, but matched in age, sex, and geographical location of residence to the IDDM patients.

Plasmids. To amplify regions of the human IA-2 molecule, polymerase chain reactions (PCR) were performed with 10 ng of IA-2 containing plasmid DNA, 5 U of Taq polymerase (Perkin Elmer Corp., Norwalk, CT), 200 μmol/l each of the triphosphate deoxyribonucleosides (dATP, dGTP, dCTP, and dTTP), 10 mmol/l of $MgCl_2$, and 0.2 pmol/l of forward and reverse primers.

Forward primers. The lowercase letters represent nucleotides that are added to create a Kozak consensus sequence (13). The numbers in the brackets indicate the positions of the first and last nucleotides of the primer corresponding to the IA-2 cDNA.

5′-agccgccaccatgGGCTGCAGCGCCGTTAGTG (164–182)
5′-gccgccaccatgGCGCGGCAGCAAGACAAG (1883–1900)
5′-gccgccacCATGGAGGATCACCTGC (2167–2183)
5′-gccgccaccatggTTATTGAGCATGACCCTCG (2381–2400)

Reverse primers. The lowercase letters represent nucleotides that are added as stop codons. The numbers indicate the positions of the first and last nucleotides of the primer corresponding to the IA-2 cDNA.

5′-tcattaCACTGAGCGCATGGGTGAGGTGCT (1804–1781)
5′-GCTGCCCGCCAAGGGGCCCCA (3039–3019)
5′-tcattaCCGAGGGTCATGCTCAATAAT (2381–2401)
5′-tcattAGCTGAGGAAGTGGAACTG (2678–2696)

The PCR products were subcloned into a vector, pCRII (Invitrogen Corp., San Diego, CA), the cloning site of which is flanked by T7 and SP6 promoters.

In vitro transcription and translation. The polypeptides of IA-2 were synthesized in vitro with a nuclease-treated rabbit reticulocyte transcription/translation system (Promega, Madison, WI) in the presence of ^{35}S-methionine (>3,000 mCi/mmol, Amersham, Arlington Heights, IL), following the manufacturer's instructions. To separate the polypeptides from free amino acids, the in vitro translated products were passed through a NAP25 column (Pharmacia Biotech, Piscataway, NJ). The radiolabeled polypeptides were eluted with PBS, and counts per minute (cpm) were obtained by liquid scintillation counting.

Immunoprecipitation assay. The procedure of Grubin et al. (14) was followed with slight modification. Briefly, ~100,000 cpm of a radiolabeled polypeptide was incubated with 5 μl of a serum sample overnight at 4°C on a rotating platform in 100 μl of precipitation buffer (20 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl,

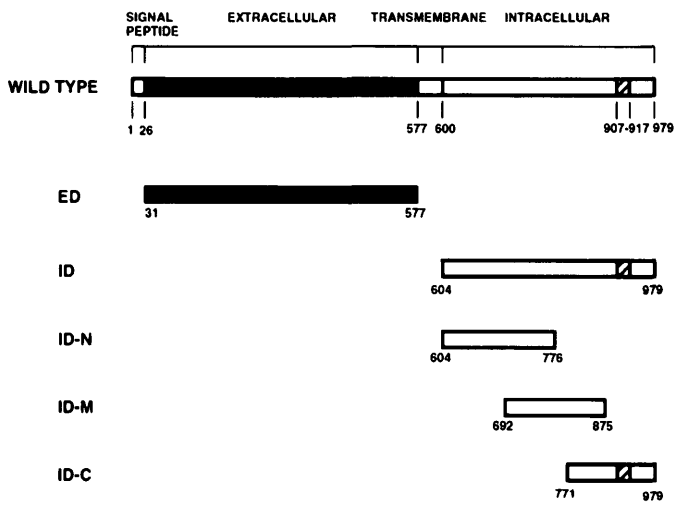

FIG. 1. IA-2 and recombinant constructs. Numbers designate the amino acid positions. ED and ID span the entire extracellular and intracellular domains, respectively. ID-N, ID-M, and ID-C represent the NH_2-terminus, middle portion, and COOH-terminus of the intracellular domain, respectively. Shaded box (amino acid 907 to 917) represents the core sequence of the PTP domain.

