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IA-2 (Islet Cell Antigen 512) Is the Primary Target of Humoral Autoimmunity Against Type 1 Diabetes-Associated Tyrosine Phosphatase Autoantigens

Ezio Bonifacio, Vito Lampasona, and Polly J. Bingley

IA-2 (islet cell Ag 512) and IA-2β (phogrin/IAR) are related autoantigens associated with type 1 diabetes. To determine the critical regions for autoantibody binding and which of these autoantigens is the primary target, mutant and chimeric constructs were used to characterize Ab epitope binding in sera from 217 new onset patients with type 1 diabetes and sequential samples from 141 islet cell Ab positive first degree relatives of patients. All 22 relatives and 121 of 129 patients with IA-2/IA-2β to characterize Ab epitope binding in sera from 217 new onset patients with type 1 diabetes and sequential samples from 141 islet regions for autoantibody binding and which of these autoantigens is the primary target, mutant and chimeric constructs were used to develop in follow-up samples. In each of these, Abs to IA-2-specific epitopes were the first detected. In three, spreading to epitopes shared between IA-2 and IA-2β in subsequent samples was seen. In the 17 relatives who developed type 1 diabetes, progression to disease was associated with reactivity to multiple IA-2/IA-2β epitopes. These data suggest that IA-2 is the primary phosphatase-like autoantigen associated with type 1 diabetes and that studying autoantibody epitope diversity may assist in disease prediction.


The novel protein tyrosine phosphatase (PTP)β-like proteins IA-2 (islet cell Ag 512) and IA-2β (phogrin/islet cell Ag-related PTP) are autoantigens associated with type 1 diabetes (1–3) and have been recognized as the target molecules of the 40/37K Abs that immunoprecipitate 40- and 37-kDa proteolytic islet polypeptides (4–7). IA-2 and IA-2β are transmembrane proteins expressed within the secretory granule membrane of neuroendocrine cells (8, 9). Their intracellular regions are characterized by the presence of a single PTP-like domain of ~300 amino acids and a short region (juxtamembrane domain) of ~100 amino acids, which links the PTP-like domain to the membrane anchoring portion. The luminal ectodomains of both proteins are ~600 amino acids, most of which are cleaved to yield mature proteins of ~64 kDa (9). The two proteins share 88% amino acid sequence homology between their PTP-like domains, ~50% homology between their juxtamembrane domains, and only ~10% homology between their ectodomains. IA-2 and IA-2β are relatively distant from classic transmembrane PTPs (10), and autoantibodies in diabetes do not recognize PTPs other than IA-2 and IA-2β (11). No phosphatase activity has been found for the IA-2 protein (12), and only weak activity has been reported for a human clone of IA-2β (3). Partial phosphatase activity can be restored by substituting residues 877 and 911 of IA-2 with those found on the classic transmembrane PTPs (13).

The finding of two structurally related autoantigens presents the possibility of studying aspects of the maturation of the autoimmune response in disease. Autoantibodies found in diabetes bind to epitopes within the intracellular portions of IA-2 and IA-2β, and there is considerable Ab cross-reactivity between the two proteins (4, 7, 11, 14). It has been shown that IA-2 can partially or completely inhibit the binding of autoantibodies to the 37-kDa tryptic islet polypeptide (4–6) and to IA-2β (2, 7, 14), indicating that some of the autoantibody epitopes are shared. Epitopes have been recognized in the intracellular region of IA-2 within both the PTP-like and the juxtamembrane domains (11, 15), while for IA-2β, the epitopes have been only partially identified (14). To determine which are the critical regions for initial autoantibody binding and which of the PTP-like autoantigens is the primary target of humoral autoimmunity, we have analyzed IA-2 and IA-2β autoantibody epitopes of sera from patients with type 1 diabetes and examined changes in epitope recognition in sequential islet Ab containing samples from relatives of patients. These studies suggest that IA-2-specific epitopes are the initial targets of the diabetes-associated humoral autoimmunity against the PTP autoantigens and have demonstrated intra- and intermolecular spreading to epitopes shared with the structurally related IA-2β protein, allowing identification of the potentially critical residues involved in this initial humoral response.

