Humoral autoreactivity to an alternatively spliced variant of ICA512/IA-2 in Type I diabetes

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Abstract

Aims/hypothesis. The receptor tyrosine phosphatase like-protein ICA512/IA-2 occurs as a proteolytically-processed 65,000 M_r type 1 transmembrane glycoprotein in beta cells and is a major autoantigen of Type I (insulin-dependent) diabetes mellitus. We investigated whether alternative splicing could affect humoral autoreactivity to the molecule.

Methods. Genomic and cDNA sequence analysis showed the presence of a ICA512 variant in islets and lymphoid tissues with an in-frame deletion of exon 13 which produces a secreted form lacking aa 557–629 including the transmembrane domain (aa 577 to 600). The alternatively spliced protein is detectable by western blotting in normal islets and translated into a protein that is processed to a series of soluble forms of 25,000–35,000 M_r. Radioimmuno-precipitation assays for anti-ICA512 autoantibodies were developed with the widely used ICA512.bdc construct (which has exon 13 deleted) and a series of full-length and modified ICA512/IA-2 molecules.

Results. The assays showed that ICA512.bdc and ICA512_{604–979} gave the best discrimination between diabetic and control sera. With ICA512_{604–979} a somewhat greater proportion of patients expressing antibodies were detected than with ICA512.bdc in the groups studied (70.5 % vs 63.2 % of prediabetic/new-onset and 25.0 vs 13.9 % in patients with diabetes > 20 years). Conversely, a small proportion (3 % recent-onset and 6 % > 20 years) had antibodies to ICA512.bdc but not ICA512_{604–979}.

Conclusion/interpretation. Important epitopes lie within the exon 13 region and others can be generated by the alternative splicing. As the Δexon 13 variant is probably secreted by the beta cell, it could be recognized by the cellular and humoral arm of the immune system in the absence of cellular damage. [Diabetologia (2000) 43: 1293–1301]

Keywords Insulin-dependent diabetes mellitus, alternative splicing variant, ICA512/IA-2.

A number of autoantigens have been identified in Type I (insulin-dependent) diabetes mellitus, including insulin [1], glutamic acid decarboxylase (GAD)

[2–6], carboxypeptidase H [7], the glycolipid GM2–1 [8], islet cell autoantigen 69 [9] and the protein tyrosine phosphatase (PTP)-like proteins ICA512 [10–14] and phogrin (IA-2β) [15, 16]. Autoantibodies (AA) as such have yet to be shown to play a major part in the pathogenesis of the disease and could represent “epiphenomena” of a beta-cell attack. Nevertheless, they provide valuable markers to predict Type I diabetes and confirm the diagnosis of autoimmunity [17–20].

The combined measurement of ICA512, GAD65 and insulin autoantibodies is the most reliable marker for the diagnosis and prediction of Type I diabetes.

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Abbreviations: PTP, Protein tyrosine phosphatase; JM, juxta membrane; TM, transmembrane; RIPA, radioimmunoprecipitation assay.

We initially cloned ICA512 by screening a human islet cDNA expression library using a pool of sera from patients with Type I diabetes [10, 21] and IA-2 from a subtractive cDNA library [12]. Discrepancies between the sequences of IA-2 and ICA512 were subsequently attributed to sequencing errors and it was resolved that both cDNAs encode a 979-amino acid protein with a molecular mass of 106,000 M_r with a single transmembrane (TM) region (aa 577–600). The protein is found in cells of the diffuse neuroendocrine system within the membranes of secretory granules as a proteolytically-processed 65,000 M_r form (aa 418–979) [22].

Our initial investigations of humoral autoimmunity to ICA512 used a cloned partial sequence (clone HB-1; nt 837–3613) obtained from a human islet cDNA expression library. We, and others, have developed radioimmunoprecipitation assays (RIP) for anti-ICA512 using in vitro transcribed and translated products derived from the HB-1 sequence subcloned into pGEM vector (designated ICA512.bdc hereafter). The predominant epitopes of ICA512 have been localized in its cytoplasmic segment which incorporated both the PTP domain (amino acids 687–979) and the juxta-membrane (JM) amino acids 601–686 [14, 23, 24]. We report here that both the ICA512.bdc sequence and the parental clone (HB-1) lack the TM domain of the protein as a result of alternative splicing that deletes exon 13 (aa 577–629) [25]. Because the JM region incorporates important immunological epitopes and an alternatively spliced variant could contain unique conformational epitopes, we have investigated a series of in vitro produced ICA512 variants as targets of Type I diabetes-associated autoantibodies.

Subjects and methods

Unless otherwise stated, all molecular cloning procedures were done by standard protocols and reagents were of analytical grade and purchased from Sigma (St. Louis, Mo., USA).

