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Mapping of Epitopes for Autoantibodies to the Type 1 Diabetes Autoantigen IA-2 by Peptide Phage Display and Molecular Modeling: Overlap of Antibody and T Cell Determinants[1]

James A. Dromey,* Sarah M. Weenink,* Günther H. Peters,† Josef Endl,‡ Patrick J. Tighe,§ Ian Todd,§ and Michael R. Christie[2]*

IA-2 is a major target of autoimmunity in type 1 diabetes. IA-2 responsive T cells recognize determinants within regions represented by amino acids 787–817 and 841–869 of the molecule. Epitopes for IA-2 autoantibodies are largely conformational and not well defined. In this study, we used peptide phage display and homology modeling to characterize the epitope of a monoclonal IA-2 Ab (96/3) from a human type 1 diabetic patient. This Ab competes for IA-2 binding with Abs from the majority of patients with type 1 diabetes and therefore binds a region close to common autoantibody epitopes. Alignment of peptides obtained after screening phage-displayed peptide libraries with purified 96/3 identified a consensus binding sequence of Asn-x-Glu-x-x-(aromatic)-x-x-Gly. The predicted surface on a three-dimensional homology model of the tyrosine phosphatase domain of IA-2 was analyzed for clusters of Asn, Glu, and aromatic residues and amino acids contributing to the epitope investigated using site-directed mutagenesis. Mutation of each of amino acids Asn^{858}, Glu^{836}, and Trp^{799} reduced 96/3 Ab binding by >45%. Mutations of these residues also inhibited binding of serum autoantibodies from IA-2 Ab-positive type 1 diabetic patients. This study identifies a region commonly recognized by autoantibodies in type 1 diabetes that overlaps with dominant T cell determinants. ***The Journal of Immunology,*** **2004, 172: 4084–4090.**

An understanding of the molecular basis of immune recognition of autoantigens in human autoimmune disease is essential for the development of Ag-specific immunotherapy for these disorders. Many autoimmune diseases have been shown to be associated with both Ab and T cell reactivity to target autoantigens, which have been identified largely from studies of Abs. However, the dominant determinants for immune recognition of these Ags are in most cases unknown and the relationship between autoantibody and T cell responses in the autoimmune disease process is currently unclear.

One of the major targets of the autoimmune response to islets in human type 1 diabetes is IA-2, a protein tyrosine phosphatase (PTP)[3]-like protein localized to the secretory granule membranes of islets and other neuroendocrine cells (1–3). The presence of Abs to IA-2 (IA-2A) in nondiabetic individuals is strongly predictive of future development of diabetes and is associated with rapid progression to disease (4, 5). T cell reactivity to IA-2 has been demonstrated using lymphocytes from peripheral blood of diabetic patients (6, 7), and the use of synthetic peptides has allowed the identification of T cell determinants on the protein, with some consensus as to the major regions recognized (8–12). Further analyses of autoantibody responses have used deletion mutants and chimeric constructs of IA-2 with the closely related IA-2β to further define IA-2 recognition. These studies have shown that diabetes-associated IA-2 autoantibodies bind exclusively to the cytoplasmic domain of the molecule (amino acids 601–979) and that epitopes within this region are diverse (1, 13, 14). These include two distinct linear epitopes within the juxtamembrane domain (amino acids 611–620 and 621–630, respectively), conformational epitopes in the PTP domain toward the C terminus of the molecule (931–979) and within the central region (795–889) of the PTP domain (15–19). PTP domain Abs can be both specific to IA-2 and cross-reactive with IA-2β (13). Autoantibodies detected early in the disease process are commonly IA-2 specific and directed to juxtamembrane domain epitopes (20), whereas these are less frequent by the time of diabetes onset when Abs to epitopes common to the PTP domains of IA-2 and IA-2β predominate (2, 13, 16, 19).

Because autoimmune patients frequently have a mixture of Ab specificities, precise definition of individual autoantibody epitopes is difficult with patient sera alone. The availability of mAbs to autoantigens from disease patients has facilitated characterization of autoantibody epitopes. Human mAbs to IA-2 have been generated by Epstein-Barr viral transformation of peripheral B lymphocytes from diabetic patients and shown to recognize epitopes within the juxtamembrane and PTP domains of the protein (21). One such human mAb (96/3), recognizes a conformational epitope within amino acid 777–979 of the IA-2 PTP domain, cross-reacts

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[3] Abbreviation used in this paper: PTP, protein tyrosine phosphatase.

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with epitopes on IA-2β, and was shown to very effectively compete for IA-2 binding with Abs in sera from a majority of type 1 diabetic patients (21). The epitope for the 96/3 Ab therefore lies within a region of IA-2 frequently targeted by Abs in type 1 diabetes and further characterization of this epitope would provide information valuable for our understanding of the Ab response in the disease.