Materials and Methods

Serum samples were obtained within 2 wk of type 1 diabetes onset from 217 patients who presented at the San Raffaele Hospital between 1988 and
allow in vitro transcription/translation. The amplified cDNA was cloned with mutagenized primers in different combinations: IA-2a and phogrin-IC-3,5 ers phogrin-IC-5,9 from reverse-transcribed RIN 5AH cells total RNA, with the specific prim-15ular portion (amino acids 629-1004) of the rat protein was PCR amplified (phogrin; GenBank accession no. Z50735), a cDNA encoding the intracel-lular region of IA-2b was amplified utilizing the QuickChange (Stratagene) method using mutagenesis-specific oligonucleotides. The following amino acid residues were modified: aa 877 Ala > Asp; aa 911 Asp > Ala; aa 877 Ala > Asp + aa 911 Asp > Ala.

In vitro translation and immunoassay

Purified plasmid DNA of the constructs was obtained by Qiagen spin column preparation (Diagen, Hilden, Germany) and in vitro translated and translated utilizing the TnT SP6-coupled rabbit reticulocyte lysate system (Promega in the presence of [35]S)methionine (Amersham), according to the manufacturer’s instructions. Unincorporated [35]S)methionine was removed by gel chromatography on a NAP5 column (Pharmacia, Uppsala, Sweden). The correspondence of the molecular size for all translated pro-teins, that predicted from the amino acid sequence was verified by SDS-PAGE and autoradiography. The presence of PTP-like domain protein in the standard Ab radiobinding assay. The following chimeric IA-2/IA-2β PTP domains. These were obtained and tested upon insertion of single nucleotide changes into the cytoplasmic portion of the IA-2 cDNA by the QuickChange (Stratagene) method using mutagenesis-specific specific oligonucleotides. The following amino acid residues were modified: aa 877 Ala > Asp; aa 911 Asp > Ala; aa 877 Ala > Asp + aa 911 Asp > Ala.

Cloning of IA-2 and IA-2β constructs

Constructs used in this study are shown in Figure 1. The IA-2a-749-979 construct was used to identify IA-2 Abs. The IA-2a-749-979 and IA-2b-645-682 constructs were used to identify Abs binding to the juxtamembrane region of IA-2, and the IA-2a-847-979 construct was used for Abs binding to the PTP-like domain of IA-2. These were prepared in the pSP64 poly(A)IA-2a-749-979 or pGEM-T (IA-2a-749-979 and IA-2b-645-682) cloning vectors (Promega, Madison, WI) under the control of the SP6 promoter as de-scribed previously (11).

Human IA-2β and rat phogrin. cDNA clones encoding the intracellular portion of human IA-2β (amino acids 662-1033) corresponding to the ICAAR cDNA sequence (GenBank accession no. Y08569) (16), the jux-tamembrane membrane domain (amino acids 662-744), and PTP-like domain (amino acids 741-1033) were subsequently obtained after PCR amplification (Per-kinElmer, Norwalk, CT) of reverse transcribed (Amersham, Aylesbury, U.K.) total RNA extracted from human islets using the following specific primers in different combinations: IA-2β-IC-5, 5'-GGTTACCATGCT CAGCAACAGGCTAAGAGA-3', EST T33358-3', 5'-GGGTGAT CCTAAACTGCGCTGACTACG-3', IA-2β-IM-3', 5'-GGGGTACCCT CAGGAGGATGCATTGTTGGA-3', and IA-2β-PPT-5', 5'- CAACATGGAACCCCTC-3'.

The IA-2βIC-5' primer contains an artificial in-frame start codon fol-lowed by an alanine codon to allow efficient expression of the amplified product. A cDNA clone containing a single point mutation, which encodes a stop codon at amino acid 999, was also isolated during the clone screen-ing procedure. The products were cloned into the pGEM-T vector. The IA-2β-662-1033 construct was used to identify IA-2β Abs. The IA-2β-662-744 construct was used for identification of Abs binding to the juxtamembrane region of IA-2β, and the IA-2β-741-1033 construct was used for Abs binding to the PTP-like domain of IA-2β. For bacterial expression, the cDNAs were inserted into the pGEM-T vector. For bacterial expression, the cDNAs were inserted into the pGEM-T vector. The following chimeric IA-2/IA-2β PTP domains. These were obtained and tested upon insertion of single nucleotide changes into the cytoplasmic portion of the IA-2 cDNA by the QuickChange (Stratagene) method using mutagenesis-specific specific oligonucleotides. The following amino acid residues were modified: aa 877 Ala > Asp; aa 911 Asp > Ala; aa 877 Ala > Asp + aa 911 Asp > Ala.