Nomenclature. The term ICA512 is used to designate the product of the ICA512/IA-2 gene. ICA512 and IA-2 refer to the same gene product. ICA512.bdc refers to a specific cDNA clone used extensively in assays of autoreactivity in Type I diabetes. It is truncated NH2 terminally and carries a deletion of exon 13 and thus can be designated IA2/ICA512_{256–556 630–979}.

Islets, tissues and cell lines. Islets were isolated from 5 to 7-day-old newborn Sprague-Dawley rats and BALB/C mice as described previously [26]. Human islets were isolated from two human cadaveric donors (gift from Dr. Camillo Ricordi, University of Miami, Medical School) by collagenase digestion as described previously [27]. Rat pituitary, pancreatic islets, brains, thymus, spleen and adrenal tissues were also isolated, homogenized and digested into single cells. The mouse and rat insulinoma cell lines, β TC3, MIN6, and INS cells were maintained in tissue culture in Dulbecco's Modified Eagle's

Medium (DMEM) or RPMI 1640 medium (Gibco BRL, Paisley, UK) containing 10% FCS (Sigma Chemical Co., Poole, Dorset, UK) until they were subconfluent.

RT-PCR analysis. We extracted RNA from the above tissues using Trizol reagent (Life Technologies, Long Island, N.Y., USA) and isolated Poly(A) + mRNA using oligo(dT)-cellulose (Life Technologies) following the manufacturer's instructions. We generated cDNA from total RNA by reverse transcription and then subjected it to PCR amplification (RT-PCR) run for 35 cycles in a Thermoline thermocycler (1 min at 94°C, 1 min at 55°C, 2 min at 72°C). The PCR products were visualized on ethidium bromide stained 2% agarose gels. The primers IA-2-F 5'-AGA CAG GGC TCC AAA TCT TGC-3' and IA-2-R 5'-GCC TGG GCT GCG TCG CTG AAC-3' were used to amplify both the full length IA-2 mRNA (370 bp) and the alternatively spliced transcript lacking exon 13 (151 bp). Parallel reactions using template without reverse transcriptase enzyme were run to control for genomic DNA contamination. The quality of the RNA was checked in all tissues studied by evaluating the expression of β -actin mRNA.

Sequencing and Southern blot analysis of human genomic DNA. Genomic DNA was isolated from a healthy person and nucleotide sequence spanning exon 13 and the flanking introns was analysed in both directions with a ABI 373A sequencer (Applied Biosystems, Foster City, Calif., USA) using primers 5'-AGACAGGGCTCCAAATCTTGC-3' and 5'-GCCTGGGCTGCGTCGCTGAAC-3' corresponding to 1704–1724 and 2053–2033, of ICA512 cDNA together with internal primers. Southern blotting of genomic DNA was done after digestion with Apa I, Sph I and Nco I. The probe was derived from the intracellular portion of ICA512 cDNA (974 bp, nt 2124–3097, aa 655–979) and labelled with digoxigenin (DIG, Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Patients and sera. Sera were obtained from 121 healthy control subjects with no family history of diabetes, 279 subjects with Type I diabetes from the Joslin Diabetes Center and the Barbara Davis Center. The latter were comprised of 51 (27 male and 24 female) relatives of patients with Type I diabetes followed to onset of disease, 71 (36 male and 35 female) new-onset Type I diabetic patients tested within 7 days of diagnosis, 34 (18 male and 16 female) recent-onset Type I diabetic patients (duration < 1 year), 52 (25 male and 27 female) intermediate-duration diabetic patients (duration 1–10 years), and 71 patients with long-duration diabetes (duration > 10 years). The median age of the patient groups at sampling was 10.4 years (range: 1.1–37.4 years), 9.2 years (range: 0.4–41.5 years), 10.4 years (range 1.2–51.7 years), 16.0 years (range 2.5–43.5 years) and 30.2 years (range: 11.3–77.8 years), respectively. The median age of control subjects was 20.2 years (range: 7.1 to 51.4 years). Sequential sera samples were also obtained from 50 subjects of the DAISY (Diabetes Autoimmune Study in the Young) cohort who were followed from the finding of autoantibody positivity (GAD, ICA512.bdc or IAA) to overt diabetes [28]. Of these 40 were siblings or offspring of patients with Type I diabetes and 10 were newborns from the general population. The median age of relatives at initial assay was 2.8 years (range 0–8.0 years) and that of the non-relative newborns was 0.8 year (range 0–2.7 years). The mean follow-up of the former was 2.0 $\pm$ 1.6 years and the latter was 1.4 $\pm$ 1.1 years. All sera were stored at –20°C until use. Diagnosis of Type I diabetes accorded with the criteria of the ADA commission [29]. All subjects gave informed consent and protocols were approved by the Institutional Review Boards of the University of Colorado.

