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# Two Distinctly HLA-Associated Contiguous Linear Epitopes Uniquely Expressed Within the Islet Antigen 2 Molecule Are Major Autoantibody Epitopes of the Diabetes-Specific Tyrosine Phosphatase-Like Protein Autoantigens<sup>1</sup>

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The related tyrosine phosphatase-like proteins islet Ag (IA)-2 and IA-2 $\beta$  are autoantigens of type 1 diabetes in humans. Autoantibodies are predominantly against IA-2, and IA-2-specific epitopes are major autoantibody targets. We used the close homology of IA-2 and IA-2 $\beta$  to design chimeras and mutants to identify humoral IA-2-specific epitopes. Two major IA-2 epitopes that are absent from the related autoantigens IA-2 $\beta$  and IA-2 $\Delta$  13 splice variant ICA512.bdc were found contiguous to each other within IA-2 juxtamembrane amino acids 611–620 (epitope JM1) and 621–630 (epitope JM2). JM1 and JM2 are recognized by sera from 67% of patients with IA-2 Abs, and relatives of patients with type 1 diabetes having Abs to either JM epitope had a >50% risk for developing type 1 diabetes within 6 years, even in the absence of diabetes-associated HLA genotypes. Remarkably, the presence of Abs to one of these epitopes was mutually exclusive of the other; JM2 Abs and not JM1 Abs were found in relatives with HLA DR3/4, DR4/13, or DR1/4 genotypes; and the binding of autoantibodies to the JM2 epitope, but not the JM1 epitope, markedly affected proteolysis of IA-2. This is a unique demonstration of HLA-associated B cell responses to epitopes within a single autoantigen in humans and is consistent with modification of Ag processing by specific Ab-influencing peptide presentation by HLA molecules. *The Journal of Immunology*, 2002, 168: 4202–4208.

Islet cell Ag 512 (islet Ag (IA)<sup>3</sup>-2) and IA-2 $\beta$  are novel proteins of the protein tyrosine phosphatase (PTP) family (1–4). Both are transmembrane proteins expressed within secretory granules in brain and neuroendocrine cells including the pancreatic islet  $\beta$  cells (4, 5). The amino-terminal intraluminal domains show only modest homology to each other (20%), whereas their cytoplasmic domains are closely related (80%), being characterized by a single PTP-like domain of ~300 amino acids and a short juxtamembrane (JM) portion of 100 amino acids which links the PTP domain to the transmembrane region (1–4). These PTP-like proteins have no or only weak phosphatase activity against common PTP substrates (2, 6) and are postulated to have a regulatory role in secretory granule exocytosis (7–9).

IA-2 and IA-2 $\beta$  are also major autoantigens of type I diabetes (1, 6, 10–12). Autoantibodies to these proteins can be detected in

sera of 60–80% of patients with recent onset type 1 diabetes and their detection in sera of nondiabetic individuals together with other islet autoantibodies such as GADA and IAA is highly predictive for a future development of the disease (13, 14). Humoral autoimmunity is directed to the cytoplasmic portion of the two proteins (15). Autoantibodies specific to the IA-2 JM region and PTP-like domain, specific to the IA-2 $\beta$  PTP-like domain, and cross-reactive between the IA-2 and IA-2 $\beta$  PTP-like domains have been identified (15–20). Those directed against IA-2-specific epitopes are frequently detected in the early humoral response, while IA-2 $\beta$ -specific Abs are relatively infrequent, suggesting that humoral autoimmunity to IA-2 $\beta$  is secondary to that of IA-2 and arises via epitope spreading (21). Therefore, characterization of the IA-2-specific epitopes may be an important step toward the understanding of the maturation of the autoimmune response against these PTPs.

We previously reported the presence of IA-2-specific epitopes within the first 82 JM IA-2 intracellular amino acids (15). Part of this region is encoded by exon 13 of the IA-2 gene and is deleted in an alternatively spliced form of IA-2, ICA512.bdc (22). In this study we have used chimeric IA-2/IA-2 $\beta$  constructs and IA-2 peptides to identify minimal epitopes involved in Ab binding and show that most of the IA-2 JM Ab reactivity is directed against two potentially linear contiguous epitopes with distinct HLA-DR allele associations that are absent in the alternatively spliced ICA512.bdc.

## Materials and Methods

### Sera

First, to define the minimal epitopes of the IA-2 JM Abs, sera from 19 patients with IA-2 JM Abs were used. These were selected from a cohort of 217 patients with new onset type 1 diabetes previously assayed for IA-2 and IA-2 $\beta$  Ab epitope reactivity (16). Next, to determine the prevalence of

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<sup>3</sup> Abbreviations used in this paper: IA, islet Ag; JM, juxtamembrane; PTP, protein tyrosine phosphatase; CI, confidence interval.