Competition experiments

Identification of IA-2 and IA-2β PTP-like domain-specific and cross-reactive Abs was performed with competition by the alternate bacterially expressed PTP-like domain protein in the standard Ab radiobinding assay. The pTyr-His bacterial expression vector (Xpress expression and purifi-cation system, Invitrogen) containing IA-2β-847-979 and IA-2β-847-979 was transformed into the INVXe Escherichia coli strain. After culturing to early log phase, protein expression was induced by the addition of 1 mM isopropyl-b-D-thiogalactopyranoside. Bacteria were harvested 4 h after induction and resuspended in 10 mM HEPEs, 150 mM NaCl (pH 7.4) containing 10 mM benzanidine, 0.1% aprotinin, and 2% Triton X-100, followed by incubation for 2 h at 4°C with shaking. Insoluble material was removed by centrifugation at 15,000 × g for 15 min. Competition was conducted by preincubating the sera with the IA-2 or IA-2β PTP-like do-main preparations containing ~5 µg of recombinant protein, or control E. coli extract, for 1 h at 4°C before adding, respectively, radiolabeled IA-2β or IA-2β PTP-like domain and conducting an immunoassay as described above. Sera were considered to contain cross-reactive PTP-like domain Abs if binding to radiolabeled IA-2β-847-979 was at least partially inhibited (>10%) by unlabeled IA-2β-847-979, and binding to radiolabeled IA-2β-847-979 was at least partially inhibited (>10%) by unlabeled IA-2β-847-979. Sera were considered to contain IA-2-specific PTP-like domain
Abs if binding could only be detected against the IA-2 687–979 protein and not IA-2β 741–1033 or if binding to IA-2 687–979 remained above 1.5 U after competition with unlabeled IA-2β 741–1033. Sera were considered to contain IA-2β-specific Abs if binding to IA-2β 741–1033 remained above 1.5 U after competition with unlabeled IA-2 687–979 indicating the presence of Abs. These two sera did not immunoprecipitate the 37-kDa islet tryptic polypeptide from IA-2β, and therefore these sera also contained IA-2β-specific Abs only (Table II).

**Results**

**IA-2 and IA-2β Ab epitopes in type 1 diabetes and prediabetes**

IA-2 Abs were detected in 129 (59%) of 217 patients and in 22 (16%) of 141 relatives with ICA ≥ 10 Juvenile Diabetes Foundation (JDF) units; Abs to IA-2β were found in 90 (41%) patients and 14 (10%) of the relatives (Table I). None had Abs to IA-2β in the absence of IA-2 Abs. Among those with IA-2 Abs, reactivity was evenly distributed between epitopes found within the juxtamembrane region (84 (65%) of patients and 17 (77%) of 22 relatives) and the PTP-like domain (112 (87%) patients and 17 (73%) relatives); 67 (52%) patients and 12 (55%) relatives with IA-2 Abs had reactivity to both regions. None of the sera binding IA-2β Abs had reactivity to both regions. None of the sera binding IA-2 Abs had IA-2β-specific Abs if binding to IA-2β 741–1033 remained above 1.5 U after competition with unlabeled IA-2β 741–1033. Sera were considered to contain IA-2β-specific Abs if binding to IA-2β 741–1033 remained above 1.5 U after competition with unlabeled IA-2 687–979.

**Statistical analysis**

A Kaplan-Meier survival analysis was used to determine the cumulative risk of the development of type 1 diabetes. Follow-up time was calculated from the date of the first sample found to contain IA-2 Abs to the date of diabetes onset or last contact. Diabetes-free survival was compared with the log rank test. The 5-yr risks are given as the cumulative risk with a 95% confidence interval (CI) calculated from the SE. The Statistical Package for Social Sciences (SPSS, Chicago, IL) was used for statistical analyses.

**FIGURE 2.** IA-2β reactivity is contained within the PTP-like domain. Ab reactivity to the intracellular region (IA-2β662–744), the juxtamembrane region (IA-2β662–744), and the PTP-like domain of IA-2β (IA-2β741–1033) by sera with IA-2β Abs (lanes 1–8) was examined.