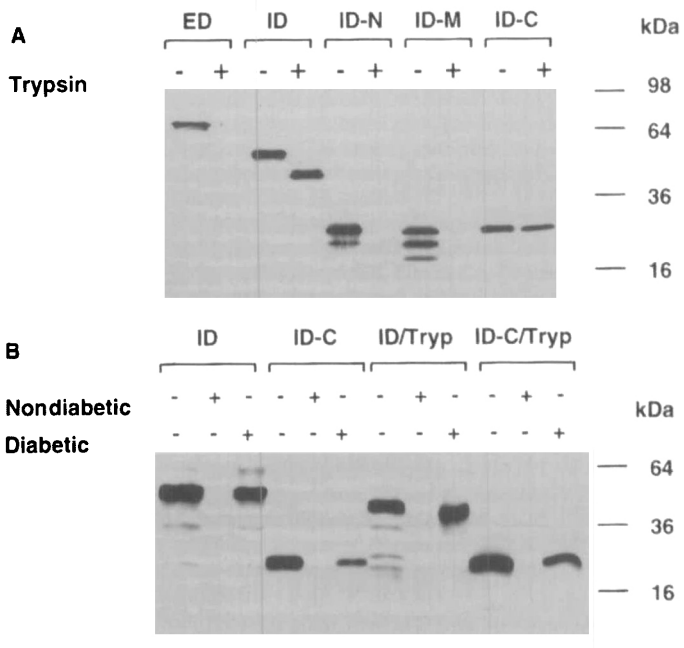

FIG. 2. Tryptic fragments of intracellular domain and their reactivity with IDDM serum. *A*: radiolabeled peptides of IA-2, trypsin-treated (50 mg/ml) and untreated, were subjected to SDS-PAGE and autoradiography. *B*: radiolabeled peptides, trypsin-treated and untreated, were immunoprecipitated with a IA-2–positive diabetic sera and subjected to SDS-PAGE and autoradiography. Nondiabetic sera served as the negative control.

1% Triton X-100, and 0.1% aprotinin). Fifty microliters of protein A agarose (Life Technologies, Gaithersburg, MD) were added, and the incubation was continued for another hour. After washing three times with the precipitation buffer, the beads were transferred to multiscreen-HV 96-well filtration plates (0.45 μm; Millipore, Marlborough, MA). The plates were washed four more times in a multiscreen vacuum manifold apparatus, and the precipitated beads were punched into scintillation vials for counting. For each polypeptide, counts of the nondiabetic group were averaged. The mean plus three standard deviations was used as a base value. The count per minute of each diabetic sample was divided by the base value and expressed as n-fold increase.

RESULTS

The intracellular and extracellular domains of IA-2 were synthesized in a rabbit reticulocyte transcription/translation system (Fig. 1) and visualized by SDS-PAGE and autoradiography (Fig. 2*A*). To localize the antigenic sites with which autoantibodies to IA-2 in patients with IDDM react, 33 diabetic sera that we previously showed immunoprecipitated IA-2 were incubated with the intracellular and extracellular domains of IA-2. As seen in Fig. 3, all 33 sera immunoprecipitated the intracellular domain of IA-2. Reactivity ranged from 5- to ~35-fold above background. In contrast, none of the sera reacted with the extracellular domain of IA-2.

To further define the antigenic regions within the intracellular domain, the COOH-terminus (ID-C), NH_2-terminus (ID-N), and middle portion (ID-M) of the intracellular domain were synthesized in the rabbit reticulocyte transcription/translation system (Fig. 1). On SDS-PAGE, multiple bands were observed, ranging from 18 to 25 kDa, probably due to different internal transcription start sites (Fig. 2*A*). Immunoprecipitation with the same sera that were used in Fig. 3 revealed that 32 of the 33 sera that reacted with the intracellular domain of IA-2 also reacted with one or more of the recombinant peptides (Table 1). Of the 33 sera, 31 (94%) reacted with the ID-C, 13 (40%) reacted with the ID-N, and 13 (40%) reacted with the ID-M. Fourteen of the sera reacted only with the ID-C, and one serum reacted only with the ID-N. None of the sera reacted only with the ID-M. Of the 33 sera, eight reacted with all three intracellular domains and nine reacted with two of the intracellular domains.

Recent experiments showed that treatment of full-length IA-2 with trypsin resulted in a 40-kDa fragment that could be immunoprecipitated by IDDM sera (5–8). To determine which regions of the IA-2 molecule were trypsin sensitive, the intracellular and extracellular domains, ID-C, ID-M, and ID-N were treated with trypsin, run on SDS-PAGE, and visualized by autoradiography. Figure 2*A* shows that the extracellular domain, ID-N, and ID-M were digested to completion, and no bands were detected. In contrast, the intracellular domain was cleaved into a 40-kDa fragment, whereas the ID-C was totally resistant to trypsin cleavage. Both the 40-kDa tryptic fragment and the trypsin-treated ID-C remained susceptible to immunoprecipitation by diabetic, but not by nondiabetic, sera (Fig. 2*B*).