Knowledge of three-dimensional protein structure is necessary to fully define conformational Ab epitopes, but structures of many autoantigens, including IA-2, are not yet crystalographically resolved. Nevertheless, useful information on conformational epitopes can be derived by homology and molecular modeling, particularly when combined with peptide phage display to identify potential Ab contact residues (22–24). The PTP domain of IA-2 is well suited to homology modeling because comparison of known structures of vertebrate PTP domains has revealed a highly conserved secondary structure, even in cases of low sequence identity (25). In this project, we have used a combination of peptide phage display and molecular modeling to characterize the epitope for the 96/3 monoclonal IA-2 autoantibody and have investigated the contribution of residues implicated in 96/3 binding to serum autoantibody reactivity in type 1 diabetes.

Materials and Methods

Abs and sera

The EBV-transformed B cell clone 96/3, secreting Abs to IA-2 and derived from a patient with type 1 diabetes, was maintained as previously described (21), and tissue culture supernatant was collected as a source of Ab. Secreted human mAbs were purified from 96/3 B cell culture supernatants by ammonium sulfate precipitation and affinity chromatography (26). Bovine Ig was also purified using the same methodology from FCS used in the tissue culture medium for the purpose of removing phage reactive with any trace amounts of bovine IgG contaminating the human mAb preparations. Tissue culture supernatant from an EBV-transformed human B cell clone (b96) secreting mAbs to glutamate decarboxylase (23) was used as a negative control for Ag-binding experiments.

A rabbit polyclonal Ab to IA-2 was generated by immunization with recombinant protein representing the cytoplasmic domain of IA-2. Mouse mAbs 76F and 76B recognizing epitopes within the juxtamembrane- and ecto-domains of IA-2, respectively, were gifts from Dr. E. Bonifacio (San Rafaele Institute, Milan, Italy). Mouse monoclonal IA-2 Abs, 4H6, 3C12, 2D8, 2E11, N25, 4C11, and 2F9 were gifts from Dr. N. Morgenthaler (Brahms, Berlin, Germany) and Dr. B. Ziegler (University Greifswald, Karlsburg, Germany). Sera from recent onset type 1 diabetic patients having IA-2 Abs exclusively to the juxtamembrane domain or PTP domain were selected from a previous study of IA-2 autoantibody epitopes (13).

Screening of random peptide phage display libraries

Two phagemid libraries expressing random peptides fused to the N terminus of the pVIII coat protein of M13 phage were kindly provided by Dr. G. Smith (University of Missouri, Columbia, MO). One library (f88–4/15 mer) contained linear 15-mer peptides whereas the second (f88–4/Cys^4) contained 14-mer peptides with conformation constrained by the presence of cysteine residues within the peptide at positions 5 and 10. The two libraries were mixed (3×10^{11} transducing units of each library) and phage reactive to bovine IgG was removed by incubation in tubes (Nunc Maxisorb; Roskilde, Denmark) coated with purified bovine IgG. Unbound phage was then subjected to three rounds of biopanning with purified 96/3 Ab using procedures previously described (27). Phage were incubated for 1 h in tubes coated with purified 96/3 Ab. Tubes were washed six times with PBS, pH 7.3, and bound phage was eluted by incubating with 0.2 M glycine, 0.1% BSA, pH 2.2, for 5 min at room temperature. Eluates were neutralized and recovered phage was amplified in late log phase *Escherichia coli* strain K91 kan. Amplified phage were then subjected to a further two rounds of biopanning, to enrich for 96/3 Ab reactive phage clones. Phage from the third round of biopanning were screened for specific reactivity by immunoblotting as described (27). Positive phage clones were isolated, the DNA insert was amplified by PCR, and sequences were determined by automated DNA sequencing.

Molecular modeling

A model structure of the PTP domain of IA-2 and of single amino acid mutants were generated using the x-ray crystallographic structure of hPTP1B, solved in complex with a hexapeptide DADEpYL-NH_2 (28) as a template. The choice of template was justified by recent analysis demonstrating highly homologous secondary structure folding of vertebrate PTP catalytic domains, even with low sequence identity (25). Coordinates were obtained from the Protein Data Bank (Ref. 29; entry code 1ptu). The three-dimensional models were generated using the MODELLER package (30). The quality of the model structures were assessed by an analysis of how PTP structural motifs and single conserved residues were maintained in the model structures, by the application of Pro-check (31) and WHAT IF (32) and for the appropriate location of highly conserved charged and hydrophobic residues. Model structures were visualized using the Protein Explorer web-based software (www.proteinexplorer.org).

Structural fluctuations and dynamics play a role in biomolecular function (33, 34) and generally protein flexibility can experimentally be deduced from x-ray crystallography or nuclear magnetic resonance relaxation experiments (35, 36). Because the model IA-2 structure is based on homology modeling, which provides a static picture of the protein, multiple molecular dynamics simulations were performed to gain insight into protein flexibility (37, 38). The simulations were used to analyze the effects of single amino acid substitutions on the protein fold. A detailed description of the parameters used in the simulations and the set-up of the simulations has been previously described (39). The analyses of the simulation results were performed using visual molecular dynamics (40).