**FIGURE 3.** Cross-reactivity between IA-2 and IA-2β PTP-like domains. Binding to the PTP-like domains of IA-2 and IA-2β is shown after competition with control buffer (lanes), and with IA-2β PTP-like domain (lane for IA-2 or IA-2 PTP-like domain (lanes for IA-2β). Sera contain combinations of Abs which are specific for the IA-2 PTP-like domain (sera 2, 3, 4, 6), cross-reactive between the IA-2 and IA-2β PTP-like domains (sera 4, 5, 6), and specific for the IA-2β PTP-like domain (sera 4, 5) as indicated.

**Ab epitopes in seroconverters**

Of the 22 first-degree relatives with IA-2 or IA-2β Abs, 4 had undetectable Ab levels in their initial sample and developed IA-2/IA-2β Ab reactivity during follow-up (shown in Table III).
each of the 4 relatives Abs were reactive with IA-2-specific epitopes, either in the juxtamembrane region or the PTP-like domain, in the first sample in which Abs were detected. In 3 of these subjects (A, B, and D in Table III), binding to IA-2 could not be inhibited with rIA-2-β (data not shown). One had Abs to IA-2-specific PTP-like domain epitopes only (A), one to IA-2 juxtamembrane epitopes only (D), and one to both IA-2-specific PTP-like domain and juxtamembrane region epitopes (B). In relatives A and B, subsequent samples had additional Abs that bound to epitopes shared between the PTP-like domains of IA-2 and IA-2. In relative C, Abs binding to IA-2-specific PTP-like domain epitopes were detected concurrently with weak Abs to PTP-like domain epitopes shared between IA-2 and IA-2. Both Ab nucleotides increased in titer in subsequent samples.

Table II. Combinations of Abs to IA-2/IA-2β specificities

<table>
<thead>
<tr>
<th>IA-2/IA-2β Ab Specificity</th>
<th>Patients at Diabetes Onset (n = 217)</th>
<th>ICA+ Relatives (n = 141)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>11 (5%)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>32 (15%)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>7 (3%)</td>
<td>1 (0)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>14 (6%)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>10 (5%)</td>
<td>3 (1)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>2 (1%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>20 (9%)</td>
<td>4 (2)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>15 (7%)</td>
<td>5 (2)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>10 (5%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>8 (4%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>0 (0%)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>JM IA-2PTP X-react βPTP</td>
<td>88 (41%)</td>
<td>119</td>
</tr>
</tbody>
</table>

a JM, IA-2 juxtamembrane Abs; IA-2PTP, IA-2-specific PTP-like domain Abs; X-react, Abs binding both IA-2 and IA-2β PTP-like domains; βPTP, IA-2β-specific PTP-like domain Abs.

b Numbers in parentheses indicate how many have developed type 1 diabetes.

Regions and residues unique for IA-2-specific PTP domain Ab binding

To identify which IA-2 residues were involved in the binding of the IA-2-specific PTP-like domain Abs, IA-2/IA-2β chimeric constructs were made. Sera with IA-2-specific PTP-like domain Abs only showed binding to chimeras IA-2687–889/IA-2β943–1033 and IA-2β741–848/IA-2β795–889/IA-2β943–1033, which contained IA-2 residues 795 to 889, but not to chimeras IA-2β741–942/IA-2β890–979 and IA-2β849–942/IA-2β890–979 which lacked these residues (Fig. 4). The IA-2β-specific Abs showed binding to IA-2β741–942/IA-2β890–979 and none of the other constructs, indicating that IA-2β-specific residues within both regions 741–848 and 849–942 were necessary for Ab binding. Binding epitopes shared between IA-2 and IA-2β bound all constructs. A comparison of IA-2 and IA-2β PTP-like domains is shown in Figure 5. Within IA-2β741–848, IA-2β-specific residues 804, 813, 821, 822, 862, 876, 877, 878, 880, and 886 represent nonconserved changes, and residues 815, 818, 820, 834, 836, 860, 867, 882, and 884 represent conserved differences between IA-2 and IA-2β.