DISCUSSION

The fact that IA-2 has an extracellular domain makes it a potential immune target. Antibodies directed against membrane proteins on the surface of pancreatic β-cells could lead to membrane damage and β-cell destruction (15). Immunoprecipitation of radiolabeled β-cell lysate with IDDM sera, however, has failed to demonstrate molecules in the molecular size range of intact IA-2 (i.e., 106 kDa) (M.S.L., A.-L.N., unpublished observations). It is therefore possible that the extracellular domain of IA-2 is processed and/or broken down so that very little of it is present on the surface of β-cells. If this is the case, the extracellular domain of IA-2 may not be a major target involved in the immunological destruction of β-cells. The present study, in fact, shows that the autoantibodies in IDDM sera are directed to the intracellular, and not to the extracellular, domain of IA-2.

Autoantibodies to antigens expressed intracellularly are found in a number of autoimmune diseases, but as in the case

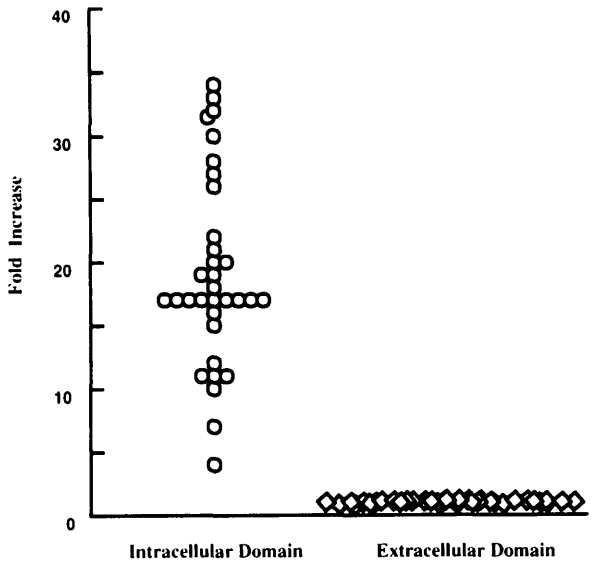

FIG. 3. Reactivity of 33 IDDM sera with intracellular and extracellular domains of in vitro translated recombinant IA-2. Fold increase represents the counts per minute precipitated by diabetic sera divided by the mean counts per minute plus three standard deviations precipitated by nondiabetic sera.

of autoantibodies to IA-2, their role in pathogenesis is not known (15). Although the current data clearly point to ID-C as the major autoantigenic determinant, the formal possibility that ID-N and ID-M might be antigenic but lose conformation when expressed in vitro, cannot be excluded. The fact that some IDDM sera react with ID-N and ID-M argues that conformational changes are not the primary explanation for the poor reactivity of most IDDM sera with these recombinant peptides. However, attempts to further localize the antigenic site(s) within the 208–amino acid ID-C by expressing even smaller peptides in vitro and immunoprecipitating them with IDDM sera has not been successful. It is quite possible that these even smaller recombinant peptides do lose their conformation and are not recognized or precipitated by IDDM sera. The demonstration here that 94% of IDDM sera do react with ID-C points to the importance of this portion of the molecule as the major antigenic determinant and explains the lower sensitivity of earlier immunoassays that used truncated forms of IA-2 lacking the correct terminal portion of the molecule (amino acid 915–979) (4).

Although only a small number of samples have been tested thus far, we have no evidence to support the idea that the immune response spreads during the course of the disease, in any significant way, to other regions of the IA-2 molecule. Sera from diabetic subjects tested 1 year after the onset of clinical symptoms reacted with the same region of the IA-2 molecule (i.e., ID-C) as sera taken one or more years before the appearance of clinical symptoms (B.Z., M.S.L., A.-L.N., unpublished observations). Studies are now underway to determine whether T-cells from IDDM patients react with IA-2, and if so, whether there is epitope spread at the T-cell level.

Recent studies have shown that the 40-kDa tryptic fragment from insulinoma cells, with which IDDM sera react, is derived from IA-2 (5–8). Precisely where on the IA-2 molecule this fragment is located, however, has not been established. The present experiments show that trypsin readily cleaves the extracellular domain, ID-N, and ID-M into fragments that could not be detected on SDS-PAGE. Trypsin, however, failed to cleave ID-C, arguing that it is from this region of the IA-2 molecule that the 40-kDa tryptic fragment is derived.