Site-directed mutagenesis

Single amino acid substitutions were made by site-directed mutagenesis of cDNA representing the cytoplasmic domain of IA-2 (IA-2ic) within the pSP64 poly(A) vector using a QuikChange Site-Directed Mutagenesis kit (Stratagene, Cambridge, U.K.), according to manufacturer's instructions. All mutations were confirmed by restriction enzyme digestion and automated DNA sequencing.

Ab-binding analysis

Ab binding to the wild-type and mutated IA-2 cytoplasmic domain was analyzed by radioligand binding assays as previously described (13). IA-2ic cDNAs were transcribed and translated in vitro in the presence of [^{35}S]methionine using a TNT-coupled reticulocyte lysate system (Promega, Southampton, U.K.). The level of radioactivity incorporated into the translated protein was determined by precipitation with 10% trichloroacetic acid and scintillation counting. Aliquots (20 μl) containing equivalent amounts (~20,000 cpm) of wild-type or mutant translated protein were incubated with 5 μl of mAbs or test sera overnight at 4°C in immunoprecipitation buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 20 mM methionine, 0.5 mg/ml BSA, and 0.5% Triton X-100). Protein A-Sepharose (5 μl) was used to isolate immune complexes, the immunoprecipitates were washed five times by vacuum filtration with immunoprecipitation buffer and subsequently twice with water. The quantity of immunoprecipitated radiolabeled Ag was determined by liquid scintillation counting.

Results

Identification of residues critical for 96/3 Ab binding by peptide phage display

Two phage libraries expressing either 15-mer linear peptides or 14-mer peptides constrained by a pair of cysteine residues within the sequence were mixed in equal proportions and screened with purified 96/3 Ab by three rounds of biopanning. The number of phage eluted after each round of screening increased consecutively (2.87×10^2; 5.63×10^4; and 5.0×10^5 per 10^{11} phage screened, for first, second, and third rounds, respectively), consistent with specific enrichment of phage displaying peptide sequences reactive with the Ab. Immunoblotting of eluted phage from the third round of biopanning identified 13 colonies from a total of 130 screened that bound strongly to the 96/3 Ab in the blotting assays. These phage clones were amplified and the oligonucleotide inserts were sequenced to identify five different peptide sequences expressed by phage that were reactive to the 96/3 Ab (Table I). Three of these peptide sequences were represented in multiple phage clones. All inserts contained cysteine residues at positions 5 and 10 of the peptide sequence, indicating that the selected phage clones were

Table I. *Phage-displayed peptide sequences of 13 clones isolated by library screening with human mAb 96/3 after three rounds of biopanning*

Sequence	No. of Clones Displaying This Peptide
NP**E**DC**F**KT**G**CNSPT	6
LM**E**PC**Y**AW**G**CTGPK	3
IM**E**QC**F**EI**G**CPATN	2
NDSQC**W**KL**G**CIAEK	1
S**N**V**E**CDGLNCDWII	1

derived from the cysteine-constrained library and that a constrained peptide conformation is important for Ab binding. Alignment of the five different peptide sequences revealed that asparagine, glutamic acid, aromatic, and glycine residues were commonly represented at specific locations within the phagotopes (bold and underlined in Table I), consistent with a peptide motif for Ab binding of NXEXX(aromat)XXG.

Localization of 96/3 Ab binding residues on IA-2 by molecular modeling

Previous studies with deletion mutants have shown that the 96/3 Ab binds a conformational epitope within amino acids 777–979 of the PTP domain of IA-2 (21). The NXEXX(aromat)XXG motif is not represented as a linear sequence within the 777–979 region of IA-2 (nor elsewhere within the IA-2 sequence), as would be expected if protein folding is important for Ab binding. Further definition of the conformational epitope requires structural information of the target protein. Although there is no published crystal structure of IA-2, the region of the protein recognized by the Ab has high sequence similarity with members of the PTP family whose structures are known (25). Alignment of structures of PTPs that have been resolved indicates a highly conserved fold for their PTP domains. Hence, the PTP domain of the IA-2 molecule should have a similar fold and is therefore well suited to structural modeling to explore possible Ab binding sites at the protein surface.

