The Core Cysteines, (C909) of Islet Antigen-2 and (C945) of Islet Antigen-2β, Are Crucial to Autoantibody Binding in Type 1 Diabetes

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Cysteines are thought integral to conformational epitopes of islet antigen-2 (IA-2) autoantibodies (IA-2A), possibly through disulfide bond formation. We therefore investigated which cysteines are critical to IA-2A binding in patients with newly diagnosed type 1 diabetes. All 10 cysteines in the intracellular domain of IA-2 were modified to serine by site-directed mutagenesis, and the effects of these changes on autoantibody binding in comparison with wild-type control were investigated by radiobinding assay. Mutation of the protein tyrosine phosphatase (PTP) core cysteine (C909) in IA-2 caused large reductions in autoantibody binding. In contrast, little or no reduction in binding was seen following substitution of the other cysteines. Modification of the core cysteine (C945) in IA-2β also greatly reduced autoantibody binding. Lysine substitution of glutamate-836 in IA-2 or glutamate-872 in IA-2β resulted in modest reductions in binding and identified a second epitope region. Binding to IA-2 PTP and IA-2β PTP was almost abolished by mutation of both the core cysteine and these glutamates. The core cysteine is key to the major PTP conformational epitope, but disulfide bonding contributes little to IA-2A epitope integrity. In most patients, at disease onset, >90% of antibodies binding to the PTP domain of IA-2 recognize just two epitope regions. ***Diabetes*** **62:214–222, 2013**

The protein tyrosine phosphatase (PTP)-like proteins islet antigen-2 (IA-2) and IA-2β are major type 1 diabetes autoantigens (1,2) localized to secretory granule membranes of islets and other neuroendocrine cells (3–5). Autoantibodies to the intracellular domains of IA-2 (IA-2ic) and IA-2β can be detected in the serum of 60–80% of patients with recent-onset type 1 diabetes (5–8) and in combination with antibodies to the other islet antigens—glutamate decarboxylase, zinc transporter 8, and insulin—they are valuable markers for predicting disease (9,10).

IA-2 and IA-2β are transmembrane proteins of 979 and 1015 amino acids, respectively. The intracellular domains include a juxtamembrane (JM) region and a PTP-like region (7,11). Despite having no (IA-2) or only weak (IA-2β) phosphatase activity (2,12,13), their PTP-like regions share 88% amino acid sequence homology, including the core cysteine of the PTP signature motif (I/VHCXXGXXRS/T) essential for phosphatase activity in catalytically active members of the PTP protein family (14) (Fig. 1). In contrast, the JM regions of the two proteins share <50% homology. During the type 1 diabetes prodrome, autoantibodies to the PTP-like region may be preceded by a response to epitopes in the JM region but, as the disease progresses, reactivity spreads so that antibodies to PTP epitopes usually predominate (3,15). Autoantibodies bind to PTP epitopes common to both IA-2 and IA-2β; 50–80% of patient sera that react with IA-2 also recognize IA-2β, whereas 95% of sera that react with IA-2β also bind IA-2 (1,5,6,16,17).

Two linear epitopes have been identified in the JM region [amino acids 611–620 and 621–630 (12,18)], but the conformational epitopes found in the PTP region are more diverse (6,19–22). PTP residues shown to be important for antibody binding include tryptophan 799, glutamate 836, asparagine 838, tyrosine 855, asparagine 858, and glutamine 862 (23–25). Studies of IA-2 crystal structure suggest that these residues form one epitope region (26). A second major PTP epitope region includes residues 876–880, which overlap the WPAE loop (25,27), as well as alanine 877 and aspartate 911, which are in close proximity (3).

Disulfide-bond formation in the PTP region was thought to be critical for maintaining antigenic structure, as reduction and alkylation of cysteine residues abolished PTP autoantibody binding and removed resistance to digestion with trypsin (28). Analysis of IA-2ic expressed in *Escherichia coli* suggested the formation of at least one disulfide bridge (29), although its location was unknown. We also showed that azide and high concentrations of Tween-20 reduced IA-2A binding to the PTP domain (30), effects we ascribed to disruption of disulfide bonds and/or modification of the core cysteine. In other PTPs, this cysteine is susceptible to oxidation by reactive oxygen species (14,31). Oxidation inhibits PTP activity and is increasingly recognized as a mechanism for reversible regulation of PTP function (14,32), although the oxidized form of the core cysteine in IA-2 and IA-2β has yet to be determined.

