# Definition of Multiple ICA512/Phogrin Autoantibody Epitopes and Detection of Intramolecular Epitope Spreading in Relatives of Patients With Type 1 Diabetes

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**The related tyrosine phosphatase-like proteins, islet cell antigen 512 (ICA512) and phosphatase homologue in granules of insulinoma (phogrin), are major targets of autoantibodies in patients with type 1 diabetes. In the current study, we have examined the overlapping specificities and antigenic epitopes of autoantibodies to ICA512 and phogrin and determined whether intramolecular epitope spreading occurs during the development of diabetic autoimmunity. ICA512 autoantibodies and phogrin autoantibodies were detected in 65–70% (*n* = 110) of patients with new-onset type 1 diabetes and 60–65% (*n* = 42) of prediabetic relatives of patients with type 1 diabetes. Of the sera, 10% reacted with ICA512 but not phogrin, whereas only 1% of sera reacted with phogrin but not ICA512. The binding of phogrin autoantibodies in 88 dual (ICA512 and phogrin) autoantibody-positive sera could be completely blocked by excess recombinant ICA512, whereas the blocking of ICA512 autoantibodies with recombinant phogrin was only partial (mean inhibition of 58.9 ± 3.7%, mean ± SE). Binding and competition analysis using multiple chimeric ICA512/phogrin constructs demonstrated that a major unique epitope for ICA512 autoantibodies is localized to amino acids 762–887. A conformational epitope associated with the carboxy-terminal 31 amino acids of ICA512 was recognized by one-third of sera, and a minor epitope is located on amino acids 601–762 of ICA512. The major epitopes for phogrin-selective autoantibodies were localized to amino acids 640–922 of phogrin. Sequential serum samples were analyzed in 22 relatives who expressed ICA512/phogrin autoantibodies. Intramolecular epitope spreading was found for 5 of 13 relatives who have progressed to type 1 diabetes. Among nine relatives who have remained nondiabetic, three demonstrated a decrease in the number of epitopes recognized. These studies highlight the complexity of autoantibody recognition of ICA512/phogrin and are consistent with the hypothesis that ICA512/phogrin may be recognized as a consequence of β-cell destruction. *Diabetes* 47:733–742, 1998**

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AA, autoantibodies; DAISY, Diabetes Autoimmunity Study in the Young; GAA, autoantibodies to glutamic acid decarboxylase 65; IAA, insulin autoantibodies; ICA512, islet cell antigen 512; PCR, polymerase chain reaction; phogrin, phosphatase homologue in granules of insulinoma; PTP, protein tyrosine phosphatase.

The autoimmunity of type 1 diabetes is characterized by T-cell–mediated destruction of pancreatic β-cells and the presence of circulating autoantibodies directed against several β-cell antigens, including insulin (1), GAD65 (2), GM2-1 ganglioside (3), glima38 (4), and 37/40-kDa tryptic fragments that were recently identified as originating from islet cell antigen 512 (ICA512) (IA-2) and phosphatase homologue in granules of insulinoma (phogrin) (IA-2β) (5–8). ICA512 was initially cloned by Rabin et al. (6) from an islet expression library screened with islet cell autoantibody–positive sera. A longer clone of ICA512, termed IA-2, was obtained independently by Lan et al. (7). Wasmeier and Hutton (8) identified phogrin as a novel insulin granule membrane protein from a rat insulinoma expression library screened with an anti-insulin granule membrane sera. Sequences identical to human phogrin (9) have been independently cloned from human fetal brain, termed ICAAR (10), and human colon carcinoma cell, termed IAR (11). ICA512 and phogrin are type 1 transmembrane glycoproteins that are localized in dense cored secretory granules of peptide-secreting endocrine cells and neurons (8,12). They are both members of the protein tyrosine phosphatase (PTP) superfamily and exhibit 30% overall sequence identity and 80% within the PTP domain.

Sensitive radioassays for autoantibodies to these proteins (9,13–15) have been developed using in vitro transcribed and translated ICA512 and phogrin. The presence of autoantibodies (AA) to phogrin (phogrin AA) are correlated to ICA512 autoantibodies (ICA512 AA), but the quantitative relationship is not strong ($r$ = 0.82), suggesting that multiple epitopes within the two molecules are recognized to different extents by the sera from different patients (14).

Although anti-islet AA probably do not directly contribute to the pathogenesis of type 1 diabetes, they provide powerful predictive markers among relatives of patients with type 1 diabetes (16). Humoral autoimmunity, especially to insulin, GAD65, and ICA512, appears to develop in a sequential manner indicative of intermoleculer epitope spreading and also to persist over many months to years (17). We now report, using chimeric ICA512/phogrin constructs, that intramolecular epitope spreading also occurs.



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## RESEARCH DESIGN AND METHODS

**Serum samples**. Sera were obtained from 110 (56 male and 54 female) patients with new-onset type 1 diabetes within 7 days of diagnosis (mean age 10.7 years; range 1.2–32.1 years); 42 (19 male and 23 female) relatives of patients with type 1 diabetes, who were followed to the onset of overt diabetes (mean age 17.4 years; range 3.9–69.0 years); and 104 healthy control subjects with no family history of diabetes (mean age 22.2 years; range 7.1–51.4 years). All were from the Joslin Diabetes Center and the Barbara Davis Center. The mean duration of follow-up in the prediabetic group was 2.3 years (range 0.1–9.7 years). Sera from 15 relatives with high-risk HLA alleles (DR 3/4, DQB1*0302) who were first studied at <1.5 years of age in the Diabetes Autoimmunity Study in the Young (DAISY) were also studied along with 66 sequential serum samples from 22 relatives from our prospective studies of first-degree relatives who expressed AA to ICA512 (ICA512 AA) or phogrin (phogrin AA) on follow-up. Among the latter, the mean follow-up period was 4.4 years (range 0.9–12.0 years), and 13 of these relatives have subsequently developed type 1 diabetes.

The diagnosis of type 1 diabetes was according to National Diabetes Data Group criteria, with either fasting hyperglycemia or oral glucose tolerance testing (18). Subjects gave informed consent, and protocols were approved by the Institutional Review Boards of the University of Colorado. Sera were stored at –20°C until use.

**cDNA cloning of ICA512 and phogrin.** Figure 1 illustrates the sequence homology between human islet ICA512 and human islet phogrin and the constructs used in the current study. The cDNA encoding ICA512 (amino acids 256–979) and COOH-terminus–truncated ICA512 (amino acids 389–948) were amplified by polymerase chain reaction (PCR) and subcloned directly into the pCRII vector (Invitrogen, San Diego, CA) as previously described (15). A PTP domain ICA512 (amino acids 687–979) construct in the pGEM-T vector (Promega, Madison, WI) was kindly provided by E. Bonifacio (University of Milan, Italy). The cytoplasmic domain of phogrin (amino acids 640–1015) cDNA was cloned by PCR as described previously (9). The amino acid numbering is based on the deposited amino acid sequence of human ICA512/IA-2 (GenBank accession number L18983) and human phogrin (GenBank accession number U66702).