Fig. 1. In vitro translation of ICA512 constructs. A schematic representation of the full-length ICA512 and Δ exon 13 variant is shown. The signal sequence, cysteine-rich domain, transmembrane domain and PTP domain are shaded. The hatched region preceding the PTP domain is conserved between ICA512 and phogrin but not other PTP members

cDNA cloning. We (Fig. 1) generated seven constructs of the ICA512 molecule by PCR using *Taq* polymerase (Ampli Taq, PE Biosystems, Foster City, Calif., USA) from the full-length ICA512 cDNA (ICA512₁₋₉₇₉) provided by Dr. E. Bonifacio (University of Milan, Italy) or the partial clone (nt 837–3613 encoding aa 389–948) described previously (Table 1) [11, 30]. Constructs were generated by PCR of cDNA sequence spanning the start codon to the PTP domain (ICA512₁₋₆₈₉), the PTP domain alone (ICA512₆₈₇₋₉₇₉), the complete cytoplasmic region (ICA512₆₀₄₋₉₇₉), ICA512.bdc (ICA512₂₅₆₋₉₇₉; Δ 577–629), and “full length” Δ exon 13 form deletion (ICA512₁₋₉₇₉; Δ 577–629). A Kozak consensus start codon was built into the primer sequence as needed and the constructs cloned directly into the pCRII vector (Invitrogen, San Diego, Calif., USA) or pGEM-T vector (Promega, Madison, Wis., USA). The sense

and antisense primers for the deletion constructs we made were as follows.

ICA512 ₁₋₆₈₉	5'-GAGTTCGGAAAGATGCGG-3'
	5'-AAGCCAACATGGACATCT-3'
ICA512 ₆₈₇₋₉₇₉	5'-ACCATGGCCAACATGGACATC-3'
	5'-GGTACACAGAGAGATGCTCA-3'
ICA512 ₆₀₄₋₉₇₉	5'-ACCATGGCGCGGCAGCAAGA-3'
	5'-GGTACACAGAGAGATGCTCA-3'
ICA512 ₂₅₆₋₉₇₉	5'-ACATGCCTGGCCACTCCTA-3'
	5'-GGTACACAGAGAGATGCTCA-3'
ICA512 ₁₋₉₇₉	5'-GAGTTCGGAAAGATGCGG-3'
	5'-GGTACACAGAGAGATGCTCA-3'

The nucleotide sequence of each construct was confirmed by automated sequencing in both directions. The amino-acid numbering is based on the deposited amino-acid sequence of human ICA512 (Gene Bank accession number L18983).

Western blotting. Groups of 1600 human islets were homogenized in 100 μ l of PBS containing 2 μ mol/l Pepstatin A, 10 μ mol/l E-64 and 0.1 mmol/l PMSF. Homogenates were centrifuged for 2 h at 259,000 g in a Beckman TLS 55 rotor. Samples (100 μ g protein) of the crude homogenate, the soluble fraction (supernatant) and the resuspended membrane fraction (pellet) were electrophoresed on 12.5 % SDS-PAGE gels, transferred to nitrocellulose and immunoblotted with rabbit antibodies (1:500) generated to the luminal domain of the mature protein (aa 389–576; Fig. 1). Blots were developed with an alkaline phosphatase-conjugated secondary antibody using a proprietary fluorescent substrate (Vistra ECF reagent; Amersham International, Amersham, Bucks, UK) and quantified by fluorescence imaging (Storm 860; Molecular Dynamics, Sunnyvale, Calif., USA).

Autoantibody radioimmunoprecipitation assays (RIPA). The cloned constructs were transcribed and translated in vitro in the presence of ³⁵S-methionine (Amersham International; > 37 TBq/mmol) using the TNT-coupled rabbit reticulocyte system (Promega) (Fig. 1). Radioimmunoprecipitation assays were done in a 96-well plate format as described previously [30]. In brief, ³⁵S-labelled protein (20,000 cpm) was incubated with patients' sera at a 1:25 dilution overnight at 4°C in 100 μ l TBST buffer (20 mmol/l TRIS-HCl, pH 7.4, 150 mmol/l NaCl, 0.1 % bovine serum albumin, 0.15 % Tween 20 containing 0.1 % aprotinin and 10 mmol/l benzamidine). Samples (50 μ l) were transferred to MultiScreen-DV 96-well filtration plate (Millipore, Burlington, Mass., USA) and 25 μ l of 50 % (w/v) Protein A-Sepharose (Pharmacia, Uppsala, Sweden) in TBST added. After incubation for 45 min at 4°C, immunoprecipitates were washed nine times with ice-cold TBST using the Millipore vacuum-operated 96-well plate washer (Millipore, Bedford,