A three-dimensional model of the PTP domain of IA-2 was generated using human PTP1B as a template. The predicted surface of the PTP domain of IA-2 was then analyzed for clusters of amino acids represented within the putative 96/3 binding motif (Asn, Glu, Phe, Trp, Tyr). Five asparagine residues were located within the minimal 96/3 epitope (777–979), four of which were surface-accessible (Fig. 1). Only two of these were located within 9Å (the maximum separation predicted from the NXE binding motif) of glutamic acid residues on the surface of the model IA-2 structure. Thus, Asn^{858} was adjacent to both Glu^{836} and Glu^{863} (but >9Å from Glu^{800}) and Asn^{971} was located close to Glu^{968} (Fig. 1, *A* and *B*). Each of these Asn residues was also in proximity to aromatic residues; Asn^{858} was close to Trp^{799} and Tyr^{835}, and Asn^{971} near Phe^{961}. Hence, the model structure identifies two regions potentially important for the 96/3 epitope; one including Asn^{858} together with Glu^{863} or Glu^{836} and Trp^{799} or Tyr^{835} (Fig. 1*A*), and the second bounded by Glu^{968}, Asn^{971}, and Phe^{961} (Fig. 1*B*).

Identification of critical 96/3 binding residues by site-directed mutagenesis

To further evaluate which of the residues identified by modeling are likely to be components of the 96/3 Ab epitope, the influence of mutations of specific residues on the ability of the Ab to bind IA-2 was determined in radioligand binding assays. Initial studies were performed to identify which of three glutamic acid residues (at positions 836, 863, and 968) might be involved by mutating each to lysine, thereby altering the charge of the side chain and introducing a potential repellent force between Ab and Ag. Binding of 96/3 Ab to each mutant was compared with that to wild-type IA-2. Neither mutations to Glu^{863} nor to Glu^{968} were found to affect 96/3 Ab reactivity, whereas the Glu^{836} mutation abolished binding (Fig. 2*A*). In contrast, binding of a rabbit polyclonal Ab to IA-2 was not affected by any of the mutations. Hence, Glu^{836} is implicated as a component of the 96/3 epitope.

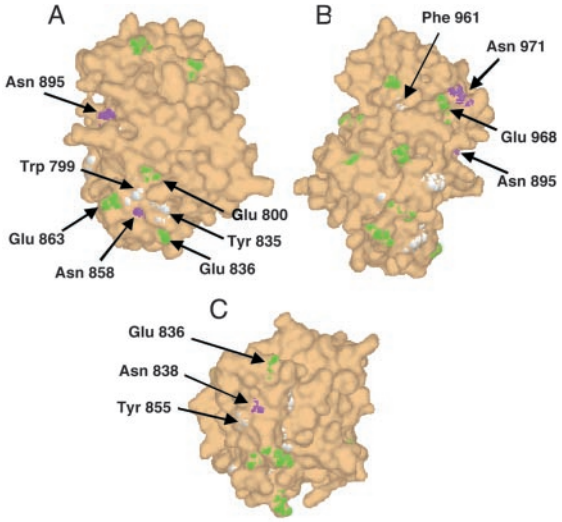

FIGURE 1. Location of potential 96/3 Ab contact residues on the surface of the model IA-2 structure. Three views (*A–C*) of the model PTP domain of IA-2 are shown with surface-localized glutamate (colored green), asparagine (magenta), and aromatic (white) amino acids within the 777–979 region of IA-2 highlighted.

To determine whether the amino acids in the vicinity of Glu^{836} (Asn^{858}, Trp^{799}, Tyr^{835}) are also involved in Ab binding, these residues were individually mutated to alanine and the mutants were tested for binding to 96/3 in radioligand binding assays. Both the Asn^{858} to Ala and Trp^{799} to Ala mutations were found to inhibit reactivity of the 96/3 Ab by >40% compared with wild-type IA-2 whereas the Tyr^{835} to Ala mutation had limited effect (Fig. 2*B*). None of the mutations affected binding of the rabbit polyclonal IA-2 Ab. The results suggest that the region bounded by Glu^{836}, Asn^{858}, and Trp^{799} represents the 96/3 epitope.

To further define the influence of the single amino acid substitutions on IA-2 immune reactivity, immunoprecipitation reactions were performed between the mutants and eight mouse mAbs to IA-2. Three of these Abs (76F, 4H6, 3C12) recognized epitopes within the juxtadomain and five (2D8, 2E11, N25, 4C11, 2F9) were directed to the IA-2 PTP domain. A mouse mAb (76B) to the IA-2 ectodomain, which is deleted from the IA-2 constructs tested, was included as a negative control. In contrast to the results with the 96/3 Ab, none of the mouse mAbs show substantially reduced binding with any of the mutants (Fig. 2*C*). One Ab (2E11) showed enhanced binding relative to the wild-type constructs with two mutants (Asn^{858} to Ala and Trp^{799} to Ala). However, the results with the mouse Abs show no evidence of loss of immune reactivity that may result from any major changes in protein conformation in the mutant IA-2 constructs.