**Construction of chimeric PTP-like molecules.** The chimeric cytoplasmic domain of ICA512/phogrin constructs used in this study (Fig. 2) were generated by interchanging segments of ICA512 cytoplasmic region designated 512N (amino acids 601–762 of ICA512), 512M (amino acids 762–887), and 512C (amino acids 887–979) with corresponding segments of the phogrin cytoplasmic domain, PhN (amino acids 640–798), PhM (amino acids 798–923), and PhC (amino acids 923–1015). The primary sequences for ICA512 between amino acids 762–770 and amino acids 887–895 are identical to the phogrin sequences between amino acids 798–806 and amino acids 923–931, respectively, a fact that facilitated the generation of these constructs and ensured that perturbation of the secondary structure of the chimeras was minimized. The sense and antisense primers for the regions were as follows.

| | |
|---|---|
| ICA512–5′ | 5′ TCTGTGTCATATGCAGCATGC 3′ |
| ICA512–3′ | 5′ GGTACACAGAGATGCCCA 3′ |
| Phogrin-5′ | 5′ CCTCATGCATAGCTCTCA 3′ |
| Phogrin-3′ | 5′ TTCCTGACAACATCCGTG 3′ |
| Phogrin-5′ (*Sac*I) | 5′ TGAGCTCCTCATGCATAGCTCTCA 3′ |
| Phogrin-3′ (*Nde*I) | 5′ TGCCATATGCTGGCGCTGGGGAAGGGCCTT 3′ |
| 512N-3′ | 5′ AATGGGGCTAGCGTTGATGATATCGCTG 3′ |
| 512M-5′ | 5′ CAGCGATTACATCAACGCTAGCCCCATT 3′ |
| 512M-3′ | 5′ GTTTACCTTTCTGCGGAAGTCCAGCAGG 3′ |
| 512C-5′ | 5′ CCTGCTGGACTTCCGCAGAAAGGTAAAC 3′ |

To create the PhN-512M-512C chimera, the $\text{phogrin}_{640\text{–}798}$ segment was generated from a phogrin template by PCR using Phogrin-5′ and 512N-3′ primers and the $\text{ICA512}_{762\text{–}979}$ segment from a ICA512 template using 512M-5′ and ICA512-3′ primers. The PCR products were purified from a low-melting agarose gel using QIAEX II DNA Purification System (QIAGEN, Chatsworth, CA) and then combined together in a PCR reaction and amplified with Phogrin-5′ primer and ICA512-3′ primer. This PCR product was ligated into pGEM-T vector (Promega) for in vitro transcription and translation. The chimeric 512N-512M-PhC, PhN-PhM-512C, and 512N-PhM-PhC constructs were amplified by a similar strategy. The chimeric PhN-512M-PhC and 512N-PhM-512C cDNAs were amplified sequentially with PhN-512M and PhC cDNA or 512N-PhM and 512C cDNA, respectively, using the same approach but using the chimeric clones as templates. The amino acid sequence of 512N, 512M, and 512C showed 59.1, 79.8, and 90.3% identity with the corresponding regions of phogrin, respectively (7,9).

A tandem phogrin/ICA512 cytoplasmic tail construct was also generated by PCR (Fig. 1*B*). A PCR product was amplified using a ICA512-3′ primer and ICA512-5′ primer which contained an *Nde*I restriction site upstream of the amino acid position 601 of ICA512. This was ligated into pGEM-T vector (Promega) in SP6 promoter orientation. In a separate reaction, the cytoplasmic domain of phogrin cDNA was amplified using a 5′ primer introducing a start codon at amino acid position 640 of the phogrin sequence and a *Sac*I restriction site (Phogrin-5′ [*Sac*I] primer), and a 3′ primer that deleted the translational stop codon and introduced a *Nde*I restriction site (Phogrin-3′ [*Nde*I] primer). The tandem construct was generated by ligation of the *Sac*I/*Nde*I digested phogrin fragment into the above pGEM-T /ICA512 vector digested with *Sac*I and *Nde*I. All constructs were confirmed by restriction enzyme digestion and nucleotide sequencing by automated sequencing using a sequencer (ABI 373A; Applied Biosystems, Foster City, CA) and by SDS-PAGE and autoradiography of the chimeric proteins after in vitro transcription and translation (data not shown).

**Radioimmunoassay for AA to ICA512, phogrin, and chimeric ICA512/phogrin molecules.** The constructs were transcribed and translated in vitro in the presence of [$^{35}$S]methionine (Amersham, Amersham, U.K.; >1,000 Ci/mmol) using the TNT-coupled rabbit reticulocyte system (Promega) with SP6 RNA polymerase. Precipitation of the translation products with 25% (wt/vol) trichloroacetic acid was used to determine the extent of incorporation of the labeled amino acids.

The immunoprecipitation radioassays for AA to ICA512, phogrin, and ICA512/phogrin chimeric molecules were determined in duplicate, as described previously (15), in a 96-well microtiter plate format. Immunocomplexes were isolated on Protein-A sepharose (Pharmacia, Piscataway, NJ), and radioactivity was determined with Top Count 96-well plate beta counter (Packard, Downers Grove, IL). Positive and negative control sera were included, and the antibody levels were expressed as an index defined as follows: (counts per minute in the unknown sample – negative control)/(positive control – negative control). Classification of a sample as positive for ICA512 AA radioassays was based on the 99th percentile of sera from 208 healthy control subjects. This corresponded to an index of 0.160 for $\text{ICA512}_{389\text{–}948}$AA, 0.048 for $\text{ICA512}_{256\text{–}979}$AA, and 0.039 for $\text{ICA512}_{687\text{–}979}$AA, respectively (15). The cut-off values for $\text{phogrin}_{640\text{–}1015}$ AA and combined PTP AA were based on the 99th percentile of sera from 104 healthy control subjects, and were 0.110 and 0.035, respectively. For AA to the chimeric ICA512/phogrin constructs, standard deviation scores were calculated for each serum based on the mean value and SD of indexes with 70 normal control serum (SD score = (antibody index of test serum – mean index of healthy control sera)/SD of the indexes of healthy control sera). Positive was defined as a standard deviation score ≥3. For all radioimmunoassays, sera from the same group of normal controls were used to determine the cut-off values.

**IAA and GAA assays.** The insulin autoantibody assay (IAA) was performed by a fluid-phase radioassay using competition with cold insulin and precipitation with polyethylene glycol, as described previously (19). The 99th percentile of the normal range for IAA is 42 nU/ml. The GAD65 autoantibody assay (GAA) was based on immunoprecipitation of in vitro transcribed and translated human islet $^{35}$S-labeled GAD65, as described previously (14). The levels of GAA were expressed as an index as in the case of ICA512 and phogrin. The 99th percentile cut-off in the normal controls was an index of 0.032.

**Competition studies.** Competition studies of AA binding of in vitro translated $^{35}$S-labeled proteins were performed using affinity-purified recombinant $\text{ICA512}_{601\text{–}979}$ and $\text{phogrin}_{640\text{–}1015}$ proteins (Fig. 1*B*). These were generated by cloning cDNA encoding $\text{ICA512}_{601\text{–}979}$ or $\text{phogrin}_{640\text{–}1015}$ in the PinPoint Xa1-T bacterial expression vector (Promega) by PCR. Recombinant protein expression was induced by the addition of 100 μmol/l of isopropyl-β-galactopyranoside in the XL-1 blue bacterial host in the presence of 2 μmol/l of biotin. After overnight culture at room temperature, the bacteria were recovered and resuspended in 50 mmol/l Tris (pH 8.0) and 100 mmol/l NaCl containing 1 mmol/l EDTA, 1 mg/ml lysozyme, and 1 mmol/l phenylmethylsulfonyl fluoride followed by incubation for 20 min at 37°C. Cells were lysed by freezing and thawing twice; $MgCl_2$ (5 mmol/l) and DNAse I (10 μg/ml) were added; and the lysate was incubated at 37°C for 20 min. Insoluble material was removed by centrifugation at 9,000*g* for 30 min, and the recombinant protein was affinity-purified using a SoftLink Soft Release Avidin Resin (PinPoint Protein Purification System; Promega). Bacteria transformed with PinPoint Xa1 vector without insert were used as a control. The protein concentration was determined by BCA Protein Assay (Pierce, Rockford, IL). Purity of the recombinant protein was analyzed on 10% SDS-PAGE with Coomassie blue staining. The overall yield was 3–5 mg/l of bacterial culture. Competition studies were carried out by preincubation of sera with the purified recombinant proteins or control buffer for 3 h at room temperature followed by the addition of in vitro translated $^{35}$S-labeled ICA512, phogrin, or chimeric ICA512/phogrin in Tris-buffered saline/Tween 20. Samples were then processed in the AA radioassay as above.