Abs to individual IA-2 JM epitopes, sera from 106 IA-2 Ab-positive first-degree relatives of patients with type 1 diabetes were used. These included 20 children participating in the German BABYDIAB study (23), 42 relatives from the Munich family study (24), and 43 relatives from the Barts Oxford family study (25). The 106 IA-2 Ab-positive relatives were from 105 unrelated families and included one sibling pair. Broad Ab reactivity to the IA-2 JM and PTP regions was previously reported in a portion of subjects from these cohorts (16, 21). The relatives had a median age of 11.8 years at Ab testing (interquartile range: 5.2–19.2 years), and 54 developed type 1 diabetes during follow-up (median time to diabetes or last contact if no diabetes: 3.3 years; range: 0.1–14.5 years). All relatives also had autoantibodies to insulin and/or glutamic acid decarboxylase.

#### IA-2/IA-2 $\beta$ constructs

Constructs used in this study are shown in Fig. 1. The IA-2<sub>604–979</sub> (IA-2IC) and IA-2 $\beta$ <sub>741–1033</sub> (IA-2 $\beta$ IC) constructs (16) were used to construct the chimeras. All the chimeric constructs were obtained by in-frame joining of different portions of IA-2 and IA-2 $\beta$  using the Seamless (Stratagene, La Jolla, CA) method with appropriate oligonucleotide primers. The correct in frame sequence of joining boundaries was confirmed by DNA sequencing.

#### Ab measurements

Abs to each construct were measured by radio binding assay to [<sup>35</sup>S]methionine-labeled, in vitro transcribed and translated proteins as previously described (16). Purified plasmid DNA of the constructs were obtained by Quantum Prep (Bio-Rad, Hercules, CA) spin columns and in vitro transcribed and translated using the TnT SP6-coupled rabbit reticulocyte lysate system (Promega, Madison, WI) in the presence of 40  $\mu$ Ci of Redivue L-[<sup>35</sup>S]methionine (Amersham Pharmacia Biotech, Uppsala, Sweden). Unincorporated [<sup>35</sup>S]methionine was removed by gel chromatography on a NAP5 column (Amersham Pharmacia Biotech). In all cases the expected size of the product was confirmed on SDS-PAGE. A total of 20,000 cpm of product in TBST (25  $\mu$ l of 50 mM Tris, 150 mM NaCl, 1% Tween 20 (pH 7.4)) was incubated overnight at 4°C with 2  $\mu$ l of sera and an excess of bacterially expressed intracellular IA-2 $\beta$  to compete out binding to the non-IA-2-specific epitopes of the chimeras and mutants as previously described (16). The quantity necessary for complete inhibition of binding to the IA-2 $\beta$  was determined by titration against sera with high-titer IA-2 $\beta$ -binding Abs. Protein A-Sepharose (Amersham Pharmacia Biotech) was subsequently added for 1 h, washed five times with 800  $\mu$ l ice-cold TBST, and counted in a TopPlate beta counter (Canberra Packard, Groningen, The Netherlands). To control for binding efficiency a serum with Abs to PTP epitopes shared between IA-2 and IA-2 $\beta$  and no reactivity to IA-2- or IA-2 $\beta$ -specific epitopes was included in each assay. This serum should not be influenced by mutations from IA-2 to IA-2 $\beta$  because binding is equivalent to both proteins. Sera from IA-2 Ab-negative normal control subjects

were also included in each assay. Positivity against each protein was defined by the upper limit of normal control sera.

Further definition of epitopes within the IA-2 JM region was performed by competition with 20-mer peptides corresponding to IA-2 601–620, IA-2 611–631, IA-2 621–641, and IA-2 631–651. Serum (2  $\mu$ l) was preincubated with 1  $\mu$ g of peptide for 1 h at 4°C before testing in the radiobinding assay.

#### HLA class II typing

HLA DR and DQ typing was performed on DNA using sequence-specific oligonucleotides in 80 of the IA-2 Ab-positive first-degree relatives and in the 19 IA-2 JM Ab-positive patients with type 1 diabetes.

#### Statistical analysis

Analyses of the associations of JM Ab specificities with HLA class II alleles and genotypes were performed using Fisher's exact test. Kaplan-Meier survival analysis was used to determine the risk for developing type 1 diabetes. For all statistical methods the Statistical Package for Social Sciences (SPSS, Chicago, IL) was used.

## Results

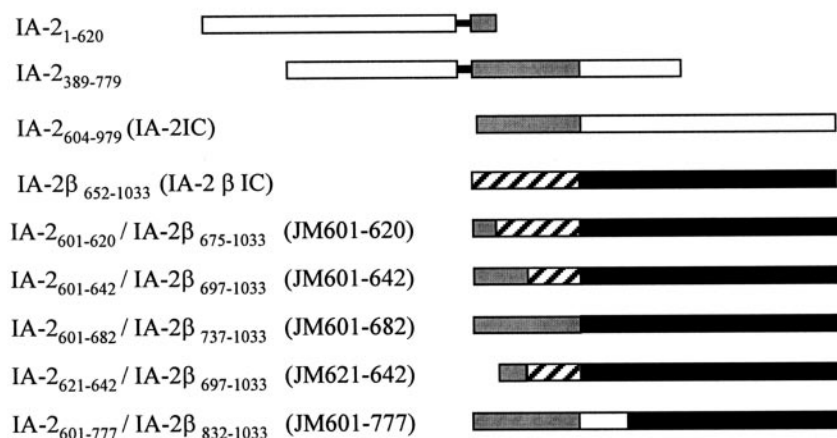
### Characterization of IA-2 JM epitopes