Conformational changes in mutated IA-2 model structures

The possible influences of the single amino acid substitutions on IA-2 protein conformation were further investigated by introducing the mutations into the model IA-2 structure and performing simulations on each model structure. The simulations showed that

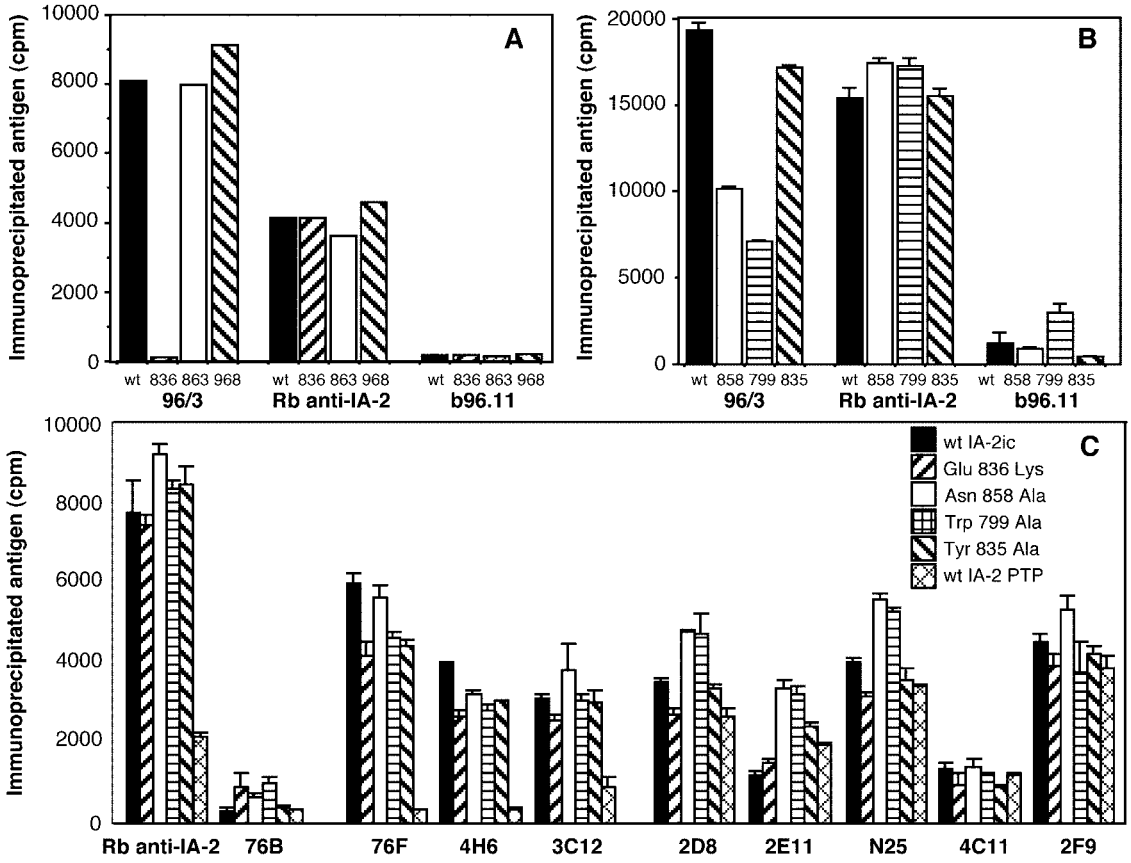

FIGURE 2. Influence of amino acid mutations on Ab binding to the IA-2 cytoplasmic domain (IA-2ic). *A*, Glu residues that potentially contribute to the epitope for 96/3 Ab were mutated to Lys and radiolabeled wild-type (wt, ■) or mutated (white or hatched bars) IA-2ic generated by in vitro transcription and translation. Radioactivity (in cpm) of translated protein immunoprecipitated by 96/3 Ab, a rabbit polyclonal Ab to IA-2 (Rb anti-IA-2), or a human mAb to glutamate decarboxylase (b96.11) is shown. *B*, Amino acids Asn^{858}, Trp^{799}, and Tyr^{835} were each mutated to Ala and tested in immunoprecipitation assays as in *A*. *C*, The ability of nine mouse monoclonal IA-2 Abs to immunoprecipitate wild-type IA-2ic, mutant IA-2ic, or wild-type IA-2 PTP domain is shown. Results are shown as means of duplicate (*A*) or triplicate (+SEM) (*B* and *C*) observations.

the overall fold is maintained and that in all model structures similar patterns in the average root mean square displacement as a function of residue number are found (data not shown). Although several potential conformational changes were identified, those appeared at localized sites in the protein within the proposed binding site of the Ab. In the model IA-2 structure, Trp^{799} was predicted to be in close contact with Arg^{865} and interactions between these two residues are expected to stabilize the conformation of this region (41). This interaction is lost in the model Trp^{799} to Ala mutant structure, and during the course of the simulations, the orientation of the neighboring residues, Glu^{836} and Tyr^{835}, are altered. Glu^{836} is surface-exposed in the wild-type IA-2 model structure and simulations indicate that the Glu^{836} to Lys mutation introduces conformational changes in neighboring residues including Tyr^{835} and Asn^{858} and in a flexible loop region (Lys^{754}-Asp^{763}) located close to the 96/3 Ab binding region. The effects of Tyr^{835} to Ala and Asn 858 to Ala substitutions on local conformational changes are less pronounced.