**Statistical analysis**. A standard deviation score was calculated for each serum according to the formula: (antibody index of test serum – mean index of healthy control sera)/SD of the indexes of healthy control sera. The correlation between antibody levels was analyzed using Spearman's rank-correlation test. A $P$ value <0.05 was considered statistically significant. Amino acid sequence comparisons were performed using the BESTFIT program of Genetics Computer Group (GCG package version 7 from Madison, WI).

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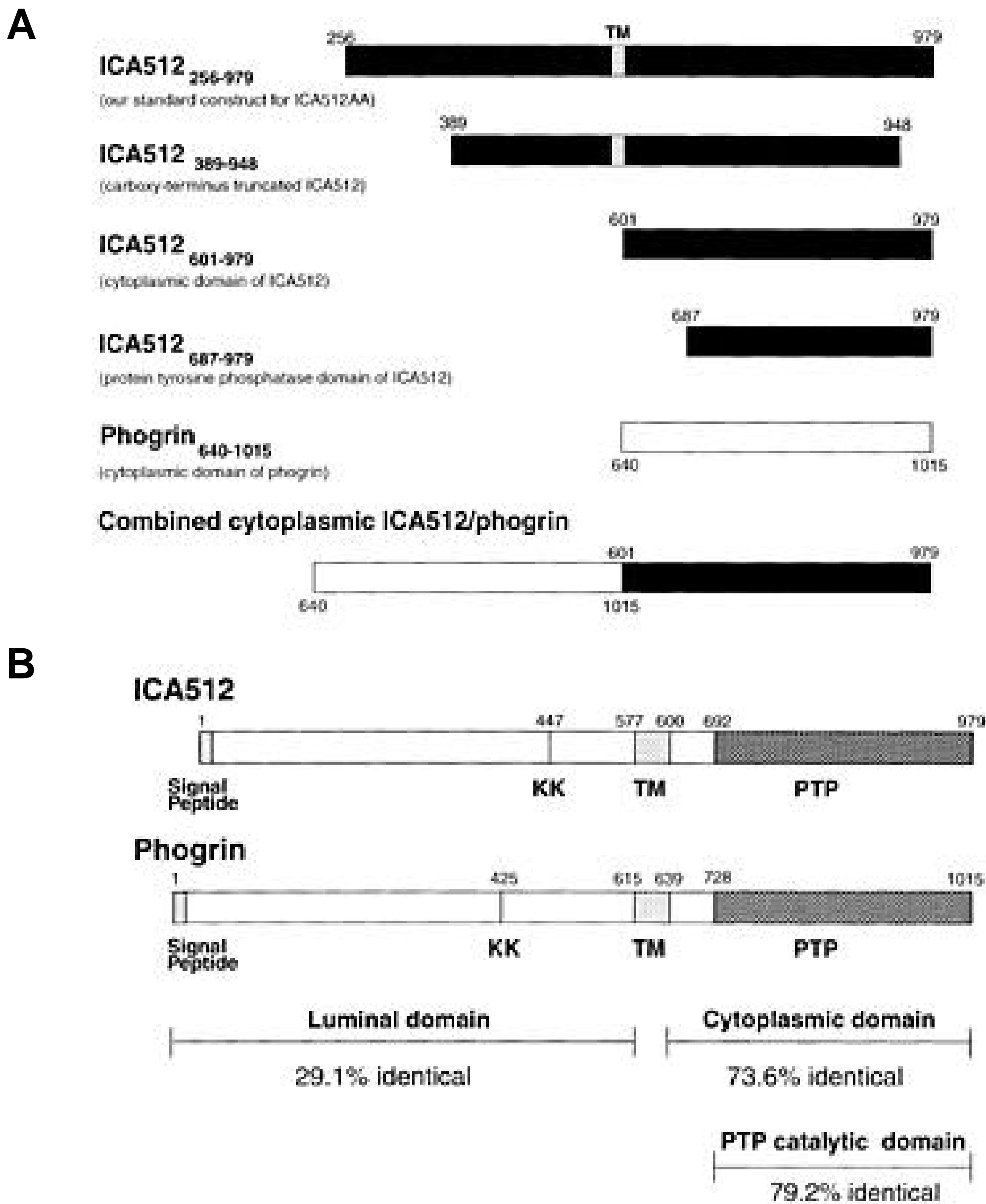


**FIG. 1. Nucleotide sequence identity between ICA512 and phogrin (*A*) and the amino acid boundaries of ICA512 or phogrin constructs used in this study (*B*). The amino acid sequence is numbered according to the deposited sequence of IA-2 (GenBank accession number L18983) and phogrin (GenBank accession number U66702). KK, post-translational proteolysis site; TM, transmembrane region.**

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## RESULTS

**Prevalence and concordance of ICA512 AA and phogrin AA.** Of 110 patients with new-onset type 1 diabetes, 73 expressed $ICA512_{256–979}$ AA (66.4%) versus 63 of the 110 who were $phogrin_{640–1015}$ AA–positive (57.3%; Table 1). Thirty (71.4%) and 27 (64.3%) of 42 first-degree relatives followed to diabetes were positive for $ICA512_{256–979}$ AA and $phogrin_{640–1015}$ AA, respectively. In 90 patients positive for phogrin AA, 88 (97.8%) were also positive for $ICA512_{256–979}$ AA. Eleven of 110 (10.0%) sera from new-onset patients and 4 of 42 (9.5%) sera from prediabetic relatives reacted with $ICA512_{256–979}$ but not phogrin. One of 110 (0.9%) new-onset patients and 1 of 42 (2.4%) prediabetic relatives were phogrin AA–positive but $ICA512_{256–979}$ AA–negative (Table 1). The indexes of phogrin AA in these two individuals (0.120 and 0.216) in relation to the 99th percentile index of normals = 0.110.

**Overlapping specificities of ICA512 and phogrin AA.** Based on the dose-response curve with dual (ICA512 and phogrin) AA-positive sera, 5 µg of recombinant $ICA512_{601–979}$ and $phogrin_{640–1015}$ completely inhibited the serum binding to $^{35}$S-labeled ICA512 $_{601–979}$ and phogrin $_{640–1015}$, respectively (data not shown). For 82 of 88 dual AA-positive sera, absorption with 10 µg of the recombinant $ICA512_{601–979}$ completely blocked reactivity to $^{35}$S-$phogrin_{640–1015}$. Phogrin AA reactivity in the remaining six sera was completely blocked by preincubation with additional (25–30 µg) recombinant ICA512. In contrast, preincubation of dual AA-positive sera with 20–30 µg of recombinant phogrin completely inhibited the ICA512 AA reactivity in only 15 of 88 (17.0%) sera. Of these 15 sera, 7 sera were positive for the COOH-terminus–truncated $ICA512_{394–948}$ AA, but 8 were $ICA512_{394–948}$ AA–negative. The mean level of $ICA512_{256–979}$ AA in 88 sera positive for phogrin AA was decreased from an index of 1.36 ± 0.04 (mean ± SE) to a mean index of 0.63 ± 0.06 by addition of recombinant phogrin, with a mean inhibition of $ICA512_{256–979}$ AA reactivity of 58.9 ± 3.7% (mean ± SE).