TABLE 1
Reactivity of IDDM sera with peptides of the intracellular domain of IA-2

	Domains			
Sera	ID	ID-C	ID-M	ID-N
1	22.9	11.3		
2	17.0	2.7		
3	17.3	2.7	1.6	3.3
4	19.6	10.6	1.2	8.1
5	27.7	2.0		
6	32.5	17.9		
7	26.9	15.8	1.7	15.2
8	25.7	8.8		
9	7.0	2.3		
10	17.6	2.9	1.5	
11	16.9	8.2		
12	31.0	5.4		
13	17.8	2.9		
14	16.7	1.3		
15	31.7	3.2		1.8
16	33.6			2.5
17	17.3	19.4	2.1	1.8
18	17.0	24.9	1.2	
19	15.7			
20	9.0	6.4		1.3
21	10.2	19.2		
22	22.8	62.3	4.5	14.0
23	16.9	17.6		2.9
24	11.3	15.0	2.0	
25	6.0	6.3		
26	13.7	12.0	1.3	
27	18.8	35.7	1.8	
28	19.7	38.9	1.4	2.0
29	18.3	54.4	5.9	2.4
30	18.8	48.4	2.3	2.4
31	9.1	17.9		
32	15.6	4.9		
33	3.8	5.3		3.5

Data are n-fold increases, as indicated in the legend to Fig. 3.

Although it is clear that the autoantibody response to IA-2 is a good marker for IDDM (3) and a prognostic indicator for identifying individuals at high risk of ultimately developing clinical diabetes (M. Maclaren, M.S.L., A.-L.N., unpublished observations), the role of the immune response to this molecule in the pathogenesis of IDDM still remains to be established.

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REFERENCES

1. Lan MS, Lu J, Goto Y, Notkins AL: Molecular cloning and identification of a receptor-type protein tyrosine phosphatase, IA-2, from human insulinoma. *DNA Cell Biol* 13:505–514, 1994
2. Lu J, Notkins AL, Lan MS: Isolation, sequence and expression of a novel mouse brain cDNA, mIA-2, and its relatedness to members of the protein tyrosine phophatase family. *Biochem Biophys Res Commun* 204:930–936, 1994
3. Lan MS, Wasserfall CW, Maclaren NK, Notkins AL: IA-2, a transmembrane pro-

tein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. *Proc Natl Acad Sci USA* 93:6367–6370, 1996
4. Rabin DU, Pleasic SM, Shapiro JA, Yoo-Warren H, Oles J, Hicks JM, Goldstein DE, Rae PMM: Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. *J Immunol* 152:3183–3188, 1994
5. Payton MA, Hawkes CJ, Christie MR: Relationship of the 37,000- and 40,000-Mr tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512). *J Clin Invest* 96:1506–1511, 1995
6. Passini N, Larigan D, Genovese S, Appella E, Sinigaglia F, Rogge L: The 37/40-kilodalton autoantigen in insulin-dependent diabetes mellitus is the putative tyrosine phosphatase IA-2. *Proc Natl Acad Sci USA* 92:9412–9416, 1995
7. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E: Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol* 155:5419–5426, 1995
8. Lu J, Li Q, Xie H, Chen Z-J, Borovitskaya AE, Maclaren NK, Notkins AL, Lan MS: 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. *Proc Natl Acad Sci USA* 93:2307–2311, 1996
9. Christie MR, Tun RYM, Lo SSS, Cassidy D, Brown TJ, Hollands J, Shattock M, Bottazzo GF, Leslie RDG: Antibodies to GAD and tryptic fragments of islet 64K antigen as distinct markers for development of IDDM. *Diabetes* 41:782–787, 1992
10. Baekkeskov S, Aanstoot H-J, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Oleson H, DeCamilli P: Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 347:151–156, 1990
11. Baekkeskov S, Landin M, Kristensen JK, Srikanta S, Bruining GJ, Mandrup-Poulsen T, De Beaufort C, Soeldner JS, Eisenbarth G, Lindgren F, Sundkvist G, Lernmark A: Antibodies to a 64,000-Mr human islet cell antigen precede the clinical onset of insulin-dependent diabetes. *J Clin Invest* 79:926–934, 1987
12. Atkinson M, Maclaren NK, Scharp DW, Lacy PE, Riley W: 64,000 Mr autoantibodies as predictors of insulin-dependent diabetes. *Lancet* 335:1357–1360, 1990
13. Kozak M, An analysis of 5′ noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res* 15:8125–8132, 1987
14. Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, Karlsen AE, Boel E, Michelsen B, Lernmark A: A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* 37:344–350, 1994
15. Atkinson MA, Maclaren NK: The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* 331:1428–1436, 1994