Contribution of putative 96/3 contact residues to IA-2 binding of serum Abs from diabetic patients

Previous studies have demonstrated that the monoclonal IA-2 Ab 96/3 competes for IA-2 binding with serum Abs in the majority of patients with type 1 diabetes (21) and therefore binds a region close to common autoantibody epitopes. It was therefore of interest to examine the influence of mutations in the putative 96/3 contact residues on serum Ab binding. Because a proportion of the sera of diabetic patients sera also include Abs directed to epitopes in the juxtamembrane domain (19), sera from 10 recent onset type 1 diabetic patients were selected from a previous study of IA-2 epitopes (13) with Abs only to the PTP domain. Two sera with Abs specifically to the IA-2 juxtamembrane domain were selected as control sera expected not to be affected by the PTP domain mutations. All sera were tested in radioligand binding assays against wild-type IA-2 and each of the IA-2 variants with mutations in amino acids within the proposed 96/3 binding domain. The Trp^{799} to Ala mutation was found to reduce binding in all sera with Abs to the IA-2 PTP domain (Fig. 3, patients 1–10) and mutations in other residues contributing to the 96/3 epitope also had inhibitory effects with selected sera. Thus, eight sera (from patients 2–6, 8–10) showed reduced binding to the Asn^{858} to Ala mutant and four (patients 7–10) to the Glu^{836} to Lys mutant; three were affected by all three mutations. In contrast, mutation in the noncontributing Tyr^{835} residue (that lies adjacent to Glu^{836}) had very limited effect on immunoprecipitation of the protein (Fig. 3). Sera with Abs specific to the IA-2 juxtamembrane domain (Fig. 3, patients 11 and 12) were little affected by the mutations.

Discussion

Previous studies on diabetes-associated autoantibodies to IA-2 have established that epitopes for these are diverse, located within the cytoplasmic PTP- and juxtamembrane domains of the IA-2

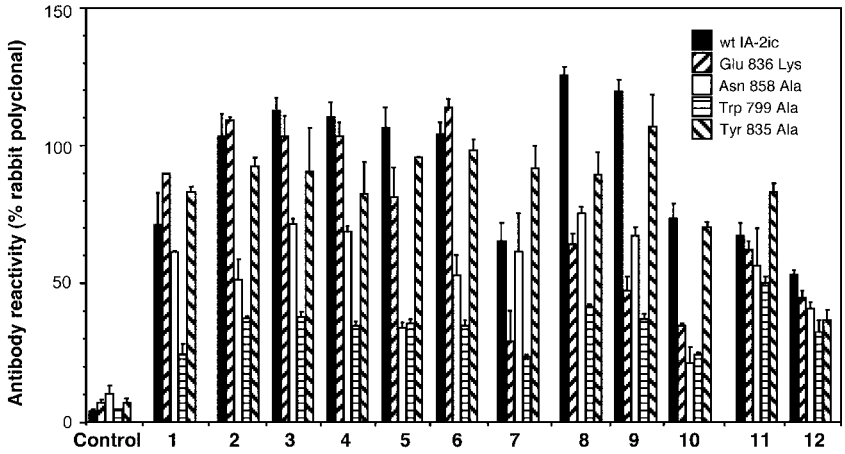

FIGURE 3. Influence of mutations to specific residues within the putative 96/3 epitope on IA-2 immunoprecipitation by serum Abs of type 1 diabetic patients. Radiolabeled wild-type IA-2ic (■) or IA-2ic with mutations introduced into Glu^{836}, Asn^{858}, Trp^{799}, or Tyr^{835} were used in radioligand binding assays with sera from a normal healthy control individual, or from type 1 diabetic patients with Abs to the IA-2 PTP domain (1–10) or IA-2 juxtamembrane domain (11, 12). Results are expressed relative to cpm precipitated by a rabbit polyclonal IA-2 Ab whose reactivity is unaffected by the mutations (Fig. 2). Results are means + SEM of three independent experiments.

molecule and that there is epitope spreading in the early phase of the autoimmune response (14, 20). Although no universal pattern of epitope spreading has been identified, autoantibodies to two linear epitopes within the juxtamembrane domain are most commonly detected as the first Ab specificity early in the response (19, 20). These are usually followed by Abs to conformational epitopes in the PTP domain that can be either specific to IA-2 or cross-reactive with the closely related IA-2β. IA-2 juxtamembrane domain Abs can disappear or decrease in titer as other autoimmune responses become established (20) and by the time of diabetes onset PTP domain Abs predominate (13–20). More precise mapping of epitopes within these regions is required to understand the extent of diversity of epitope recognition within the IA-2 PTP domain in the established autoimmune response.