Table 1. Comparison of 99 % index, control mean index, SD score and intra-assay and inter-assay variances between different constructs

Construct	99 % Index	Control mean index	Diabetes SD score	Inter-assay	Intra-assay
(1–979)	0.111	0.004 $\pm$ 0.037	7.2 $\pm$ 0.8	6.5 (9)	10.1 (10)
(1–556, 630–979)	0.319	–0.011 $\pm$ 0.0157	2.4 $\pm$ 0.2	11.2 (8)	10.2 (9)
(256–556, 630–979)	0.049	0.001 $\pm$ 0.010	59.5 $\pm$ 5.4	11.7 (9)	10.0 (9)
ICA512.bdc					
(604–979)	0.030	0.002 $\pm$ 0.009	41.4 $\pm$ 3.7	9.2 (8)	10.0 (10)
(687–979)	0.031	–0.001 $\pm$ 0.010	31.6 $\pm$ 3.1	9.9 (9)	10.0 (10)
(256–979)	0.000	–0.005 $\pm$ 0.075	4.5 $\pm$ 1.0	9.2 (8)	10.0 (10)
(1–689)	0.10	–0.037 $\pm$ 0.064	1.1 $\pm$ 5.6	11.5 (9)	12.1 (10)

Fig. 2. Expression of the ICA512 mRNA and its variant in different tissues and beta-cell lines (MIN6, β TC3 and INS-1 cells respectively). The ICA512 cDNA (370 bp) and the Δ exon 13 variant (151 bp) were amplified from the cDNAs by PCR using the primers spanning the deleted region

Mass., USA). Then, 40 μ l of scintillation liquid (Microscint-20; Packard, Meriden, Conn., USA) was added to each well and radioactivity measured with a Top Count 96-well plate scintillation counter (Packard). Positive and negative control sera were included in every assay and the antibody binding expressed as an index defined as: (unknown sample cpm-negative control cpm)/(positive control cpm-negative control cpm). "Positive" was based on the 99th centile of sera from 121 healthy control subjects.

Competition studies. The cDNA encoding ICA512₁₋₆₈₉ and ICA512₆₈₇₋₉₇₉ were expressed as glutathione S-transferase (GST) fusion proteins in XL-1 blue E.coli using the pGEX 3X vector (Pharmacia, Arlington Heights, Ill., USA). Recombinant protein expression was induced with 0.1 mmol/l IPTG (isopropyl- β -D-thiogalactopyranoside) and the soluble product purified by glutathione-Sepharose affinity chromatography. The GST portion of each molecule was cleaved by factor Xa treatment and the GST removed by a further round of glutathione-Sepharose affinity chromatography [31]. The homogeneity of the recombinant protein was confirmed on 12% SDS-PAGE with Coomassie blue staining. Competition studies were carried out by pre-absorption of sera (1:10 dilution) in 100 μ l TBST with 50 μ g of the purified recombinant proteins for 3 h at room temperature. We then mixed 40 μ l of the sample with in vitro translated ³⁵S labelled antigen (20,000 cpm of TCA precipitable protein) in 100 μ l TBST (final volume). Samples were then processed in the autoantibody immunoprecipitation assay described above.

Statistical analysis. Differences between the autoantibody prevalence for the patients with different duration of diabetes were calculated by the χ^2 test with Yates' correction (two-tailed). When the expected cell value in a 2 $\times$ 2 table was less than 5, the Fisher's exact test was used. A *p* value less than 0.05 was considered statistically significant.

Results

Sequence analysis of ICA512.bdc. Full-length DNA sequence analysis of ICA512.bdc showed that residues 1742 to 1960 of ICA512 sequence were absent, a deletion which would result in an in-frame deletion of 73 aa [aa 557-629 a region that includes the TM domain (aa 577 to 600)]. We confirmed the deletion of the same region in the original cDNA subclone (HB-1) from which ICA512.bdc was derived. The encoded protein is 906 aa in length with a molecular mass of 98,000 *M_r*.

Reverse transcriptase polymerase chain reaction (RT-PCR). We did RT-PCR analysis of mRNA from islet

Fig. 3. Southern blotting of the gene for ICA512 in human genomic DNA. A 974 bp probe encoding intracellular ICA512 cDNA was labelled by random priming with digoxigenin