To investigate the influence of disulfide-bond formation on autoantibody binding and the importance of the core cysteines, C909 in IA-2 and C945 in IA-2β, we mutated individual cysteines within the cytoplasmic region of IA-2, IA-2 PTP, and IA-2β PTP and measured changes in antibody binding by radiobinding assay.

RESEARCH DESIGN AND METHODS

Subjects. Sera were obtained from patients with newly diagnosed type 1 diabetes participating in the Bart's-Oxford (BOX) prospective study of childhood diabetes (33). Seventy sera were selected for inclusion in this study based on

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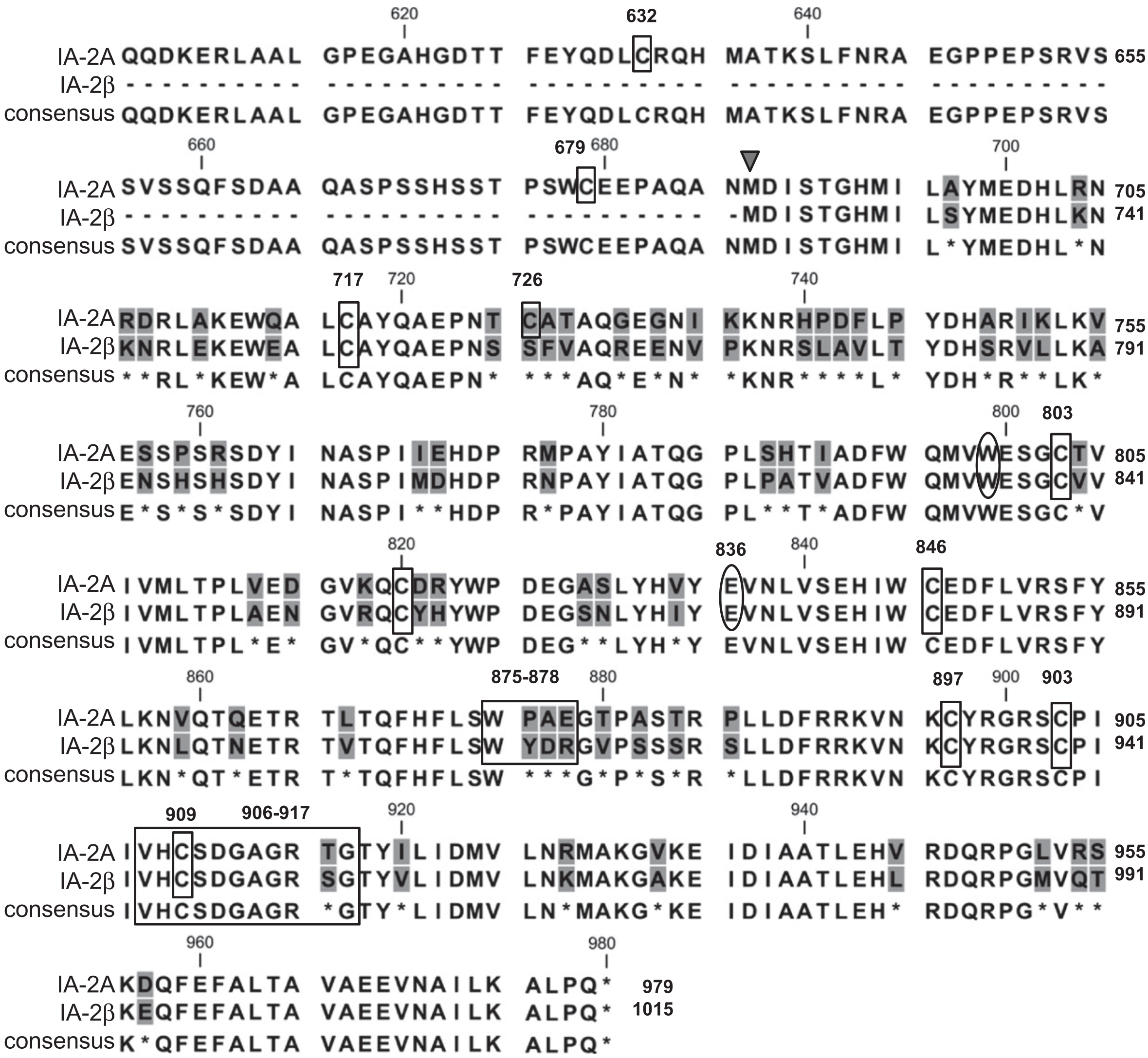