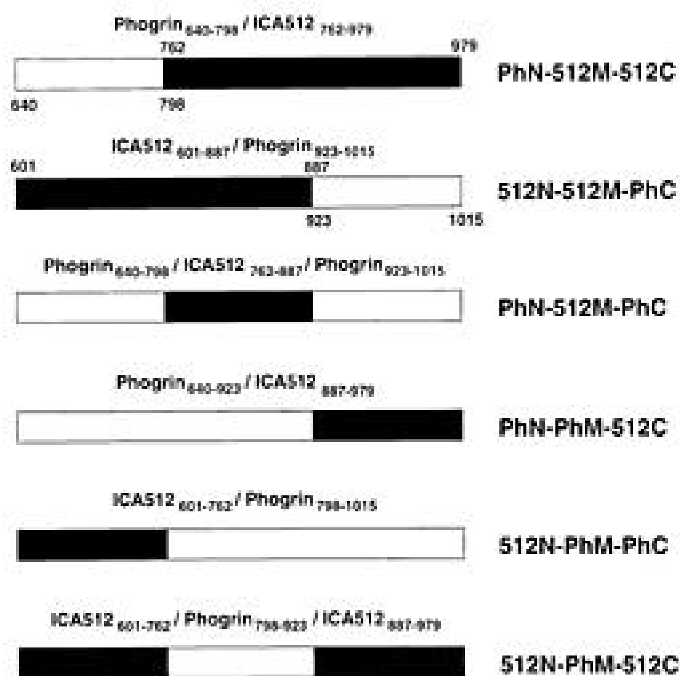


**FIG. 2. Schematic representation of chimeric ICA512/phogrin constructs of the cytoplasmic domain of molecules. Six different ICA512/phogrin chimeric constructs were prepared using sequential PCR (see METHODS). Numbers are amino acid positions according to the published human IA-2 and phogrin amino acid sequence.**

**Epitope analysis for ICA512- and phogrin-specific AA.** All sera positive for AA to COOH-terminus–truncated $ICA512_{389–948}$ were also positive for $ICA512_{256–979}$ AA and PTP-domain $ICA512_{687–979}$ AA. Furthermore, all sera that reacted with the $ICA512_{256–979}$ construct also recognized the PTP domain of $ICA512_{687–979}$. Among sera that were positive, only 26 of 73 (35.6%) new-onset patients' samples and 11 of 30 (33.3%) prediabetic relatives' samples were positive for ICA512 bearing a truncation of the 31 carboxy-terminal amino acids 949–979, indicating that a major epitope resided in this region.

Thirty-seven positive sera (15 prediabetic relatives and 22 new-onset patients) were tested for reactivity with six different chimeric ICA512/phogrin constructs based on a cut-off value for each construct defined as mean + 3 SD of the indexes of 70 normal control sera. The nomenclature for pattern of reactivity to ICA512, phogrin, and ICA512/phogrin chimeric constructs is shown in Table 2. Figure 3 illustrates representative patterns of reactivity for two such sera analyzed with or without preincubation with 20 µg recombinant phogrin, a positive control (rabbit immunized with rat phogrin COOH-terminus in complete Freund's adjuvant) and a negative control human sera. The rabbit antisera immunoprecipitated all of the six chimeric molecules (44.7–97.5% of the cpm precipitated) versus 0.9–2.1% for the negative control. The pattern of AA recognition of the six constructs by the patient sera varied dramatically, however. Sera from patient 207963 did not bind to any chimeric ICA512/phogrin construct after preincubation with recombinant phogrin, suggesting complete cross-reactivity. In contrast, sera from patient 209442 were preabsorbed by those constructs bearing the PhM segment and not in constructs with the 512M segment, suggesting that independent ICA512 and phogrin epitopes existed in this case.

In all, 11 different patterns of reactivity were distinguishable in 37 $ICA512_{256–979}$ AA–positive sera (25 positive and 12 negative for phogrin AA), which are summarized in Table 2. Most sera reacted with the chimeric constructs containing the middle segment of cytoplasmic domain of ICA512 (512M). Of 37 sera analyzed with phogrin absorption, 25 reacted with the PhN-512M-512C; 31 reacted with the 512N-512M-PhC; and 25 reacted with the PhN-512M-PhC. For constructs lacking the middle portion of ICA512, only 1 of 37 sera reacted with the 512N-PhM-PhC; 4 reacted with 512N-PhM-512C; and none of these reacted with the PhN-PhM-512C construct. In terms of the magnitude of the responses, AA to the PhN-512M-512C construct were highly correlated with those of 512N-512M-PhC AA ($r = 0.92$, $P < 0.0001$) and PhN-512M-PhC AA ($r = 0.93$, $P < 0.0001$; Fig. 4).

Of new-onset patients and prediabetic relatives, approximately one-third (37 of 103) of sera reacted with $ICA512_{256–979}$ but not COOH-terminus–truncated $ICA512_{389–948}$. For these sera, the binding to $ICA512_{256–979}$ was absorbed by preincubation of sera with the cytoplasmic domain of recombinant $ICA512_{601–979}$. These sera appear to recognize a conformational epitope(s) associated with carboxy-terminal 31 amino acids of ICA512 and additional residues within the cytoplasmic domain of ICA512. As shown in Table 2, 9 of 12 sera studied that did not react with COOH-terminus–truncated $ICA512_{389–948}$ (but did react with $ICA512_{256–979}$) did not bind to the PhN-512M-512C, PhN-PhM-512C, or 512N-PhM-512C constructs that contain the COOH-terminus of $ICA512_{887–979}$. Moreover, none of these 12 sera were absorbed by recombinant phogrin. Thus, a region of the molecule outside of the

TABLE 1
Prevalence and concordance of ICA512 AA and phogrin AA in patients with new-onset type 1 diabetes and prediabetic relatives

| AA to | | | | |
|---|---|---|---|---|
| ICA512 (amino acids 256–979) | Phogrin (amino acids 640–1015) | New-onset diabetes ($n$ = 110) | Prediabetic relatives ($n$ = 42) | Combined groups ($n$ = 152) |
| + | + | 62 (56.4) | 26 (61.9) | 88 (57.9) |
| + | – | 11 (10.0) | 4 (9.5) | 15 (9.9) |
| – | + | 1 (0.9) | 1 (2.4) | 2 (1.3) |
| – | – | 36 (32.7) | 11 (26.2) | 47 (30.9) |

Data are $n$ (%). + and –, AA-positive and -negative, respectively, based on the 99th percentile of normal controls.