CAGACAGGGCTCCAAATCTTGCAGACAGGAGTGGGACAGgtatgcgctaactggagaatcagtc
 aagtocccagagggtgatgttgcgcaagagctggaggcaaacagaggggagaactggagcc
 gaaacgtgtccgtaggcacggctggctggaggcgccctcgcccaagcagccctcccgcagtt
 ctcttttcaccagcagatggcgtaggtgtccctccctgcaggaccagccctctgtagtaaaaa
 ggcttgagaactagttgagccgccccttagtctgtctggagcgcagctctccaaattcccaagggaaca
 ccttgacagaaaggacattatactcaaaatatgttttgtttgtttgtctgagacagagtttca
 tcttgcgttccagctggagtgagtgctcgatctcggtcactgcaacctccacctgcccaggt
 tcaagcgattctcctgcctcagcctcccaagtagctgggattacaggtgcctgccaccacacccg
 ggtaaattttttttgtatttttagtagagatggggtttcaccatgttgccagggctggtctcga
 actcccgatctcaggtgatccgcaacctttggcccccaagtgctgagattacaggtgtgagc
 caccacgcccggcctatactcaaaatacctttgtcttctgcacaaactgaatgcctcacgtt
 ctacaattcaatctcaggggttctctgtcgtttttaaatagcaaacctccctgggtggggccctc
 tggcactgcactgtggttgagttgatccgatgggattgagaaggagaatctgagccatctggc
 tgtaccaccatacccctaactctccagctttgccccaggagatgtcctaacagggggtgtggcat
 gggaccggggtgaggagcagctctgaaacctccctatgcactgcctcccttgattctagccttg
 ttccacagAGGGAGGAGGCAGCTGCAGTCTTCCCAAAGTGCACAGCACCTCACCCAATGCG
 CTAGTGTCTCACTCTGGTGGCCCTGGCAGGTGTGGCTGGGTGCTGGTGGCTCTGGCTGTGG
 CTCTGTGTGTGCGGCAGCATGCGCGCAGCAAGACAAGGAGCGCTGGCAGCCCTGGGGCCTGAG
 GGGCCCCATGGTGACACTACCTTTGAGTACCAGgtgtgcagcccagaagctgattgagcaagg
 aaatgcaaggggcctgggagctctgggtcttgcgtggactaagggctggaactgaatggggagtga
 gccctcatccctgggaaggtccagtgaggctggagaagccccagtgaggaggagtagtgtaggg
 gcccttatgaacgcagtggaattgggagcagacagtgccctaaggttcttcttgggtgctgtgcttc
 tggggctctgggattccactctgtcatgtctgaggttgacataagcaccaccaagcaaaacagc
 cctggaaaggatcagggccaggcagaaagacataccccagagagctgagttccactgaaaatca
 gtcaagggcaagcctaggagcccagagttgaaccaggggccctgttgaaaagtttcaggggactt
 GGACCTCACCTGATTCTCTCCACAGGACCTGTGCCGCCAGC

Fig. 4. Nucleotide sequence of human genomic DNA around the deleted region. Intronic sequence appears in lower case

cells using primers flanking the deleted region (P1 and P2). Two bands were amplified from human islets as well as rodent islet beta-cell lines Min 6, β TC3 and INS-1 (Fig. 2). The smaller PCR product corresponded in size to the single band amplified from the ICA512.bdc clone and the original lambda clone HB-1. Sequencing of the PCR products confirmed that the bands corresponded to the deleted (151 bp) and non-deleted (370 bp) forms, respectively (data not shown). Bands of similar size were amplified from cDNA derived from the rat islets, adrenal, pituitary, brain, thymus and spleen tissue (Fig. 2).

Southern blot analysis and sequencing of human genomic DNA. A cDNA fragment of ICA512 cDNA (974 bp, nt 2124–3097, aa 655–979) which includes exon 14 was used to probe the gene in human genomic DNA digested with different enzymes which were predicted to cut once within the deleted transmembrane region. In every case a single hybridizing sequence was observed indicating that the Δ exon 13 variant arose from alternative splicing rather than being the product of an allelic variant or separate gene (Fig. 3). The human genomic sequence contained the 219 bp-deleted region (exon 13) flanked by 945 bp and 429 bp of intronic sequences (Fig. 4).

Western blotting analysis. Western blot analysis of human islet homogenates showed the presence of the major 65,000 M_r protein along with some minor components of lower molecular size (48,000 M_r , 30,000 M_r and 25,000 M_r) (Fig. 5). None of the bands were detectable after pre-absorption of the serum with recombinant GST-full-length ICA512 molecule. Fractionation of the islet tissue showed that the 65,000 M_r component was pelleted and could be solubilized with 0.1 % Triton X-100 (not shown) consistent with its known localization in dense-core granule membranes. The 48,000 M_r component behaved in a similar manner, the broadly migrating bands in the 25,000–33,000 M_r range were, however, released into the supernatant after homogenization. The Δ exon 13 mutant form of ICA512, if translated, would be expected to be produced initially as a soluble luminal protein of around 100,000 M_r and subsequently converted to a 50,000–55,000 M_r form if glycosylated and processed at the KK448 site. The soluble forms observed were clearly shorter presumably as a result of further proteolytic degradation. Western blotting analyses with a cytosolic domain antibody were uninformative principally because of its cross-reactivity with phogrin (IA-2 β). Densitometric analysis on two separate human islet preparation suggested that the 25,000–33,000 M_r forms comprised around 10–15 % of the 65,000 M_r immunoreactivity on a molar basis.