FIG. 1. Alignment of IA-2ic and IA-2β PTP sequences in pSP64 and pGEM, respectively. The IA-2 PTP sequence in pGEM is the same as IA-2ic, except it begins at amino acid 687 marked by the inverted triangle. Nonconserved amino acids are shaded. The highly conserved PTP core sequence is boxed (907–917) and differs by one amino acid between IA-2 and IA-2β. All 10 cysteines (boxed) in IA-2ic and cysteine at position at 945 in IA-2β were substituted with serine. Cysteine at position 909 and the corresponding cysteine in IA-2β (position 945) were changed to alanine. Glutamate at position 836 in IA-2 and 872 in IA-2β (circled) was changed to a lysine. Tryptophan at position 799 was changed to alanine in IA-2 PTP. The WPAE motif is also marked (boxed 875–878). The numbers above the sequence refer to the amino acid positions in IA-2ic. Sequences were obtained from GenBank accession numbers L18983 (IA-2) and Y08569 (IA-2β).

IA-2A positivity and the volume available. Patients had a median age of 10 years (range 2–19), 39 were male, and their median diabetes duration was 0 days (range −10–74). Of these sera, 58 also had glutamate decarboxylase autoantibodies and 56 zinc transporter 8 autoantibodies, whereas 53 of the 66 collected within 14 days of diagnosis had insulin autoantibodies. Local research ethics committees have approved the BOX study.

Amino acid mutations of IA-2 and IA-2β. IA-2 and IA-2β amino acid sequences of the plasmid constructs are shown in Fig. 1. IA-2ic (606–979) was cloned into the pSP64 poly(A) vector (Promega UK, Southampton, U.K.) and IA-2β PTP (723–1015) and IA-2 PTP (687–979) into pGEM (6). Mutated IA-2ic, IA-2 PTP, and IA-2β proteins were obtained by insertion of nucleotide changes using QuikChange site-directed mutagenesis (Stratagene; Agilent Technologies, Cheshire, U.K.) with oligonucleotides (Sigma-Aldrich, Dorset, U.K.) designed using the QuikChange Primer Design Program (www.genomics.agilent.com; Agilent Technologies). Mutations were verified by sequencing (University of Dundee Sequencing Service, Dundee, U.K.).

Cysteines at positions 632, 679, 717, 726, 803, 820, 846, 897, and 903 of IA-2ic were modified to serine (Fig. 1). C909 was mutated to both serine and alanine in IA-2ic, IA-2 PTP, and the equivalent position (C945) in IA-2β on the basis of sequence alignment (Fig. 1). These mutations were chosen as serine is isosteric with cysteine, but more polar and has greater hydrogen bonding potential, whereas alanine was thought unlikely to perturb gross structure. Glutamate 836 (E836) in IA-2ic and IA-2 PTP, previously shown to inhibit binding of human monoclonal IA-2 antibodies and patient sera (24), and the corresponding residue (E872) in IA-2β PTP were changed to lysine. These glutamates were also altered in combination with the IA-2 C909S and IA-2β C945S mutations to form E836K/C909S and E872K/C945S double mutants, respectively. Further, the tryptophan at position 799 in IA-2 PTP was changed to an alanine (23).

Antibody measurements. The effects of the amino acid changes on autoantibody binding were investigated in comparison with the wild-type (WT) by radiobinding assay as previously described (34). Briefly, 2 μL of serum in duplicate was incubated with 25 μL of ^{35}S-methionine–labeled antigen containing 20,000 cpm for 18–22 h at 4°C. Labels were produced using TnT SP6 rabbit reticulocyte lysate coupled transcription translation kits (Promega UK) with plasmids encoding WT or mutated IA-2ic, IA-2 PTP, and IA-2β PTP. Immunocomplexes were precipitated using protein A-sepharose (GE Healthcare, Amersham, U.K.), washed, and counted in a scintillation counter (Topcount; PerkinElmer Life Sciences, Cambridge, U.K.). The percentage reduction in binding was calculated as: 100 × ([domain cpm of WT − cpm of mutant]/cpm of WT). A negative percentage reduction corresponds to increased binding following mutation or treatment.

Monoclonal antibody binding. Monoclonal antibodies recognizing epitopes in the JM (MAb-76F4B), PTP (MAb-A5), and PTPβ (MAb-A9–19 and MAb-beta593) regions were provided by Dr. E. Bonifacio (18). Antibody binding

was assessed by radiobinding assay as described above, but using protein G-sepharose (GE Healthcare) for immunoprecipitation.