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TABLE 2
Eleven patterns of reactivity to ICA512 and chimeric ICA512/phogrin constructs in 37 ICA512 $_{256–979}$ AA-positive sera at diabetes onset

| Patterns | *n* (%) of patients with pattern | Constructs | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ICA512 (256–979) | ICA512 (389–948) | PhN-512M –512C§ | 512N-512M –PhC | PhN-512M –PhC | PhN-PhM –512C | 512N-PhM –PhC | 512N-PhM –512C |
| ICA512AA$^+$/phogrinAA$^-$ sera | | | | | | | | | |
| Ph$^-$-3* | 3 (8.1) | + | – | – | + | + | – | – | – |
| Ph$^-$-4 | 3 (8.1) | + | – | + | + | + | – | – | – |
| Ph$^-$-5 | 5 (13.5) | + | + | + | + | + | – | – | – |
| Ph$^-$-7 | 1 (2.7) | + | + | + | + | + | – | + | + |
| ICA512AA$^+$/phogrinAA$^+$ sera (tested with absorption by unlabeled recombinant phogrin) | | | | | | | | | |
| Ph$^+$-1‡ | 5 (13.5) | + | – | – | – | – | – | – | – |
| Ph$^+$-2a | 1 (2.7) | + | – | – | + | – | – | – | – |
| Ph$^+$-2b | 2 (5.4) | + | + | – | – | – | – | – | – |
| Ph$^+$-3 | 1 (2.7) | + | + | – | + | – | – | – | – |
| Ph$^+$-4 | 3 (8.1) | + | + | + | + | – | – | – | – |
| Ph$^+$-5 | 10 (27.0) | + | + | + | + | + | – | – | – |
| Ph$^+$-6 | 3 (8.1) | + | + | + | + | + | – | – | + |
| Total number of sera positive/37 sera tested | | 37/37 | 25/37 | 25/37 | 31/37 | 25/37 | 0/37 | 1/37 | 4/37 |

*Example of nomenclature; Ph$^-$-3, phogrin AA–negative, three constructs recognized; ‡Ph$^+$-1 phogrin AA–positive, one construct recognized; §cytoplasmic domain of $NH_2$-terminus phogrin, middle ICA512, and COOH-terminus ICA512 (see Fig. 2); + and –, AA-positive and -negative, respectively.

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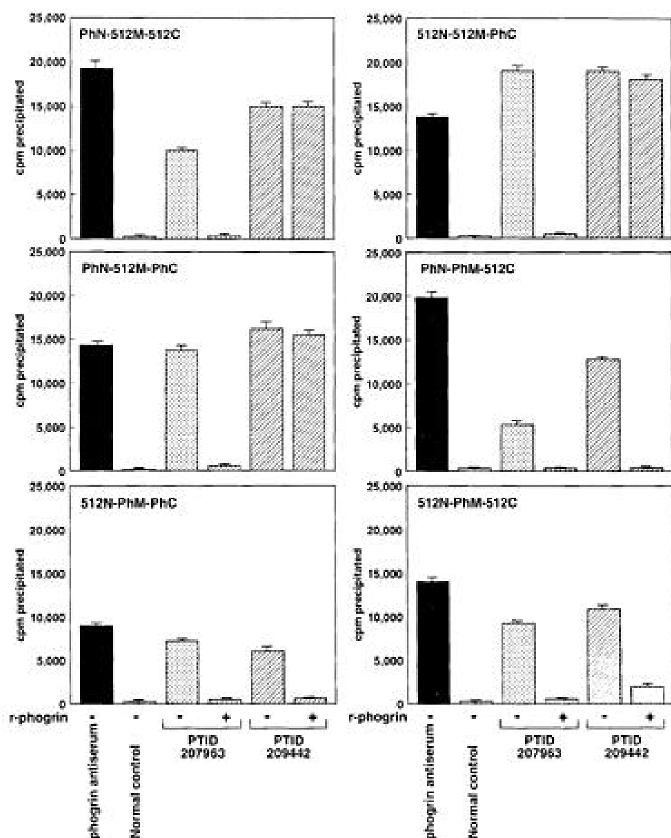


FIG. 3. Immunoprecipitation of chimeric ICA512/phogrin constructs. A rabbit antiserum against COOH-terminus of phogrin, a normal control serum, and two sera from patients containing ICA512 AA and phogrin AA (PTID 207963 and 209442) were incubated with in vitro transcribed and translated $^{35}$S-labeled ICA512/phogrin chimeric proteins. Immunocomplexes were precipitated by Protein A-sepharose, and radioactivity was determined by liquid scintillation counting. Patient sera were analyzed with (+) and without (–) absorption with 20 µg of unlabeled recombinant phogrin. Results are expressed as mean cpm ± SD for duplicate analyses.

carboxy-terminal 31 amino acids of ICA512 is important for binding, indicating a conformational and not a linear ICA512 epitope(s).

For the few sera ($n = 6$) with phogrin-selective AA, a dominant epitope appears to be contained within amino acids 640–922 of phogrin (PhN-PhM). These sera reacted only to the PhN-PhM-512C chimeric construct when sera were preincubated with recombinant ICA512. The two sera that were positive for phogrin AA but negative for ICA512 AA also reacted with this construct (data not shown).

**ICA512 AA and phogrin AA can appear in high-risk relatives early in life.** Of 15 relatives of patients with type 1 diabetes with high-risk HLA alleles—DR3/4 (DQB1*0302)—followed from birth as part of the DAISY study, 5 developed at least one anti-islet AA (GAA, IAA, and ICA512 AA) by age 1.3 years. Figure 5 illustrates intermolecular epitope spreading for two of these relatives who sequentially developed GAA, IAA, and ICA512 AA. One of these individuals (#207224) was positive for GAA at 9 months of age and subsequently developed IAA (at 1.1 years of age) and ICA512 AA (at 2.4 years of age) in the absence of phogrin AA. Another relative (#207227) was positive for IAA at 1.3 years of age and subsequently developed ICA512 AA (at 2.1 years of age), GAA (at 3.0 years of age), and phogrin AA (at 3.2 years of age).

**ICA512/phogrin intramolecular AA epitope spreading during prospective evaluation.** To analyze whether the epitopes of ICA512/phogrin change during follow-up, 66 sequential samples obtained from 22 first-degree relatives of type 1 diabetes patients who expressed ICA512 AA during follow-up were analyzed with the chimeric ICA512/phogrin constructs. Development of phogrin AA subsequent to appearance of ICA512 AA was observed in 3 of 22 relatives. Furthermore, AA reacting with COOH-terminus–truncated $ICA512_{389–948}$ appeared in two relatives and disappeared for one relative despite the continued presence of $ICA512_{256–979}$ AA. Figure 6 illustrates the representative pattern of intramolecular epitope spreading to ICA512 and phogrin for two relatives who have

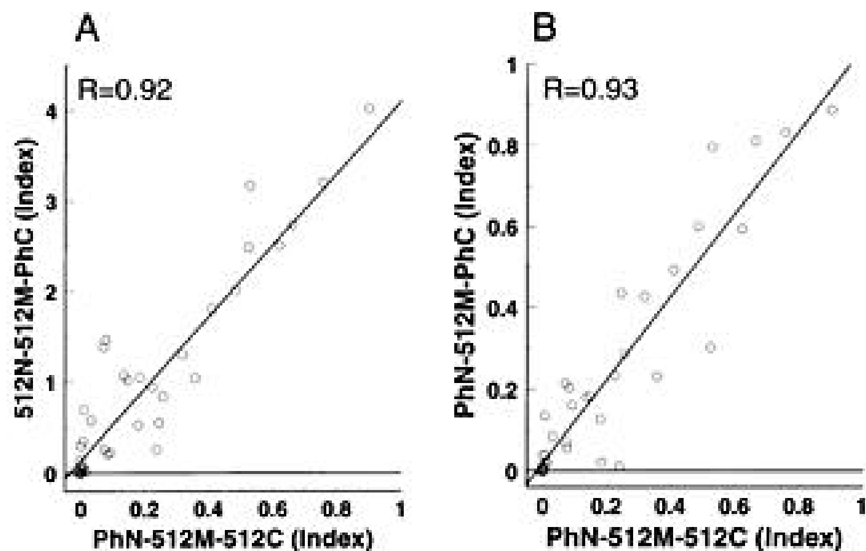


**FIG. 4. Relationship between the levels of AA to PhN-512M-512C construct and those recognizing 512N-512M-PhC (*A*) and PhN-512M-PhC (*B*) constructs. AA levels are expressed as indexes based on the binding of positive and negative control sera. Results are shown for sera from 37 patients with ICA512 AA analyzed after absorption with unlabeled recombinant phogrin. The levels of AAs to the PhN-512M-512C construct were highly correlated with those of 512N-512M-PhC AA ($r$ = 0.92, $P$ < 0.0001) and PhN-512M-PhC AA ($r$ = 0.93, $P$ < 0.0001).**