Autoantibody RIPA. The ICA512.bdc and an additional six ICA512 mutant constructs were produced

Fig. 5. Western blot analysis of fractionated human islet tissue. The crude homogenate (Hom.) was centrifuged at $290,000 \times g$ to generate a soluble (SN) and pellet (Pell). The gel loading of the Pell fraction was adjusted to an equivalent amount of membrane protein as Hom. SN was loaded to an equivalent of fivefold the amount of starting material

using the rabbit reticulocyte lysate in vitro translation system. The SDS-PAGE analysis confirmed the predicted sizes of the translation product (Fig. 1) although some heterogeneity existed possibly as the result of alternative AUG start codons in the molecule.

Sera were considered autoantibody positive when the radioactivity precipitated exceeded the 99th centile of normal control sera. The assay threshold index and the mean control index for each construct showed considerable variation even though the intra-assay and inter-assay covariance were generally in the same range (Table 1). This was related to background cpm observed in the assays with each construct and seemed to be a function of the extent of incorporation of the luminal domain sequence, a region which does not contain diabetes-related epitopes. The incorporation of the hydrophobic transmembrane domain did not seem to be a factor.

Of the prediabetic and new-onset Type I diabetic patients 70% (86 of 122), 64% (78 of 122), 63% (77 of 122), 70% (86 of 122), 57% (70 of 122) and 61% (75 of 122) had autoantibody levels exceeding the 99th centile of the healthy control group to

Fig. 6. Correlation of autoantibody titres obtained from diabetic sera using ICA512_{bdc} and ICA512₆₀₄₋₉₇₉. The cut-off index in each assay corresponds to 0.1 in each case

ICA512₁₋₉₇₉, ICA512₁₋₅₅₆, ICA512₆₃₀₋₉₇₉, ICA512_{bdc}, ICA512₆₀₄₋₉₇₉, ICA512₆₈₇₋₉₇₉ and ICA512₂₅₆₋₉₇₉, respectively. Of patients' sera 25% were negative for all of the constructs and 21% of them were positive for all of the constructs. Direct quantitative comparison of the different assays was possible only where the 99th centile index and control mean index were similar and was thus restricted to assays using ICA512_{bdc}, ICA512₆₀₄₋₉₇₉, and ICA512₆₈₇₋₉₇₉. (Table 1). The ICA512_{bdc}, ICA512₆₀₄₋₉₇₉ and ICA512₆₈₇₋₉₇₉ gave notably better discrimination between diabetic and control sera with a higher mean SD score compared with the other constructs ($p < 0.05$). The titre of autoantibodies detected by these assays showed broad correlation as illustrated in Figure 6 for ICA512_{bdc} compared with ICA512₆₀₄₋₉₇₉.

All new-onset Type I diabetic patients positive for the ICA512₆₈₇₋₉₇₉ construct were also positive for ICA512₆₀₄₋₉₇₉ and ICA512_{bdc}₂₅₆₋₅₅₆, ICA512₆₃₀₋₉₇₉ in accordance with their common possession of the PTP domain. In prediabetic and recent-onset patients (duration < 1 year), 18 (11.5%, 18/156) had autoantibodies to ICA512₆₀₄₋₉₇₉ (Table 2), without reactivity to ICA512_{bdc} suggesting that some epitopes are dependent on the preservation of the region between aa 604–629. Conversely 5/156 (3.2%) had autoantibodies to ICA512_{bdc}, but not to the ICA512₆₀₄₋₉₇₉. With increasing duration of diabetes, there was 10 years duration group 10/71 (14.1%) had autoantibodies to ICA512₆₀₄₋₉₇₉, but not to the ICA512_{bdc}₂₅₆₋₅₅₆, ICA512₆₃₀₋₉₇₉ compared with 4/71 (6%) with autoantibodies to ICA512_{bdc} but not ICA512₆₀₄₋₉₇₉.