To identify the IA-2-specific epitopes within the JM region, IA-2/IA-2 $\beta$  chimeras were made joining different portions of the IA-2 JM with the remaining part of the molecule belonging to IA-2 $\beta$  (Fig. 1). Sera from the 19 patients with IA-2 JM Abs were then tested against these chimeras in the presence of an excess of unlabeled IA-2 $\beta$ IC to inhibit binding to non-IA-2 regions of the chimeras (Table I). Eight sera (nos. 1–8) recognized JM<sub>601–620</sub>, JM<sub>601–642</sub>, JM<sub>601–682</sub>, and JM<sub>601–777</sub> chimeras, suggesting the presence of at least one epitope within IA-2 residues 601–620. In all eight sera binding could also be obtained against the IA-2<sub>1–620</sub> and to IA-2IC, indicating that an IA-2 JM epitope recognized by these sera should be contained within IA-2 residues 604–620 (IA-2 JM1 epitope). In seven of these eight sera, binding to the JM<sub>601–682</sub> chimera could be totally inhibited by competition with unlabeled JM<sub>601–620</sub> chimera, suggesting that the IA-2 JM reactivity was solely against the JM<sub>601–620</sub> epitope, whereas in one serum (no. 8) binding to the JM<sub>601–682</sub> chimera was only partially inhibited, indicating the presence of Abs to additional IA-2 JM epitopes.

### A Schematic representation of IA-2 and IA-2 $\beta$ proteins



### B IA-2/IA-2 $\beta$ chimeric constructs



**FIGURE 1.** Chimeric and mutant constructs used in this study. *A*, Schematic representation of IA-2 and IA-2 $\beta$  indicating the ectodomain (EC), transmembrane region (TM), JM region (shown in gray for IA-2 and hatched for IA-2 $\beta$ ), and the PTP-like domain. *B*, Chimeric and deletion constructs with the IA-2 and IA-2 $\beta$  portions represented according to *A*. An abbreviated nomenclature indicating the IA-2 regions of the JM chimeras is given in parentheses.

Table I. Patients with newly diagnosed type 1 diabetes and Abs against IA-2 JM

Patient	Reactivity Against IA-2 Deletion Mutants and IA-2/IA-2 $\beta$ Chimeras							
	IA-2 <sub>382-779</sub> mutant	JM <sub>601-620</sub> chimera	JM <sub>601-642</sub> chimera	JM <sub>601-682</sub> chimera	JM <sub>601-777</sub> chimera	IA-2 <sub>1-620</sub> mutant	JM <sub>621-642</sub> chimera	JM <sub>601-682</sub> inhibited with JM <sub>601-620</sub>
1	++	++	++	++	++	+	-	-
2	++	++	++	++	++	+	-	-
3	++	++	++	++	++	+	-	-
4	++	+	+	+	+	+	NT <sup>a</sup>	-
5	++	+	+	+	+	+	NT	-
6	++	+	+	+	+	+	NT	-
7	+	+	+	+	+	+	NT	-
8	++	+	++	++	++	-	+	+
9	++++	-	++++	++++	++++	-	++	NT
10	++	-	++	++	++	-	+	NT
11	++	-	++	++	++	-	+	NT
12	++	-	++	++	++	-	+	NT
13	+	-	+	+	+	-	+	NT
14	+	-	+	+	+	-	+	NT
15	+++	-	-	-	++	-	NT	NT
16	++	-	-	-	+	-	NT	NT
17	++	-	-	-	-	++	NT	NT
18	+	-	-	-	-	-	NT	NT
19	+	-	-	-	-	-	NT	NT

<sup>a</sup> NT, Not tested.

Another six sera (nos. 9–14) bound JM<sub>601-642</sub>, JM<sub>601-682</sub>, and JM<sub>601-777</sub>, but not the JM<sub>601-620</sub> chimera, indicating the presence of an epitope beyond IA-2 aa 620 and up to aa 642. These sera also bound a chimeric construct in which IA-2 aa 601–620 were deleted (JM<sub>621-642</sub>), indicating that the epitope was contained within aa 621–642 and that aa 601–620 were not involved in the formation of this epitope (IA-2 JM2 epitope).

Two sera (nos. 15 and 16) bound the JM<sub>601-777</sub> chimera, only suggesting the presence of epitope(s) within or extending to the IA-2 PTP region. Three sera (nos. 17–19), despite showing binding to the original IA-2<sub>389-779</sub> deletion construct, did not bind any of the JM<sub>601-620</sub>, JM<sub>601-642</sub>, JM<sub>601-682</sub>, and JM<sub>601-777</sub> chimeras. One of these sera (no. 17) bound a truncated IA-2<sub>1-620</sub> protein, suggesting that there is an epitope that includes residues upstream from the intracellular region. For this serum, binding to the original IA-2<sub>389-779</sub> construct was not inhibited by competition with an IA-2IC construct (data not shown), further suggesting that the nonintracellular portion of IA-2 was essential for binding to this epitope.