progressed to type 1 diabetes. One of these relatives (patient 10791) was positive for $ICA512_{256–979}$ AA and phogrin AA but negative for COOH-terminus–truncated $ICA512_{389–948}$ AA at 2.6 years of age. $ICA512_{389–948}$ AA subsequently developed at 4.3 years of age. AA reactive to the chimeric constructs that contained the middle segment of the cytoplasmic domain of phogrin (PhN-PhM-512C, 512N-PhM-PhC, 512N-PhM-512C) appeared at 3.1 years of age (Fig. 6, middle panel). Analysis of sera preabsorbed with recombinant phogrin demonstrated that AA to the chimeric constructs PhN-512M-512C and 512N-512M-PhC emerged at the same time as those reactive with the COOH-terminus–truncated $ICA512_{389–948}$ AA (Fig. 6, lower panel), suggesting that an epitope common to ICA512 and phogrin was initially recognized and that reactivity spread to a specific part of the cytoplasmic middle region of phogrin and subsequently to the middle portion of cytoplasmic domain of ICA512.

Another relative (#359) who was GAA and IAA positive at 8.1 years of age developed ICA512 AA and phogrin AA simultaneously 1 year later. With analysis using the chimeric ICA512/phogrin constructs, AA to the constructs that contained the middle portion of cytoplasmic domain of phogrin (PhM) emerged 1.5 years after the development of ICA512 AA (Fig. 6). Thus, phogrin-specific AA can develop after the appearance of ICA512 AA. With analysis with phogrin absorption, reactivity to the 512N-PhM-512C construct became positive 1.3 years before the development of overt diabetes.

Table 3 summarizes the transition of the ICA512/phogrin epitopes in 13 relatives who have progressed to type 1 diabetes. Five of these recognized additional ICA512/phogrin constructs during follow-up (follow-up period 2.2–9.0 years). The interval between intramolecular epitope spreading was as little as 8 months. Four of five relatives progressed to overt diabetes in <1.5 years from the first observation of intramolecular epitope spreading. Of nine relatives who have remained nondiabetic, four changed the epitopes recognized during follow-up (Table 4). Three of these four relatives lost recognition of one or more ICA512/phogrin chimeric constructs (patterns $Ph^+$-3 to $Ph^+$-2b, $Ph^+$-5 to $Ph^+$-2a, and $Ph^+$-5 to $Ph^+$-4 to $Ph^+$-2b). Another nondiabetic relative (#207227) showed intramolecular epitope spreading from pattern $Ph^+$-2b to $Ph^+$-3 within a short follow-up period (2.5 years). All of these 22 relatives continued to express GAA and/or IAA.

**Radioassay for AA to a combined PTP autoantigen.** Because there are a few diabetic patients or prediabetic relatives who have phogrin AA without ICA512 AA, we have developed a radioassay for PTP AA utilizing an $^{35}$S-labeled in vitro translated hybrid molecule that contains the cytoplas-

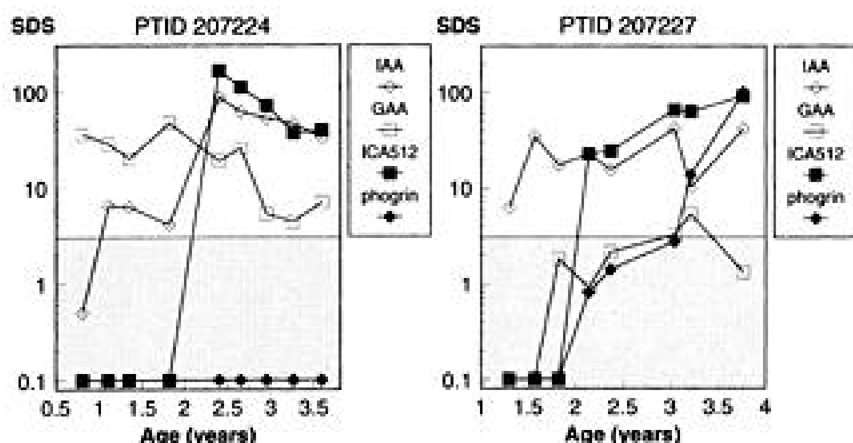


**FIG. 5. Sequential development of anti-islet AA early in life. Reactivities to different islet autoantigens (insulin, GAD65, ICA512, and phogrin) were determined by radioassays in relatives of patients with type 1 diabetes with high-risk HLA alleles, DR3/4 (DQB1*0302), followed from birth in DAISY. Shaded area indicates negative range of AA (≤3 SD score). ICA512, $ICA512_{256–979}$ AA; phogrin, $phogrin_{640–1015}$ AA.**



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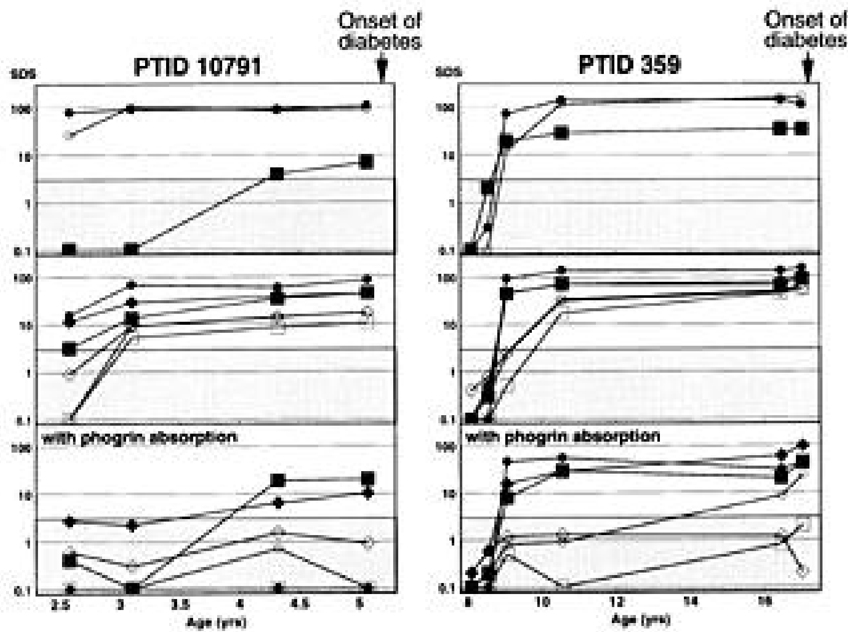


**FIG. 6. Intramolecular epitope spreading to PTP-like molecules during the development of type 1 diabetes. The level of AA was expressed as the standard deviation score (SDS). Shaded area indicates AA-negative range ( 3 SDS). Upper panel shows the time course of AA to ICA512$_{256–979}$ (●), ICA512 $_{389–948}$ (■), and phogrin (□). Middle and lower panels show the changing of the reactivity to chimeric ICA512/phogrin constructs: PhN-512M-512C (■), 512N-512M-PhC (◆), PhN-512M-PhC (●), PhN-PhM-512C (□), 512N-PhM-PhC (◇), and 512N-PhM-512C (○), without (middle) and with (lower) recombinant phogrin.**

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mic domain of phogrin (amino acids 640–1015) linked to that of ICA512 (amino acids 601–979) to detect all positive sera using a single assay. For 110 patients with new-onset type 1 diabetes and 42 prediabetic relatives, all ICA512 AA–positive and/or phogrin AA–positive sera ($n$ = 105) exhibited PTP AA levels with the chimeric protein exceeding the 99th percentile of healthy control subjects. The levels of PTP AA correlated with ICA512$_{256–979}$ AA levels ($r$ = 0.91, $P$ < 0.0001) and phogrin AA levels ($r$ = 0.75, $P$ < 0.0001; Fig. 7).