To further compare the serum reactivity against the juxta-membrane (JM) and PTP domain of ICA512 (Fig. 1), autoantibodies to ICA512₆₀₄₋₉₇₉ were evaluated in competition with excess unlabelled ICA512₁₋₆₈₉ or ICA512₆₈₇₋₉₇₉ (Table 3). Among 86 sera from 122 prediabetic and new-onset patients

Table 2. Concordance of autoantibodies to ICA512₆₀₄₋₉₇₉ and ICA512.bdc in Type I diabetic patients according to varying duration of diabetes

	Prediabetic and new-onset (<i>n</i> = 122)	Recent-onset < 1 year (<i>n</i> = 34)	Duration 1–5 years (<i>n</i> = 22)	Duration 5–10 years (<i>n</i> = 30)	Duration 10–20 years (<i>n</i> = 35)	Duration > 20 years (<i>n</i> = 36)
ICA512 ₆₀₄₋₉₇₉ + ICA512.bdc +	74 (60.7 %)	21 (61.7 %)	10 (45.5 %)	17 (56.7 %)	8 (22.9 %)	4 (11.1 %)
ICA512 ₆₀₄₋₉₇₉ + ICA512.bdc –	12 (9.8 %)	6 (17.6 %)	3 (13.6 %)	3 (10.0 %)	5 (14.3 %)	5 (13.9 %)
ICA512 ₆₀₄₋₉₇₉ – ICA512.bdc +	3 (2.5 %)	2 (5.9 %)	1 (4.5 %)	1 (3.3 %)	3 (8.6 %)	1 (2.8 %)
ICA512 ₆₀₄₋₉₇₉ – ICA512.bdc –	33 (27.0 %)	5 (14.7 %)	8 (36.4 %)	9 (30.0 %)	19 (54.3 %)	26 (72.2 %)

that reacted positively with ICA512₆₀₄₋₉₇₉, 18.6 % were blocked by ICA512₁₋₆₈₉ (JM-only epitopes) and 52.3 % with ICA512₆₈₇₋₉₇₉ (PTP-only epitopes) but the remaining 25 seemed to bear epitopes in both regions. In patients with disease of longer duration, 45 % of sera bound JM epitopes alone and 27 % PTP alone, with the remaining 27 % showing antibodies to both regions. The apparent greater persistence of autoantibodies bound to JM region did not, however, reach statistical significance ($p = 0.06$) (Table 3).

Sequential follow-up of ICA512. We followed 50 subjects of the DAISY cohort from birth. Among siblings or offspring of patients with Type I diabetes (SOC group), 20/40 (50 %) had neither autoantibodies to ICA512₆₀₄₋₉₇₉ nor ICA512.bdc whereas 15/40 (37.5 %) had antibodies to both, 4/40 (10 %) autoantibodies to ICA512₆₀₄₋₉₇₉ alone and 1/40 (2.5 %) to ICA512.bdc alone. Among a general population cohort (NEC group), seven/ten (70 %) subjects had neither autoantibody specificity, one subject had both, one ICA512₆₀₄₋₉₇₉ alone and one subject had ICA512.bdc alone. Sequential follow-up of autoantibody titre using ICA512.bdc, ICA512₆₀₄₋₉₇₉, ICA512₆₈₉₋₉₇₉ and ICA512₁₋₆₈₉ constructs did not show consistent differences in the specificities of the autoantibody reactivities during the emergence of disease but did serve to emphasize the importance of the assay sensitivity in early detection. Thus the ICA512.bdc and ICA512₆₀₄₋₉₇₉ constructs that performed best in terms of the SD score, control mean index and 99th centile index in cross-sectional analyses (Table 2) provided the most sensitive index of autoreactivity and its persistence in the four people that were examined.

Discussion

In this study we show that IA-2/ICA512 molecule is alternatively spliced in the pancreas, brain, thymus, spleen and other neuroendocrine tissues of rodents and humans to a form in which the single transmem-

Table 3. Reactivity of sera measured by antigen pre-absorption analysis using ICA512₆₀₄₋₉₇₉

Reactivity	Prediabetic and new-onset (<i>n</i> = 122)	Duration > 10 years (<i>n</i> = 71)
PTP – only	45 (52.5 %)	6 (27.2 %)
JM – only	16 (18.6 %)	10 (45.5 %)
Both	25 (29.1 %)	6 (8.4 %)
None	30	39

brane domain and some flanking sequence is deleted in frame. Studies of endogenous IA-2 in islet and pituitary have confirmed the existence of multiple molecular forms most of which are attributable to alternative sites of post-translational processing of the molecule. The major form migrates as a doublet of 60,000–65,000 M_r which has a common NH_2 sequence suggestive of cleavage by a furin/PC type endopeptidase in the secretory pathway at a dilysine motif (KK₄₄₈). The size variation here is attributed to post-translational modification or possibly alternative splicing. Other variants are found in bovine pituitary and have been attributed to proteolytic cleavage at sites aa RR₄₅, KR₁₃₃ and KK₃₈₇ in the luminal domain and FN₆₄₄ in the cytoplasmic domain respectively.