Previous studies recognized residues 877 and 911 of IA-2 as important changes from those found in PTPs with classic PTPase activity; replacement of these residues with those found in conventional PTPs partially restored PTPase activity. Replacement of residue 877 or residue 911 also altered Ab binding. Binding in sera with Abs to either IA-2-specific PTP domain epitopes or epitopes shared between IA-2 and IA-2β was reduced 28 to 52% when

Table III. IA-2/IA-2β Ab reactivity in sequential samples from seroconverters

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age at Sample (yr)</th>
<th>IA-2/1IA-2β Reactivitya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>JM IA-2/2PTP X-react βPTP</td>
</tr>
<tr>
<td>A</td>
<td>10.2</td>
<td>– – – – –</td>
</tr>
<tr>
<td></td>
<td>10.6</td>
<td>– – – – –</td>
</tr>
<tr>
<td></td>
<td>11.3</td>
<td>– – – – –</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>– ++ – –</td>
</tr>
<tr>
<td></td>
<td>12.6</td>
<td>– +++ + +</td>
</tr>
<tr>
<td></td>
<td>12.8</td>
<td>– +++ + +</td>
</tr>
<tr>
<td></td>
<td>13.2</td>
<td>– +++ + +</td>
</tr>
<tr>
<td></td>
<td>15.2</td>
<td>– +++ + +</td>
</tr>
<tr>
<td>B</td>
<td>28.1</td>
<td>– – – –</td>
</tr>
<tr>
<td></td>
<td>29.0</td>
<td>8 ++ + +</td>
</tr>
<tr>
<td></td>
<td>30.0</td>
<td>34 +++ +++</td>
</tr>
<tr>
<td>C</td>
<td>1.6</td>
<td>– – – –</td>
</tr>
<tr>
<td></td>
<td>2.7</td>
<td>– – – –</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>– ++ + +</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>– +++ + +</td>
</tr>
<tr>
<td></td>
<td>5.2</td>
<td>– +++ + +</td>
</tr>
<tr>
<td></td>
<td>5.6</td>
<td>– +++ + +</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>– +++ + +</td>
</tr>
<tr>
<td>D</td>
<td>17.2</td>
<td>– – – –</td>
</tr>
<tr>
<td></td>
<td>18.4</td>
<td>35 – – –</td>
</tr>
</tbody>
</table>

a JM, IA-2 juxtamembrane Abs; IA-2PTP, IA-2-specific PTP-like domain Abs; X-react, Abs binding both IA-2 and IA-2β PTP-like domains; βPTP, IA-2β-specific PTP-like domain Abs.

b Subject A developed type 1 diabetes at age 15.5 yr, subject B at 31.2 yr, and subject C at 8.7 yr.

FIGURE 4. Identification of PTP-like domain regions involved in IA-2-specific PTP-like binding. Ab binding to IA-2/IA-2β PTP-like domain chimeras in a serum with Abs binding epitopes shared between IA-2 and IA-2β (lane 1), sera with Abs recognizing only IA-2-specific PTP-like domain epitopes (lanes 2–7), and a serum with IA-2β-specific Abs (lane 8). Lane 2 is a serum sample from a control subject.
Ala was replaced with Asp. The replacement of Asp with Ala reduced binding by 55 to 87%, but the replacement of both Ala and Asp did not reduce binding beyond that seen with the substitution of Asp alone. Ab binding to IA-2 juxtamembrane epitopes by patient sera was not affected by this change (Fig. 6).

IA-2/IA-2β Ab epitopes and progression to IDDM in relatives
Life table analysis in relatives with ICA showed that those also having Abs to IA-2 or IA-2β had a markedly increased progression to type 1 diabetes with a 5-yr risk of 40% (95% CI, 18–62) compared with 3.7% (95% CI, 0.2–7.2) in relatives without these Abs (p < 0.0001). Among those with IA-2/IA-2β Abs, progression to diabetes was not associated with any one of the Ab epitope specificities identified. Progression was, however, increased in those with multiple epitope reactivity. The 3-yr cumulative risk in those with >2 reactivities was 79% (95% CI, 44–100) vs 23% (95% CI, 1–45) in those with 1 or 2 reactivities (p = 0.01) (Fig. 7). Two relatives with a single IA-2/IA-2β epitope reactivity developed diabetes, and in both of them, Abs were against the IA-2 juxtamembrane region (Table II).