Pretreatment of labeled IA-2 constructs with *N*-ethylmaleimide. To investigate the effect on IA-2A binding of alkylating the cysteines, the IA-2 WT and C909S-labeled proteins were pretreated by incubation with 10 mmol/L *N*-ethylmaleimide (NEM) in 20 mmol/L Tris-buffered saline (pH 7.4) with 0.15% Tween-20 (TBST) with 1% weight for volume bovine serum albumin (Tris-buffered saline with 0.15% Tween-20) for 4 h at 4°C. The NEM was removed using a NAP5 desalting column (GE Healthcare) prior to assay. The treated labels were assayed against 33 of the 70 sera, Mab-76F4B, and Mab-A5 by radiobinding assay as described above.

Molecular modeling. The crystal structures of the IA-2 PTP residues 709–979 (PDB ID: 2I1Y) (35) and the IA-2β PTP residues 750–1015 (PDB ID: 2QEP) (36) were used to model amino acid changes. InsightII was used to mutate/append the residues, and the structures were then minimized using Discover (Accelrys Inc., San Diego, CA). During initial stages of minimization, all atoms were restrained. Subsequently, all atoms were gradually allowed freedom to move, enabling backbone and side-chain flexibility as the system relaxed. Images were generated using a combination of Chimera (University of California San Francisco), Gimp (Gnu Image Manipulation Program, http://www.gimp.org/), and Powerpoint (Microsoft). The effects of serine or alanine mutations on the surface structure were investigated by measuring the root mean square deviation (RMSD) from the WT position of the WPAE loop in the models, following mutation and energy minimization. This method was also used to assess the effects of adding a sulfonate to C909 in IA-2 to mimic the effects of oxidation and of alkylation with NEM. Additional homology modeling was performed using HHPRED software (ftp://toolkit.lmb.uni-muenchen.de/HH-suite/) to predict the effect of alanine substitution for W799 and for C909 using lower sequence identity PTP homologs as templates.

Statistical analysis. Differences in levels or percentage reduction in antibody binding were compared with the WT by Wilcoxon signed-rank test using the Statistics Package for Social Sciences version 16 (SPSS, Chicago, IL). A P value <0.05 was considered significant.

RESULTS

The effect of mutating C909 in IA-2 and C945 in IA-2β to serine or alanine. Substitution of C909 with serine in IA-2 and IA-2 PTP caused profound reductions in binding by sera from IA-2A–positive patients with type 1 diabetes. For IA-2ic, the C909S mutation caused a median percentage reduction in binding of 68% (range −119–95%; $P < 0.001$) and for IA-2 PTP a median reduction of 66% (range −95–75%; $P < 0.001$) (Fig. 2). Substitution of cysteine 945 with serine in IA-2β PTP also caused major reductions in binding (median 92%, range −134–99%; $P < 0.001$). The IA-2ic C909A mutation likewise reduced binding, although the reduction was less pronounced than that observed for the C909S mutation (median 34%, range −186–72%; $P < 0.001$). The C909A and C945A mutations in IA-2 PTP and IA-2β PTP, respectively, resulted in similar reductions in binding to those seen with the corresponding serine mutations in these proteins (median 55%, range −89–94%, $P < 0.001$; median 90%, range −142–98%; $P < 0.001$, respectively) (Fig. 2).

The effect of mutations of the nine other cysteines in the IA-2 intracellular domain. To determine whether any of the cysteines pair to form disulfide bonds, each one was mutated to serine and the effect on IA-2A binding investigated. In contrast to the profound effect of the C909S mutation, substitution of the other nine cysteines with serine caused little or no reduction in binding (Fig. 3). Serine mutations at positions 632, 717, 726, and 820 resulted in slight increases in binding (median percentage increase 6% [$P = 0.042$], 2% [$P < 0.001$], 8% [$P < 0.001$], and 5% [$P < 0.001$], respectively), whereas replacing cysteines at 679, 803, 897, and 903 resulted in small reductions in binding (median percentage decrease 8% [$P < 0.001$], 4% [$P < 0.001$], 6% [$P < 0.001$], and 3% [$P = 0.03$]). The C846S mutation did not cause a significant change in median binding (0.5%; $P = 0.41$).