#### Competition with peptides identifies small contiguous IA-2 JM epitopes

Further definition of JM1 and JM2 epitopes was performed using 20-mer peptides to compete the binding to the JM<sub>601-682</sub> chimera (Fig. 2). Sera with single JM1 or JM2 specificities in the absence of Ab binding to IA-2 PTP or IA-2 $\beta$  epitopes were used in these competition studies. Binding in sera with JM1 Abs was completely inhibited by competition with IA-2 601–620 and IA-2 611–630 peptides but was unaffected by competition with IA-2 591–610, 621–640, and 631–650 peptides, indicating that the JM1 epitope is contained within IA-2 residues 611–620. Binding in sera with JM2 Abs was completely inhibited by competition with IA-2 611–630 and IA-2 621–640 peptides but was unaffected by competition with IA-2 591–610, 601–620, and 631–651 peptides, indicating that the JM2 epitope is contained within IA-2 residues 621–630.

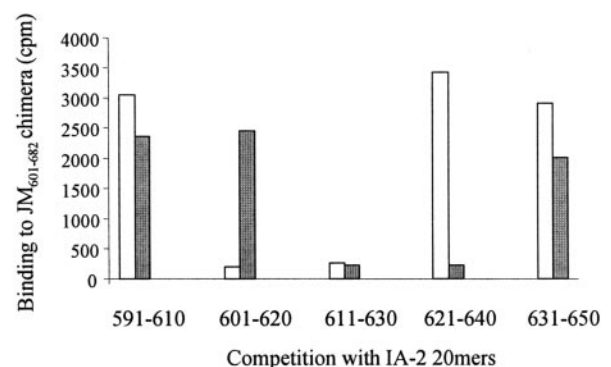
#### Abs to JM1 and JM2 epitopes are mutually exclusive of each other

We determined Ab reactivity to the distinct JM1 and JM2 epitopes in 106 IA-2 Ab-positive first-degree relatives of patients with type 1

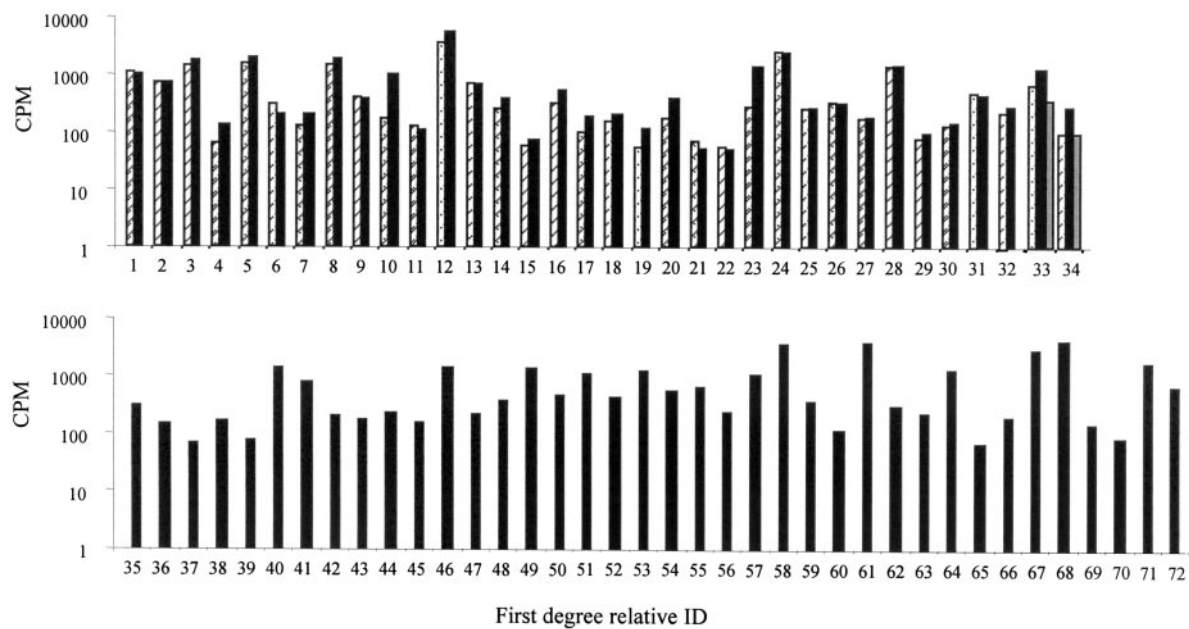
diabetes (Fig. 3). JM1 Abs were identified by binding to JM<sub>601-620</sub> and JM<sub>601-682</sub> chimeras and JM2 Abs by binding to the JM<sub>601-642</sub> and JM<sub>601-682</sub> but not the JM<sub>601-620</sub> chimera. The additional presence of JM2 Abs in sera with JM1 Abs was ascertained when binding to the JM<sub>601-682</sub> chimera was only partially inhibited by competition with excess unlabeled JM<sub>601-620</sub> chimera. Sera from 72 (68%) of these 104 relatives had Abs to JM1 and/or JM2 epitopes. IA-2 JM-1 Abs were found in 34 relatives and IA-2 JM-2 Abs were found in 40 relatives. Abs to IA-2 JM-1 and IA-2 JM-2 epitopes were almost mutually exclusive, with only two relatives having Abs to both epitopes ( $p < 0.0001$ ). Nineteen relatives had Abs to either JM1 or JM2 epitopes in the absence of Abs to the IA-2 PTP domain (data not shown).