A protein bearing the aa557–629 deletion of the Δexon 13 mutant would be synthesized as a 97,000 M_r precursor and, if processed at the KK₄₄₈ site, would generate a 53,000 M_r mature form. Newly synthesized Δexon 13 mutant would pass directly into the lumen of the endoplasmic reticulum rather than be inserted into the membrane as in the case of full-length ICA512. If it folded properly, the mutant would be secreted either constitutively as the 97,000 M_r form or in a regulated manner as a 53,000 M_r protein assuming that it was processed at KK₄₄₈ site in a storage granule. It is likely that translocation of the normally “cytoplasmic” domain of Δexon 13 into the oxidizing environment of the secretory pathway results in misfolding and proteolysis as part of the post endoplasmic reticulum editing process. Moreover, there are several sites within the “cytoplasmic” do-

main of ICA512 which would be susceptible to tryptic-like cleavages by the post-translational endoproteolytic machinery of islet cells especially in the aa 629–680 region. The PTP domain itself is insensitive to trypsinolysis both in the native protein [13] and in *in vitro* translated material [32]. The observation that only lower M_r forms of soluble ICA512 immunoreactivity were present in human islets would be explicable by such proteolytic events. The Δ exon 13 mutant mRNA content of islets approximated 30% of full-length mRNA based upon semiquantitative PCR analysis. It is probable that the relative tissue content of Δ exon 13 ICA512 protein would be less because the full-length protein is delivered efficiently to the granule and retrieved at each round of exocytosis whereas the mutant would be secreted. The finding that around 10% of the ICA512 in human islets appeared as soluble NH_2 terminal immunoreactive forms is consistent with these observations.

The cDNA clone ICA512.bdc which has been used extensively for RIP assays detects antibodies in 60–70% of prediabetic and new-onset Type I diabetic patients [30]. Most epitopes are confined to the cytoplasmic portion of ICA512 [23, 30, 33] as was confirmed in the present investigation from examination of seven different constructs. These constructs further distinguished between autoreactivity to the conserved PTP domain and the JM aa 604–687 which is unique to ICA512 and phogrin in the PTP superfamily. The latter region is deleted in the ICA512 Δ exon 13 mutant up to aa 629. In young-onset patients PTP autoantibodies have been shown to be more prevalent and to correspond to 37,000 M_r (phogrin-derived PTP domain) and 40,000 M_r (ICA512_{654–979}) autoantigens defined by trypsin digestion [23]. Juxta-membrane autoantibodies were, however, more prevalent in older-onset patients and found in association with the 40,000 M_r (ICA512-derived) fragment alone. From our study of new-onset (duration < 7 days) and long-duration (duration > 10 years) patients, it seems the JM autoantibodies are initially less prevalent than PTP antibodies (37.7 vs 79.4% of ICA512_{604–979} positive subjects) were more persistent (72 vs 54% at > 10 years duration) which is consistent with these earlier findings.

About 10% of the reactivity to ICA512_{604–979} was not detected by the RIP assay using ICA512.bdc construct in recent-onset Type I diabetic patients. We also found, however, that about 4% of Type I diabetic patients at any age or duration, had autoantibodies to ICA512.bdc but not to the ICA512_{604–979}. Although it is conceivable that an epitope upstream of aa557 is present in this construct, it is more likely that the alternatively spliced molecule has additional unique epitopes generated by the joining of exons 12 and 14 on changes in the conformation. From a practical standpoint, it is clear that assays using ICA512_{604–979}

or ICA512.bdc do not simply detect a subset of epitopes exposed on full-length ICA512 and thus assays based on different constructs could yield additional information. Ideally, these should be modelled on the three-dimensional structure of the protein which is still unresolved.

The existence of an alternatively spliced form of ICA512 that shows tissue-specific expression in lymphoid and neuroendocrine tissue raises a number of questions about the way the immune system recognizes this autoantigen in Type I diabetes. The prevailing concept that ICA512 is a secondary target of autoimmunity because the epitopes are cytosolic in location needs to be reconsidered in the light of the finding that the Δ exon 13 variant is probably secreted in beta cells. The indication that the normally “cytosolic” antigenic domain of the Δ exon 13 variant enters the secretory pathway could also mean that the molecule can access major histocompatibility complex processing compartments directly and thus be presented to the cellular arm of the immune system. The observation that lymphoid tissues seem to express only the Δ exon 13 variant could mean that the corresponding sequence is not encountered by the immune system during the process of early thymic education [34] and thus remains a cryptic epitope capable of conferring autoimmunity when expressed peripherally. Such speculations raise readily testable hypotheses that could be addressed using a deletion/mutagenesis strategy similar to that used here for the detection of humoral autoreactivity. Because cellular autoimmunity to the PTP family of proteins has been detected in the non-obese diabetic (NOD) mouse [31], such studies could be extended to an established experimental model of diabetes pathogenesis.

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