Discussion
Humoral autoimmunity to islet autoantigens is the single most consistently detected immune abnormality associated with type 1 diabetes. Over 90% of patients have islet autoantibodies at and before onset of clinical disease. These circulating autoantibodies are not considered directly pathogenic, but recent evidence showing an absence of insulitis and diabetes in B lymphocyte-deficient NOD mice suggest that B lymphocytes have a critical role in the disease process (17). Studying the early humoral responses is therefore

**FIGURE 5.** Comparison of the PTP-like domain amino acid sequences of IA-2 and IA-2β. The region containing amino acids that are potentially critical for the binding of Abs to the IA-2-specific PTP-like domain epitopes, as determined from binding studies to the PTP-like domain chimeric proteins, is shaded.

**FIGURE 6.** Effect of specific IA-2 amino acid mutations on autoantibody binding. Binding to unmutated IA-2ic (filled columns), IA-2ic Ala (open columns), IA-2ic 911 Asp (grey columns), and IA-2ic 877 Ala > Asp plus 911 Asp > Ala (dotted columns) is shown for sera with IA-2 juxtamembrane-specific Abs only (serum 1), IA-2 cross-reactive Abs only (serum 2 and 3), IA-2-specific PTP-like domain Abs (serum 4–7), and IA-2β-specific Abs (serum 8). Serum 9 is from a normal subject.

**FIGURE 7.** IA-2/IA-2β reactivity and progression to type 1 diabetes. Progression to disease in ICA-positive relatives of patients with respect to the total number of IA-2/IA-2β Ab specificities is expressed as cumulative diabetes-free survival in the Kaplan-Meier survival analysis. The numbers under the abscissa indicate the number of subjects remaining at each time point.
likely to be important for understanding the early immunization process in type 1 diabetes. The identification of two structurally related major humoral autoantigens associated with diabetes, IA-2 and IA-2β, allows the study of these responses in detail with respect to epitope spreading and has enabled us to identify potentially critical regions and residues involved in early IA-2 autoimmunity. We have determined that both the juxtamembrane region and the PTP-like domain of IA-2 contain epitopes that are specific to IA-2 and that most patients have Abs that recognize these IA-2-specific epitopes. Moreover, analysis of sequential samples from relatives who seroconverted to IA-2/IA-2β humoral autoimmunity suggests that these IA-2-specific epitopes are those to which the early Abs against IA-2 are directed. IA-2β autoimmunity appears to arise later through spreading to epitopes in the IA-2 PTP-like domain that are shared with IA-2β. These findings indicate that IA-2 is likely to be the primary target of humoral autoimmunity against diabetes associated PTP-like autoantigens.

Previous studies have suggested that autoimmunity to IA-2β rather than IA-2 may be more associated with the development of type 1 diabetes. This is based upon the suggestion that IA-2β may be more strongly expressed in β cells than is IA-2 and that Abs to IA-2β are less frequently detected in control subjects (2). Our findings do not support this view, since many patients had IA-2 Abs in the absence of IA-2β Abs, and importantly, several relatives developed the disease having only IA-2-specific epitope reactivity. We did find, however, that relatives with multiple IA-2/IA-2β epitope reactivity had a more frequent and rapid diabetes development. Thus, spreading of autoimmunity to other epitopes, including those found on IA-2β, appears to be associated with the development of clinical disease. The more rapid progression in those with multiple IA-2/IA-2β reactivity may simply reflect their identification at a later stage of preclinical disease. However, studies in offspring of parents with type 1 diabetes suggest that multiple Ag reactivity often occurs very early in life (18). Autoantibodies to multiple autoantigen determinants from different autoantigens has also been found associated with an increased likelihood of progression to disease in several studies (19–21), and therefore, both a high level and a wide range of humoral islet autoimmunity appear to be important covarates of diabetes risk. Of note is our previous demonstration that the detection of IA-2/IA-2β Abs, using extracts of rat insulinoma cell lines in relatives with ICA, was associated with a more rapid progression to diabetes than that of relatives with ICA and autoantibodies to glutamic acid decarboxylase and/or insulin only (19). Estimation of the risk associated with individual Ab specificities is problematic because of the variable presence of Abs to other autoantigens. Progression to disease in relatives, however, does occur regardless of which Ab specificities are detected, and we suggest that in relatives, the predictive value of autoantibodies appears to be less dependent upon the overall specificity of the autoantibodies than the broadness of the reactivity.