#### Abs to the adjacent IA-2 JM1 and JM2 epitopes associate with distinct HLA class II genotypes

We previously reported HLA associations in the type and broadness of IA-2 Ab reactivity that appeared in early childhood (21).



**FIGURE 2.** Competition of binding to IA-2 JM epitopes with peptides. Binding (cpm) to IA-2<sub>601-682</sub>/IA-2 $\beta$ <sub>737-1033</sub> by sera with Abs to IA-2 JM1 (open bars) or IA-2 JM2 (gray bars) epitopes in the presence of 1  $\mu$ g of IA-2 20-mer peptides. IA-2 peptides 601–620 and 611–630 completely inhibited the binding to the JM1 epitope, whereas peptides 611–630 and 621–640 completely inhibited the binding to the JM2 epitope. Data are from a representative experiment.



**FIGURE 3.** IA-2 JM1 and JM2 Abs in first-degree relatives of patients with type 1 diabetes. Binding (cpm after subtraction of the upper limit of control samples) to IA-2<sub>601-620</sub>/IA-2β<sub>675-1033</sub> (hatched bars) and IA-2<sub>601-682</sub>/IA-2β<sub>737-1033</sub> (filled bars) is shown for sera from first-degree relatives of patients with type 1 diabetes. When sera were positive against to IA-2<sub>601-620</sub>/IA-2β<sub>675-1033</sub>, the binding to IA-2<sub>601-682</sub>/IA-2β<sub>737-1033</sub> was competed with excess cold IA-2<sub>601-620</sub>/IA-2β<sub>675-1033</sub> (gray bars). *Upper panel*, Relatives with JM1 Abs; *lower panel*, JM2 Abs without JM1 Abs. Sera from an additional 34 relatives had no binding to either chimera and are not shown. IA-2 JM1 Abs were identified by binding to the JM<sub>601-620</sub> and JM<sub>601-682</sub> chimeras and an accompanying inhibition of binding to the JM<sub>601-682</sub> chimera by competition with JM<sub>601-620</sub>. JM2 Abs were identified by binding to 601-642 (data not shown) and 601-682 chimeras, but not the JM<sub>601-620</sub> chimera. The additional presence of JM2 Abs in sera with JM1 Abs was ascertained when binding to the JM<sub>601-682</sub> chimera was only partially inhibited by competition with excess unlabeled JM<sub>601-620</sub> chimeras (subjects 33 and 34).

Children with the high diabetes risk HLA DR3/4 or DR4/4 genotypes developed broad IA-2 Ab reactivity to multiple IA-2 epitopes, whereas those without these genotypes often developed Abs to the IA-2 JM region before PTP Abs. HLA DR typing was available in 83 of the 106 IA-2 Ab-positive relatives in this study, including 28 with no IA-2 JM Abs, 23 with JM1 Abs only, 31 with JM2 Abs only, and 1 with both JM1 and JM2 Abs (Table II). HLA genotypes in the 23 relatives with JM1 Abs only differed markedly from those of the relatives with JM2 Abs only or no JM Abs. Fewer than half (11 of 23) of the JM1 Ab-positive relatives had DR4-containing genotypes, whereas 30 of 31 JM2 Ab-positive and 24/28 IA-2 JM Ab-negative relatives had HLA DR4 ( $p < 0.0001$ , JM1 Ab positive vs JM2 Ab positive;  $p < 0.01$ , JM1 Ab positive vs JM Ab negative). Moreover, HLA DR genotypes found in JM2 Ab-positive or JM-negative relatives were relatively restricted when compared with those in relatives with JM1 Abs. The high diabetes risk DR3/4 genotype was present in 12 (39%) JM2 Ab-positive relatives and 11 (39%) JM Ab-negative relatives com-

pared with only one (4%) of the JM1 Ab-positive relatives ( $p < 0.005$  vs JM2 Ab positive and  $p < 0.01$  vs JM Ab negative). Six of 32 JM2 Ab-positive relatives had the less common DR4/13 genotype and five had the DR1/4 genotype, whereas only one of the JM1 Ab-positive ( $p < 0.01$ ) and one of the JM Ab-negative ( $p < 0.005$ ) relatives had these genotypes. The one relative with both JM1 and JM2 Abs was DR4/12. Consistent with our previous observation (21), none of the 14 HLA-typed relatives with IA-2 JM Abs in the absence of IA-2 PTP Abs had the HLA DR3/4 genotype.

The same associations between HLA DR genotypes and JM1 or JM2 Abs was also observed in the 19 IA-2 JM Ab-positive patients at diabetes onset used to describe the epitopes. In this cohort, none of the eight JM1 Ab-positive patients and five (84%) of the six JM2-positive relatives had the HLA DR3/4 genotype ( $p < 0.005$ ), and five (62%) of the JM1 Ab-positive patients had non-DR4-containing genotypes.