In this study, we have characterized the epitope of a human monoclonal IA-2 Ab, 96/3, that binds a region close to common IA-2 autoantibody epitopes. Screening of peptide phage libraries implicated the sequence NxExx(aromatic)xxG as a consensus motif for 96/3 Ab binding. Because glycine has no side chain, we speculated that this residue has a structural role in the phagotope, rather than contributing directly to Ab binding. Site-directed mutagenesis of potential contributing residues identified by molecular modeling demonstrated inhibitory effects of mutations in Asn^{858}, Glu^{836}, and Trp^{799} on 96/3 Ab binding. The results suggest either that these residues are involved directly in Ab binding, or that the mutations alter the alignment of neighboring residues to inhibit binding of the Ab.

Several pieces of evidence suggest that the single amino acid substitutions do not have major disruptive effects on IA-2 conformation. First, none of the substitutions were found to inhibit IA-2 binding of a rabbit polyclonal Ab to IA-2 or of eight mouse mAbs to the protein, including five directed to epitopes in the PTP domain. Furthermore, mutations of Glu^{836} and Asn^{858} did not universally inhibit binding of serum autoantibodies from diabetic patients, indicating that the conformation of several Ab epitopes is unaffected. Glu^{836}, Asn^{858}, and Trp^{799} are all predicted to be surface exposed and mutations in such residues tend generally not to affect secondary protein structure. Molecular dynamics simulations on the mutated structures suggest only limited local changes in the overall protein conformation and several other single amino acid substitutions within the IA-2 molecule in this and other (42) studies have little affect on IA-2 autoantibody binding. Together these results indicate that the IA-2 molecule is likely to tolerate mutations in single amino acids with minimal changes in protein conformation. Therefore, we propose that the region of the IA-2 molecule bounded by Trp^{799}, Glu^{836}, and Asn^{858} forms the 96/3 epitope.

The amino acids identified as contributing to the 96/3 Ab epitope lie within the 795–889 region that is proposed to be a major target for IA-2 Abs (2). The importance of the 96/3 epitope region for IA-2 PTP domain autoantibodies (21) is further supported by analysis of the influence of mutations of Trp^{799}, Glu^{836}, and Asn^{858} on IA-2 binding of serum Abs from type 1 diabetic patients. IA-2 binding of all IA-2 PTP domain-reactive sera was inhibited by the Trp^{799} to Ala mutation and substitutions of either Glu^{836} or Asn^{858} also inhibited reactivity with most sera. However, there were differences between sera in the influence of each mutation and IA-2 binding of only three sera were inhibited by all of the three mutations (Fig. 3). These results suggest that a region centered on Trp^{799} is a major target for IA-2 PTP domain autoantibodies in the sera of diabetic patients, but the contribution of other residues within the 96/3 epitope is variable and the precise epitopes recognized in this region by the Abs are diverse. Other participating amino acids in this region may include Gln^{862}, mutations of which can abolish reactivity of IA-2 PTP domain-specific Abs in some sera (42).

It is generally acknowledged that type 1 diabetes is a T cell-mediated disease but the contribution of the autoantibody response is unknown. Autoimmune diabetes can develop in individuals deficient of B cells (43), but studies in the nonobese diabetic mouse indicate that disease can be prevented or retarded by B cell depletion (44, 45). B cells with Ag-specific receptors can mediate highly efficient Ag uptake for processing and presentation to T cells and so may facilitate maintenance of autoimmune responses (46). Ab-Ag complexes can remain intact in Ag-processing compartments, where bound Ig can protect residues from proteolysis and thereby alter processing and presentation of specific determinants to T cells (47). The epitope specificity of the Ab response may therefore influence that of T cell recognition. Furthermore, maturation of the autoantibody response can be influenced by the specificity of lymphocytes providing T cell help (48). One might expect a relationship between T and B cell specificity as a result of such T-B cooperation and it is therefore of interest to compare locations of autoantibody epitopes with those of known T cell determinants.

Several groups have investigated the specificity of the T cell response to IA-2 and those peptides identified as capable of eliciting T cell responses in diabetic patients or their relatives are summarized in Fig. 4*A*. Honeyman et al. (8, 9) performed the most extensive analysis by determining proliferative responses to IA-2 peptides of lymphocytes from six IA-2 Ab-positive at-risk relatives of type 1 diabetic patients. Seventeen peptides elicited T cell responses, of which nine were clustered as two sets of overlapping peptides representing amino acids 787–814 and 841–868, respectively (orange bars in Fig. 4*A*). Lohmann et al. (11) analyzed T cell