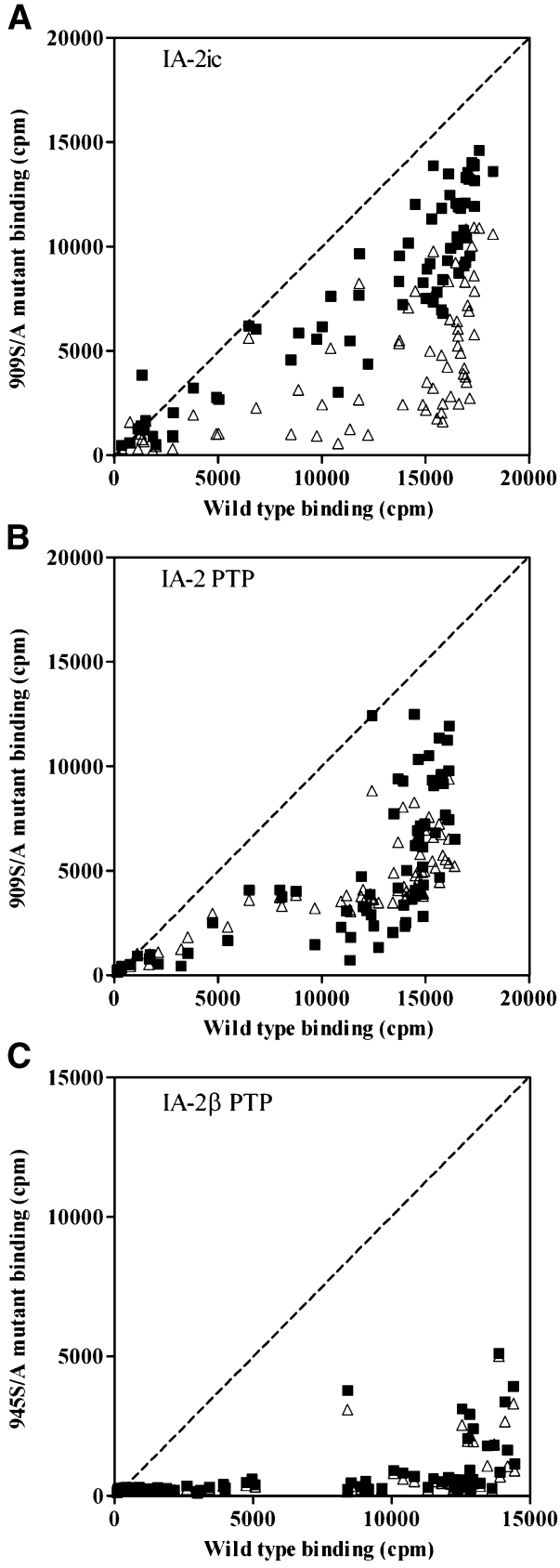


FIG. 2. Binding (cpm) shown by 70 different IA-2–positive BOX sera of labels made using IA-2ic (*A*), IA-2 PTP (*B*), and IA-2β PTP (*C*) plasmid with mutations at amino acid position 909 (945 in IA-2β) to serine (open triangles) and alanine (filled squares). The dashed line represents equivalent binding with or without the amino acid mutation.