#### Footprinting demonstrates differences in protection of Ag proteolysis between IA-2 JM1 and JM2 Abs

To demonstrate whether binding of IA-2 to JM1 and JM2 Abs may alter Ag proteolysis, footprinting studies using mild trypsin treatment of IA-2 Ag bound to JM-specific Abs were undertaken (Fig. 4). Mild trypsin digestion of IA-2IC resulted in minor bands at 40, 30, 27, and 24 kDa and a major band of small molecular mass fragments using 0.1 mg/ml trypsin, with a progressive loss of the minor bands when 1 mg/ml trypsin was used (Fig. 4A). Trypsin treatment of IA-2IC bound to Abs of the JM1 specificity yielded a similar pattern (Fig. 4B), whereas IA-2IC bound to Abs of the JM2 specificity was markedly more resistant to trypsin digestion with a major 40-kDa fragment and a relatively small amount of low molecular mass fragments remaining even when 1 mg/ml trypsin was used (Fig. 4C). The footprinting pattern of IA-2IC complexed to

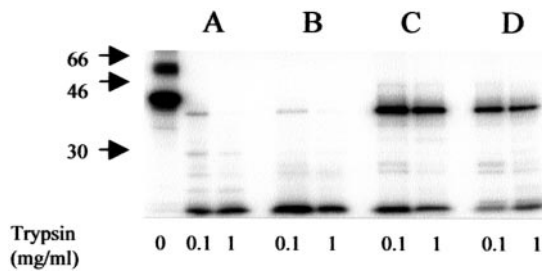
Table II. HLA DR genotypes in IA-2 Ab-positive relatives according to their JM epitope reactivity

HLA DR Genotype	IA-2 JM Autoantibody Reactivity			
	JM1 only (n = 23)	JM2 only (n = 31)	JM1 and JM2 (n = 1)	JM negative (n = 28)
DR3/4	1 (4) <sup>a</sup>	12 (39)	0	11 (39)
DR13/4	1 (4)	6 (19)	0	0
DR1/4	0	5 (16)	0	1 (4)
DR4/y <sup>b</sup>	9 (39)	8 (26)	1	12 (43)
DRx/x <sup>c</sup>	12 (52)	1 (3)	0	4 (14)

<sup>a</sup> Percentages are listed in parentheses.

<sup>b</sup> y, Alleles other than DR1, DR3, and DR13.

<sup>c</sup> x, Alleles other than DR4.



**FIGURE 4.** Modification of trypsin proteolysis by autoantibody binding to IA-2. *A*, SDS-PAGE/autoradiograph of in vitro transcribed and translated [<sup>35</sup>S]methionine-labeled IA-2<sub>604–979</sub> (IA-2IC) after treatment with trypsin at 4°C for 10 min. *B–D*, SDS-PAGE/autoradiograph of trypsin-treated in vitro transcribed and translated [<sup>35</sup>S]methionine-labeled IA-2IC, which is bound to anti-IA-2 JM1 (*B*), anti-IA-2 JM2 (*C*), and anti-IA-2-specific PTP (*D*) autoantibody.

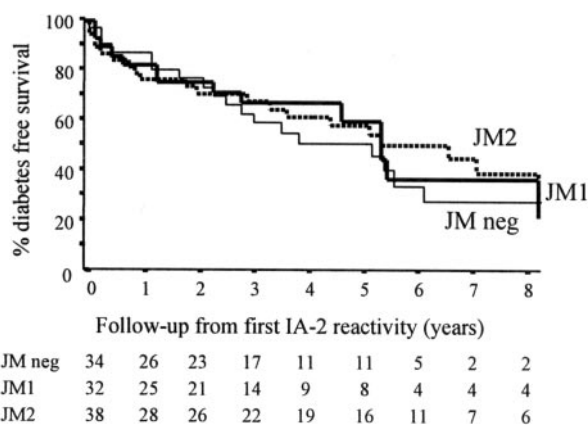
IA-2-specific PTP domain Abs gave a similar pattern to that seen with JM2 Abs (Fig. 4*B*).

#### IA-2 JM Abs and risk of progression to diabetes

We previously reported an increased risk for developing type 1 diabetes in young relatives who had Abs to the JM region of IA-2 (21). Therefore, we examined diabetes development in the 104 IA-2 Ab-positive relatives according to IA-2 JM Ab status (Fig. 5). Of the 54 relatives who developed type 1 diabetes on follow-up, 36 were IA-2 JM Ab positive. The 6-year risk after first IA-2 Ab detection was 63% (95% confidence interval (CI): 40–86%), 49% (95% CI: 33–65%), and 70% (95% CI: 51–89%) in relatives who had JM1 Abs only, JM2 Abs only, and no JM Abs, respectively. Despite having low-risk HLA genotypes, the 12 non-DR4 IA-2 JM1 Ab-positive relatives had a similar risk to develop diabetes within 6 years (67%) as the DR4 IA-2 Ab-positive relatives (data not shown).