## DISCUSSION

This study demonstrates that AA reacting with ICA512 and phogrin share several overlapping epitopes. With in vitro translated $^{35}$S-labeled ICA512 and phogrin, 98% (88 of 90) of patients with new-onset type 1 diabetes and prediabetic relatives who are positive for phogrin AA had ICA512 AA (Table 1). Only the cytoplasmic domain of human phogrin was used in the assay of phogrin AA because previous work showed that all sera positive for full-length phogrin AA reacted with the cytoplasmic domain of phogrin (9). This region shares 80% amino acid sequence identity to ICA512 and is the site of AA binding in both molecules. Nevertheless, 10% of sera from patients with new-onset type 1 diabetes and prediabetic relatives reacted with ICA512 but not phogrin. Competition studies using affinity-purified recombinant ICA512 revealed that reactivity to phogrin was completely blocked by preincubation with recombinant ICA512 in all ICA512 AA–positive/phogrin AA–positive sera. In contrast, the binding to ICA512 in most doubly positive sera was only partially blocked by preincubation with excess recombinant phogrin. This suggests that AA in patients with type 1 diabetes may develop primarily to ICA512 rather than

TABLE 3
Changing patterns of reaction to PTP molecules during the development of type 1 diabetes

| PTID | Relation to proband | HLA -DR | Patterns Construct reactivity (age testing) | | | | | Age at diabetes onset |
|---|---|---|---|---|---|---|---|---|
| **359** | **Sibling** | **4/4** | **Neg (8.1)** | **Ph$^+$-5* (9.1)** | **Ph$^+$-5 (10.6)** | **Ph$^+$-6 (16.5)** | **Ph$^+$-6 (17.0)** | **17.8** |
| **10791** | **Sibling** | **3/4** | **Ph$^+$-1 (2.6)** | **Ph$^+$-1 (3.1)** | **Ph$^+$-4 (4.3)** | **Ph$^+$-4 (5.1)** | | **5.1** |
| **4674** | **Offspring** | **1/4** | **Ph$^+$-1 (2.8)** | **Ph$^+$-5 (3.6)** | **Ph$^+$-6 (4.3)** | **Ph$^+$-6 (7.3)** | | **7.5** |
| **5582** | **Offspring** | **3/3** | **Ph$^+$-2b (8.1)** | **Ph$^+$-2b (9.1)** | **Ph$^+$-5 (10.2)** | | | **10.2** |
| **7354** | **Sibling** | **3/4** | **Ph$^-$-4 (6.8)** | **Ph$^+$-4 (8.0)** | **Ph$^+$-1 (9.1)** | **Ph$^+$-1 (9.4)** | | **10.5** |
| 360 | Sibling | 3/4 | Ph$^+$-2b (7.3) | Ph$^+$-2b (10.0) | | | | 10.0 |
| 5097 | Sibling | 3/4 | Ph$^+$-2b (9.5) | Ph$^+$-2b (12.5) | | | | 13.5 |
| 2360 | Sibling | 4/6 | Ph$^+$-3 (25.3) | Ph$^+$-3 (29.0) | | | | 29.4 |
| 2201 | Sibling | 1/8 | Ph$^+$-4 (25.3) | Ph$^+$-4 (29.3) | | | | 29.3 |
| 2279 | Sibling | 4/6 | Ph$^+$-5 (8.4) | Ph$^+$-5 (11.6) | | | | 11.7 |
| 3687 | Sibling | 3/4 | Ph$^+$-5 (8.2) | Ph$^+$-5 (9.7) | Ph$^+$-5 (13.0) | Ph$^{+-}$5 (14.1) | | 14.4 |
| 3901 | Sibling | 4/7 | Ph$^+$-5 (8.3) | Ph$^+$-5 (11.7) | | | | 13.5 |
| 4147 | Sibling | 1/4 | Ph$^+$-6 (11.2) | Ph$^+$-6 (14.4) | | | | 14.4 |

*Example of nomenclature: Ph$^+$- 5, phogrin AA–positive, five constructs recognized. Bold characters indicate the patients who showed intramolecular epitope spreading; age in years shown in parentheses. Neg, negative for AA to all chimeric ICA512/phogrin constructs. The nomenclatures for pattern of reactivity to ICA512, phogrin, and ICA512/phogrin chimeric constructs are shown in Table 2.

TABLE 4
Changing patterns of reaction to PTP molecules in ICA512 AA–positive still nondiabetic first-degree relatives of patients with type 1 diabetes

| PTID | Relation to proband | HLA-DR | Follow-up period (years) | Patterns Construct reactivity (age testing) | | | |
|---|---|---|---|---|---|---|---|
| **1230** | **Offspring** | **4/5** | **4.9** | **$Ph^+$-5* (13.1)** | **<u>$Ph^+$-2b (14.2)</u>** | **$Ph^+$-3 (14.9)** | **<u>$Ph^+$-2b (18.2)</u>** |
| **13288** | **Sibling** | **3/4** | **3.8** | **$Ph^+$-5 (13.2)** | **$Ph^+$-5 (13.9)** | **<u>$Ph^+$-2a (15.0)</u>** | **$Ph^+$-2a (17.0)** |
| **3644** | **Sibling** | **3/3** | **12.0** | **$Ph^+$-5 (10.6)** | **<u>$Ph^+$-4 (13.7)</u>** | **<u>$Ph^+$-2b (22.0)</u>** | |
| **207227** | **Sibling** | **3/4** | **2.5** | **Neg (1.3)** | **$Ph^+$-5 (2.1)** | **$Ph^+$-2b (3.2)** | **$Ph^+$-3 (3.8)** |
| 207224 | Sibling | 3/4 | 2.8 | Neg (0.8) | $Ph^+$-5 (2.4) | $Ph^+$-5 (3.0) | $Ph^+$-5 (3.6) |
| 11071 | Offspring | 3/4 | 1.8 | $Ph^+$-2b (20.5) | $Ph^+$-2b (22.6) | | |
| 8974 | Sibling | 2/4 | 3.7 | $Ph^+$-7 (8.5) | $Ph^+$-7 (12.1) | | |
| 1852 | Sibling | 4/5 | 9.5 | $Ph^+$-5 (10.9) | $Ph^+$-5 (20.4) | | |
| 205461 | Sibling | 3/4 | 0.9 | $Ph^+$-5 (9.7) | $Ph^+$-5 (10.3) | $Ph^+$-5 (10.6) | |

*Example of nomenclature: $Ph^-$-5 phogrin AA–negative, five constructs recognized; Neg, negative for AA to all chimeric ICA512/phogrin constructs. Bold characters indicate the patients who showed a change of reactivity during follow-up; underline indicates the loss of recognition of ICA512/phogrin chimeric constructs compared with previous point. Age in years is shown in parentheses. The nomenclatures for pattern of reactivity to ICA512, phogrin, and ICA512/phogrin chimeric constructs are shown in Table 2.

phogrin and that the antigens have a relationship similar to that between GAD65 and GAD67 in this respect (20).