We have identified potential key residues involved in the initial humoral response to IA-2. IA-2-specific residues in both the juxtamembrane and PTP-like domains appear to be important in early Ab recognition of Ag. The IA-2 juxtamembrane region (amino acids 601–682), in particular, appears to harbor unique epitopes not found in IA-2β, and many patients and relatives have Abs that recognize only this region of the IA-2 protein. Ab binding to IA-2/IA-2β PTP-like domain chimeras determined that the amino acids that are critical for Ab recognition of the IA-2-specific PTP-like domain epitopes are contained within residues 794–889 of the IA-2 protein. Within this region, there are a total of 19 residues that differ between IA-2 and IA-2β, 9 of which represent only conserved changes. This finding needs to be confirmed using IA-2 with specific mutations and in studies examining the epitope specificities of IA-2/IA-2β that appear in the first years of life, but their identification is of potential importance for disease prevention. Identification of residues that stimulate autoimmunity but are not essential for function may enable the genetic engineering of functionally intact β cells with a lower capacity to induce or re-stimulate autoimmunity. Knowing these residues may also be helpful in designing vaccines. Immunization with either insulin or glutamic acid decarboxylase autoantigens in animal models of diabetes can induce T cell tolerance and delay diabetes onset (22–24). Thus far, there are no studies showing that IA-2/IA-2β or peptides of these Ags can induce T cell or B cell tolerance or prevent diabetes in these models. Moreover, humoral autoimmunity to IA-2 and IA-2β does not appear to be a feature of these animals (25). It still remains unclear whether immunization with any of the diabetes-associated Ags can induce tolerance in man, especially after autoimmunity has been initiated. Nevertheless, several proposed intervention strategies are based on the principle that Ag-induced modulation of the chronic inflammation leading to diabetes is possible either through tolerance induction or, more likely, specific regulation and/or Th2-like immune activation. Since IA-2 and IA-2β are expressed within islet β cells, it is expected that induction of Th2-like immunity against these proteins may also delay disease onset. Maintenance of residues that readily promote Ab responses is likely to be useful in the development of strategies for vaccination with Ag, during which an induction of a protective Th2-like immunity is desired.

Other IA-2 residues appear to be important for structure and function as well as Ab binding (13, 15). Residue 911 has been shown to restore partial classic PTPase activity of IA-2 if changed to an alanine; this activity was further enhanced by the additional change of residue 877 to an aspartic acid. The finding that these changes also affect binding of autoantibodies to the PTP-like domain suggests that they may be important for overall PTP-like domain structure. Residue 877 is interesting because it is an aspartic acid in native IA-2β, and it would therefore be expected that the cross-reactive PTP-like domain Abs would preferentially bind to IA-2. Evidence for the presence of Abs that recognize both IA-2 and IA-2β PTP-like domains with different affinities is given by the ability of IA-2ββ741–1047 to compete for the binding of IA-2β741–1047 in the absence of direct binding to IA-2ββ741–1047 in some sera. Finally, sera from two patients in our study had Abs that bound to human but not rat IA-2β. Both of these patients had cross-reactive PTP-like domain Abs but not IA-2β-specific Abs. Therefore, residues that are not conserved between the rat and human IA-2β PTP-like domains must be involved in the Ab recognition of some of the epitopes that are shared between IA-2 and IA-2β. Since only a minority of sera with cross-reactive PTP-like domain Abs did not bind to rat IA-2β, it is likely that there are several different epitopes that are shared between IA-2 and IA-2β.

In conclusion, this study shows that autoantibody binding to the PTP-like autoantigens associated with type 1 diabetes is most frequently against IA-2 rather than IA-2β; that almost all individuals with IA-2/IA-2β Abs have Abs that bind specifically to IA-2 and not IA-2β; and that these IA-2-specific Abs are most frequently those that can be identified in the early Ab response to IA-2/IA-2β. These findings suggest that IA-2 is the primary PTP-like autoantigen in type 1 diabetes. Potentially critical residues for the recognition of the IA-2-specific epitopes have been identified, and their identification may be useful for the design of analogues that can be used for preventing destructive autoimmunity. The finding of an increased disease risk associated with increased diversity of the Ab reactivity to IA-2/IA-2β epitopes suggests that studying this diversity may assist in disease prediction and, moreover, may
be a valuable inclusion as a surrogate marker when evaluating the efficacy of intervention therapies to prevent type 1 diabetes.

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References