## Discussion

The related PTP-like proteins IA-2 and IA-2β are autoantigens of type 1 diabetes in humans. Autoimmunity is predominantly against IA-2, and IA-2-specific epitopes are major targets of type 1 diabetes-associated autoantibodies. In this study we have identified



**FIGURE 5.** Type 1 diabetes-free survival in relatives with IA-2 autoantibodies according to the presence of anti-IA-2 JM1 and anti-IA-2 JM2 Abs. Survival curves were calculated using Kaplan-Meier survival analysis. Follow-up time is calculated from the first detection of IA-2 autoantibodies in the relative to type 1 diabetes onset or last contact. The numbers in each group remaining on follow-up is shown below the x-axis. The two relatives with both JM1 and JM2 Abs are not shown.

two major contiguous epitopes of the short IA-2 JM region. The two major JM epitopes are found within IA-2 residues 611–620 (epitope JM1) and 621–630 (epitope JM2), account for most of the Ab reactivity against the JM region, and are recognized by sera from the majority of IA-2 Ab-positive relatives. Remarkable is the finding that autoantibodies to these epitopes are almost mutually exclusive of each other and associate with distinct HLA class II genotypes. To our knowledge this is the first example of HLA-associated B cell responses to epitopes within a single autoantigen in humans.

This mapping of IA-2 JM1 and JM2 humoral epitopes suggested that they are contained within short linear adjacent sequences. The complete JM1 epitope appeared to be contained within residues 611–620 and contact residues for Ab binding outside 611–620 seem unlikely. This was deduced by the observation that 1) binding could be obtained against IA-2<sub>1–620</sub>, the JM<sub>601–620</sub> chimera, and to IA-2<sub>604–979</sub>, indicating that residues 604–620 should be sufficient for Ab binding and 2) complete inhibition of binding was obtained with the overlapping peptides 601–620 and 611–630, whereas no inhibition was obtained with IA-2 peptide 591–610. Abs recognizing the JM2 epitopes bound a chimera in which IA-2 residues 601–620 were deleted and in which IA-2 residues 643–979 were replaced by the corresponding residues of IA-2β. Together with previous studies which showed that JM Abs bind the JM region when IA-2 PTP 683–979 is deleted (15), the binding data suggest that necessary residues are contained within IA-2 aa 621–642, although we cannot exclude that residues common between IA-2 and IA-2β in region 643–682 are also involved. That inhibition of binding was achieved by peptides 611–630 and 621–640, but not 631–650, suggested that the essential residues involved in Ab binding are within 621–630. This is also supported by a recent report identifying an epitope within IA-2 aa 623–631 (19).

The location of these two epitopes is intriguing from the viewpoint of IA-2 function. It was recently reported that IA-2 contains a PDZ domain that includes residues 663–700 within the JM region, and that this is involved in the binding of IA-2 to β2-syntrophin which in turn links IA-2 and secretory granules to the cytoskeleton (7, 8). Release of the secretory granule is postulated to result through cleavage by μ-calpain and involving residues 643–659 (8). Cleavage would result in a truncated protein containing the two epitopes and would dissociate these JM epitopes from those contained within the PTP domain of IA-2. In our experience, IA-2 autoantibodies rarely if ever require both the JM and the PTP domain for Ag recognition and usually do not bind epitopes that span across the JM-PTP junction. Therefore, it could be postulated that autoreactivity is generated through the distinct intracellular portions that result from cleavage rather than the intact molecule. Remarkably, both these functional sites lie outside the binding sites of IA-2 autoantibodies. Similarly, autoantibodies to GAD and to insulin, the other major diabetes-associated autoantigens, rarely bind epitopes that are close to the functional domains of the proteins (26, 27). These observations imply that autoantibodies in type 1 diabetes do not directly interfere with protein function, and that functional regions of proteins may normally be protected from Ab binding.

The JM region of IA-2 appears to be highly immunogenic and to act as a distinct autoantigen from the PTP-like domains of IA-2 and IA-2β (15). It is unique to IA-2, having only weak homology to the corresponding region of IA-2β, and whereas autoantibodies binding to the IA-2 PTP-like domain often cross-react with IA-2β, autoantibodies binding to the JM region of IA-2β have not been identified (16, 17). Moreover, an alternatively spliced form of IA-2 (ICA512.bdc) that lacks exon 13 is also recognized by IA-2 PTP

Abs (22), but the deletion of residues 557–629 as a result of the exon 13 splicing would remove both JM1 and JM2 epitopes. Because these epitopes are important autoantibody targets, the use of ICA512.bdc IA-2 alone for autoantibody measurements is not advocated. Indeed, 19 of the 106 IA-2-positive relative sera, including 6 of the 54 who developed type 1 diabetes, had IA-2 Abs recognizing only the JM1 or JM2 epitopes (data not shown) and would not be expected to bind the alternatively spliced IA-2.