We have recently analyzed ICA512 epitopes in patients with type 1 diabetes using overlapping ICA512 constructs (15) and localized the major epitope within the cytoplasmic domain of ICA512 around residues 687–979. The present study using radioassays with different chimeric ICA512/phogrin constructs (Table 2) permits further definition of the reactivity in this region. At least 11 patterns of the reactivity to ICA512/phogrin chimeric molecules were discerned in 37 sera obtained from patients at the onset of diabetes. Most patients had AA against an epitope(s) contained within amino acids 762–887 of ICA512 after phogrin preabsorption, suggesting that this is a major target for ICA512-specific AA. Accordingly, the extent of immunoprecipitation of the radiolabeled PhN-512M-512C construct was highly correlated with that with 512N-512M-PhC AA and PhN-512M-PhC AA (Fig. 4).

ICA512 constructs with the carboxy-terminal amino acids 949–979 deleted were recognized by fewer sera from new-onset patients and prediabetic relatives. The nonreactive sera in this case did not bind to the chimeric constructs that contain 512C region (Table 2), suggesting that they recognize a conformational epitope(s) associated with the carboxy-terminal 31 amino acids in the native ICA512 molecule. Only 1 serum that was phogrin AA–negative reacted with the 512N-PhM-PhC, and 4 of 37 sera reacted with 512N-PhM-512C, suggesting that minor epitope(s) for ICA512-specific AA are located to amino acids 601–762, a region that is largely outside of the PTP homology domain but that is nevertheless highly homologous between ICA 512 and phogrin.

None of the sera reacted with the PhN-PhM-512C construct after preabsorption with phogrin. Furthermore, six sera whose reactivity to phogrin was not blocked by preincubation with 10 µg of recombinant ICA512 and two sera positive for phogrin AA but negative for ICA512 AA also reacted with this construct. These results suggest that the epitope(s) recognized by phogrin-selective AA are primarily located at amino acids 640–922 of the phogrin molecule. The binding to $ICA512_{256–979}$ in 15 of 88 (17%) patients positive for $ICA512_{256–979}$ AA and phogrin AA was completely absorbed with either recombinant $phogrin_{640–1015}$ or $ICA512_{601–979}$. Moreover, 7 of these 15 sera did and 8 did not react with $ICA512_{389–948}$. These results suggest that a subset of sera recognizes an epitope(s) common to ICA512 and phogrin. The location of phogrin-specific epitopes, however, remains to be determined using a number of phogrin AA–positive/ICA512 AA–negative sera.

These findings support and extend a recent report by Lampasona et al. (21) using immunoprecipitation with different fragments of ICA512 that found *1*) that antigenic epitopes were related to amino acids 601–682 of the juxtamembrane region; *2*) an epitope at amino acids 687–979 in the tyrosine phosphatase-like domain; and *3*) that all sera with AA recognizing only the juxtamembrane region of ICA512 had 40 kDa (ICA512) AA but not 37 kDa (phogrin) AA. Zhang et al. (22) recently reported that the major epitope of ICA512 is located at the COOH-terminus (amino acids 771–979) and that minor antigenic determinants are located at the $NH_2$-terminus (amino acids 604–776) and middle portion (amino acids 692–875) of the cytoplasmic domain of the molecule. We cannot exclude the possibility that some antigenic epitopes in the native molecule might be disrupted in the chimeric constructs because of differences in tertiary structure. Nevertheless, the data obtained so far should provide an initial framework toward deletion and mutagenesis studies aimed at the precise localization of amino acids participating in ICA512 and/or phogrin AA recognition.

We have recently reported the sequential development of AA to different islet autoantigens (insulin, GAD65, and ICA512) in first-degree relatives of type 1 diabetes (17). The appearance of humoral autoimmunity initially appeared limited to insulin and GAD65, followed by a later response to ICA512 (intermolecular epitope spreading). In the present study, we have shown that of 13 relatives who have progressed to type 1 diabetes, 5 exhibited intramolecular epitope spreading with recognition of additional ICA512/phogrin constructs during the preclinical phase. Furthermore, three of nine relatives who have remained nondiabetic lost reactivity to one or more ICA512/phogrin chimeric constructs with follow-up. In contradiction to our findings, a study by Zhang et al. (22) recently concluded that there was little intramolecular epitope spreading of AA recognition of ICA512 during the course of the dis-



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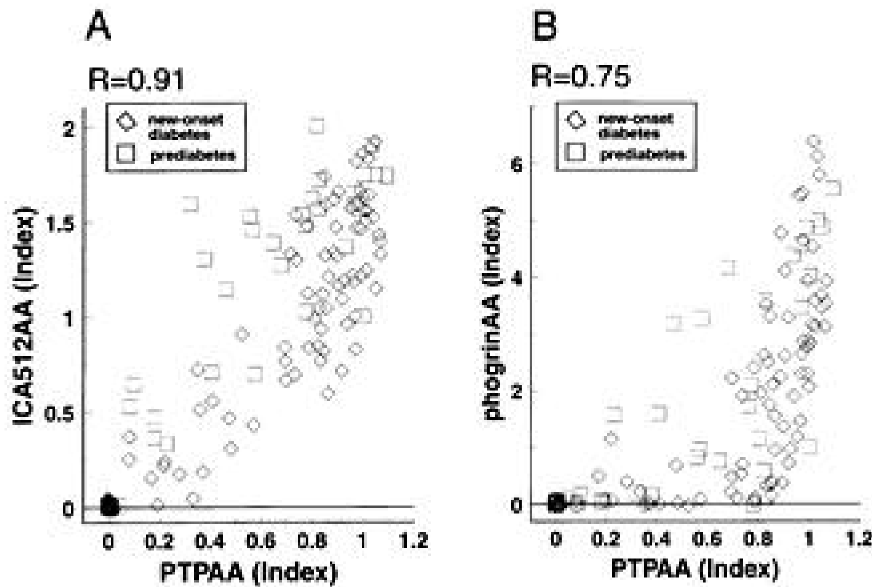


**FIG. 7. Correlation between the levels of combined PTP AA and those of $ICA512_{256–979}$ AAs (*A*) or phogrin AA (*B*). Sera from 110 patients with new-onset type 1 diabetes and 42 prediabetic relatives were studied. AA levels are expressed as indexes based on the 99th percentile of sera from normal control subjects (*n* = 208 for ICA512AA and *n* = 104 for phogrin AA and combined PTP AA). The levels of PTP AA correlated with ICA512 AA levels (*r* = 0.91, *P* < 0.0001) and phogrin AA levels (*r* = 0.75, *P* < 0.0001).**

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ease. However, these studies used overlapping ICA512 constructs without phogrin preabsorption. Because the majority of the sera used by these authors probably have phogrin AA that cross-react with ICA512, the number of molecule-specific epitopes that could have been identified would have been limited. We conclude from the present study that intramolecular epitope spreading does occur for the secretory granule PTP family members. Autoimmunity directed toward these molecules may be the result of chronic autoimmune destruction of the β-cell and attendant presentation and immune recognition of ICA512/phogrin by elements of the inflammatory infiltrate. Such a mechanism would be consistent with the seeming paradox that disease-specific antibodies are associated with an antigen that has a broader tissue distribution than the pancreatic islet. It might also account for the relative complexity of the humoral immune responses observed. In practical terms, our study illustrates the feasibility of using chimeric ICA-512/phogrin constructs to map PTP epitopes in diabetes and the utility tandem or hybrid ICA512/phogrin construct should have to increase the range and sensitivity of AA radioassays to this family of molecules.

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