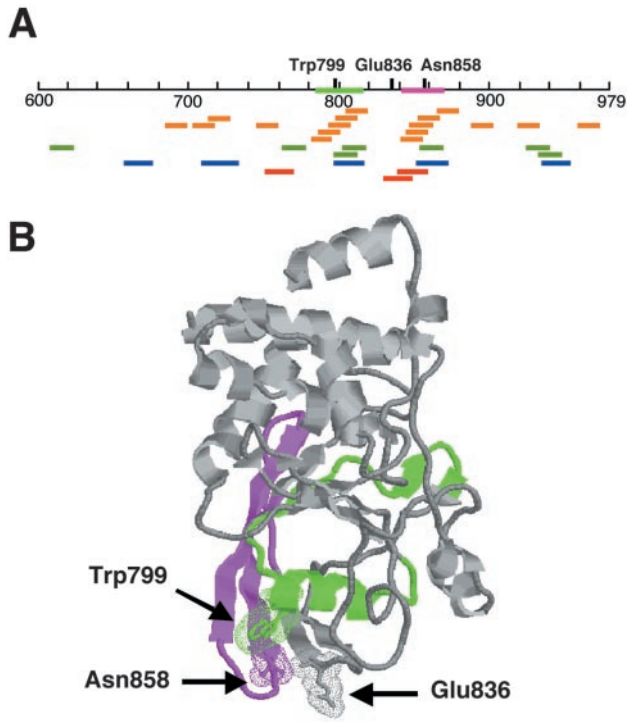

FIGURE 4. Relationship of residues within the 96/3 Ab epitope to determinants recognized by autoreactive T cells. *A*, Linear representation of the cytoplasmic domain of IA-2 (residues 601–979) with peptides stimulating T cell responses represented as colored bars in the figure. Peptides shown are those identified in studies by Honeyman et al. (orange bars, Ref. 9), Lohmann et al. (green bars, Ref. 11), Peakman et al. (blue bars, Ref. 10), and Hawkes et al. (red bars, Ref. 12) to induce positive proliferative responses of PBLs or IA-2-responsive T cell lines. Amino acid residues participating in 96/3 Ab binding were located within two clusters of overlapping peptides represented by amino acids 787–817 and 841–869 shown to stimulate T cell responses in several studies. These regions are shown (787–817 in green, 841–869 in magenta) on the linear representation of IA-2 in *A* and on the three-dimensional model structure of the IA-2 PTP domain in *B*. The amino acids contributing to the 96/3 epitope are indicated above the linear representation of IA-2ic in *A* and by arrows on the model PTP domain in *B*.

reactivity to peptides predicted to be HLA-DR*0401 or DQ*0302 binders and detected T cell responses in diabetic patients to seven (green bars in Fig. 4*A*), four of which (797–809, 803–815, 854–866, 925–937) overlapped with those identified by Honeyman et al. (8). A further two studies have used different approaches to identify naturally processed and presented IA-2 peptides. One identified processed IA-2 peptides eluted from HLA-DR4 molecules that were capable of stimulating T cell responses in diabetic patients (10) and the other mapped determinants recognized by T cell lines generated by stimulation of lymphocytes from diabetic patients with recombinant IA-2 (12). These studies confirmed that peptides in the regions 797–817 and 841–869 were naturally processed and presented to T cells. The observation that multiple independent studies were able to identify T cell responses in diabetic patients or at-risk relatives to a series of overlapping peptides representing amino acids 787–817 and 841–869 (Fig. 4*A*) suggests that there is a focus of T cell reactivity to determinants within these regions. The areas of overlap are relatively broad, and the four studies fail to identify a single common T cell determinant that may be dominant as a consequence of preferential binding to MHC. Rather the clustering of stimulatory peptides over extended regions of the IA-2 molecule points to an influence of Ag processing; for example, these regions may be more stable in Ag processing compartments.

The regions representing overlapping peptides capable of stimulating T cell responses lie within the 795–889 region previously proposed to contain major IA-2 autoantibody epitopes and that includes the residues implicated in this study as targets of major IA-2 PTP domain Abs. This suggests that there is overlap of T cell and Ab determinants. In the model IA-2 PTP domain, the 841–869 region is predicted to form three anti-parallel β-sheets and the Asn^{858} and Glu^{836} residues are located on exposed loops linking these strands (Fig. 4*B*). Trp^{799} is on an exposed region of α-helix within the 787–817 region and aligned close to Asn^{858} and Glu^{836} (Fig. 4*B*). Ab binding to these residues has the potential to influence proteolytic processing of exposed regions of the 787–817 and 841–869 regions and thereby contribute to T-B cooperation. Demonstration of an association between IA-2 PTP domain Abs and the activity of T cells responsive to peptides in these regions would lend support to such a mechanism. Unfortunately, although activated autoreactive T cells access the blood, current techniques to analyze peripheral blood T cell responses to peptides do not accurately reflect the activity of disease-relevant T cells residing largely in the target tissue (49, 50) and it is therefore not easy to test for such associations. It should, however, be possible to investigate whether the regions identified are particularly stable during processing by APCs, and whether Abs to epitopes within these regions, such as 96/3, alter the pattern of processing and presentation to T cells. If T cell and Ab responses in type 1 diabetes are demonstrated to become focused on regions identified in this study, then these should be valuable targets for immune intervention to prevent and treat the disease.

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