The uniqueness of the immunogenic JM region to IA-2 and not the related IA-2 $\beta$  and ICA512.bdc proteins has potentially interesting implications for tolerance loss. Autoantigens have been detected in human thymus during fetal life (28). Remarkable is that transcription of the alternatively spliced ICA512.bdc form (which lacks the JM epitopes), but not IA-2, could be detected in human thymus (29). Therefore, the IA-2 JM epitopes and not those present on the remainder of the molecule or on IA-2 $\beta$  could be “absent” during thymic education, and IA-2 JM autoreactive T cells could escape deletion or anergy, leading to an increased susceptibility to autoimmunity. So far, only isolated reports of IA-2 T cell epitopes have appeared (30, 31), and only a limited number of potential epitopes have been suggested, none of which reside in the IA-2 exon 13 region (30–32). Nevertheless, Ab responses suggest that the JM and PTP regions of IA-2 represent quite distinct entities for autoantigen recognition (15); therefore, T cell epitopes within the JM region are likely. An important role of the JM region of IA-2 early in tolerance loss to IA-2 is further suggested by our previous report that the IA-2 JM epitopes are the first IA-2 Ab epitopes that are recognized in almost 50% of subjects developing type 1 diabetes-associated autoantibodies (21). Moreover, in examining the age of first autoantibody appearance in the BABYDIAB cohort (21), we found that children who develop IA-2 JM Abs did so earlier and progressed to clinical diabetes more rapidly than children who developed islet autoantibodies without IA-2 JM specificities (median age of first IA-2 Ab appearance was 1.9 years in IA-2 JM-positive children vs 3.1 years in IA-2-positive JM-negative children;  $p = 0.02$ , Mann-Whitney  $U$  test).

Striking in our study was the observation that, despite the high prevalence of Abs to JM1 and JM2 epitopes, the simultaneous presence of Abs to both epitopes was rare in individual patients, suggesting that sustained expansion of both JM1- and JM2-responsive B cells was inhibited. An important factor in determining which of the B cells are expanded appeared to be related to HLA class II genes. HLA DR4-containing haplotypes were almost always found in relatives and patients with IA-2 JM2 Abs, but were present in only half of the relatives with IA-2 JM1 Abs. Moreover, none of the genotypes found in JM2 Ab-positive relatives were found in JM1 Ab-positive relatives. DR3/4, DR13/4, and DR1/4 were the predominant genotype (74%) of IA-2 JM2 Ab-positive relatives, whereas only 9% of the IA-2 JM1 Ab-positive relatives had these genotypes. HLA DR3 and DR13 almost always share the same allele at the *DRB3* locus, potentially explaining why both were associated with IA-2 JM2 Ab positivity. The mechanism leading to HLA-associated Ab reactivity to distinct epitopes is not entirely clear. Recent analysis of HLA class II genotype structure and diabetes risk strongly indicated that risk was related to autoantigen peptide binding to HLA class II molecules (33). Peptides are generated by proteolytic enzymes within vesicles of the endocytic pathway, and it has been postulated that selective responses may arise through modified processing of Ag due to its binding to specific B cell receptors (34). It is known that the dissociation rate of monovalent Ag from high-affinity Ab is slower than the time taken for Ag capture, endocytosis, and processing by professional APCs. Thus, when high-affinity Abs drive Ag uptake, either di-

rectly via B cell membrane Ig or indirectly via FcRs, the substrate for processing may frequently be an Ag/Ab complex. Ab-bound Ag can both prevent the generation of peptides and generate new “cryptic” epitopes and thereby alter the response of Ag-specific T cell clones (34, 35). In this study we find that proteolysis of IA-2 Ag bound by JM2 Abs in vitro differs from that of IA-2 bound by JM1 Abs or unbound Ag. Binding of IA-2 JM2 Abs protected against proteolysis of IA-2, a finding consistent with the relatively restricted HLA class II genotypes found in subjects with these Abs as compared with those with IA-2 JM1 Abs. It is conceivable that B cell receptor binding to the JM2 autoantibody epitopes preferentially generates T cell epitopes that can bind to HLA class II molecules expressed in DR3/4, DR13/4, or DR1/4 APCs and result in T cell help for JM2-specific B cells, whereas B cell receptors binding to JM1 autoantibody epitopes either do not generate these peptides or generate peptides that are preferentially bound by other class II molecules. Although the mechanism by which a HLA-associated epitope-specific Ab response occurs is speculative, the observation is consistent with processing and presentation of Ab-bound IA-2 Ag having an important role in the determination of the survival of specific B cell clones.

In conclusion, this study has demonstrated how chimeric construct screening together with point mutation can be used to identify specific autoantibody epitopes. This approach has identified two major diabetes-related adjacent potentially linear epitopes within the IA-2 JM region that are absent from the related autoantigens IA-2 $\beta$  and ICA512.bdc. Relatives with Abs to either JM epitope have a high risk for developing type 1 diabetes, even in the absence of diabetes-associated HLA genotypes. However, the presence of Abs to one of these two epitopes was mutually exclusive of the other; the epitope specificity of Abs was strongly linked to HLA class II genotype; and the binding of Abs to one of the epitopes, but not the other, markedly affected proteolysis of IA-2. These findings are consistent with processing of specific Ab-bound Ag being a determinant for peptide presentation by HLA class II. This approach, together with the generation and characterization of mAbs (36) and their screening with peptide libraries (37), should lead to the definition of minimal epitopes that could be potentially used in the modeling of molecular mimicry.

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