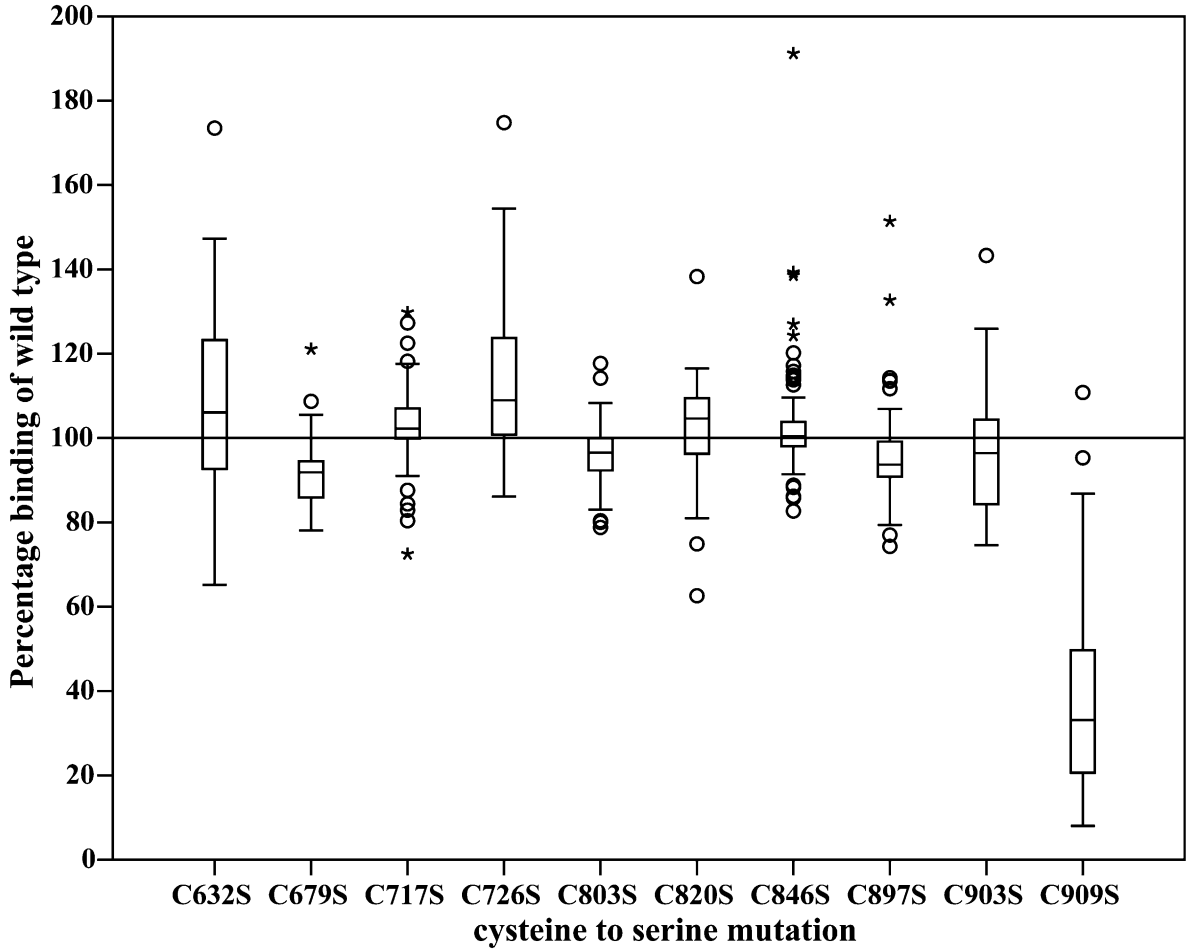


FIG. 3. A box and whisker plot showing percentage binding by 70 IA-2A–positive patient sera of IA-2ic labels containing the 10 different cysteine-to-serine mutations in comparison with binding of WT label. Mutation of C909 resulted in 68% reduction in binding, but mutation of the other nine cysteines caused little reduction or slight increases in binding by the sera. The median percentage binding of WT is represented by the line in the box. The box contains the interquartile range. The region within the whiskers represents 80% of the sera, whereas outliers are sera between 1.5 (open circle) and 3 (asterisk) interquartile ranges from the end of the box.

Effect of mutating E836 in IA-2 and E872 in IA-2β, with and without mutation of the core cysteine to serine. To identify more clearly the epitope region affected by substitutions of the core cysteine, we investigated the effect of lysine substitution at E836. This substitution was previously shown to abolish binding of monoclonal antibody 96/3 and reduce binding of sera from patients with type 1 diabetes (23,24). The mutation E836K in IA-2ic and IA-2 PTP resulted in modest reductions in binding by patient sera (median 10%, range −0.8–93%, $P < 0.001$; and 18%, range −16–98%, $P < 0.001$, respectively) (Fig. 4). The corresponding mutation E872K in IA-2β also caused a moderate reduction in binding (median 20%, range −25–88%; $P < 0.001$). However, the double mutation E836K/C909S in IA-2ic caused a 75% median reduction in binding (range −9.5–97%; $P < 0.001$), whereas the E836K/C909S and E872K/C945S double mutations in IA-2 PTP and IA-2β PTP caused median reductions in binding of 94% (range −174–99%; $P < 0.001$) and 93% (range −134–98%; $P < 0.001$), respectively.

Effect of alanine mutation of W799 in IA-2 PTP. To understand the relationship of the previously reported W799A mutation to the epitopes identified by C909 and E836, we compared the reduction in binding of the W799A-labeled PTP mutant with those of the C909S and E836K PTP-labeled constructs in a subset of 58 patient samples for which sufficient serum was available. The median reduction in binding compared with WT was 73% (range −97–81%) for W799A ($P < 0.001$) and 61% (range −88–74%) for C909S ($P < 0.001$) (Fig. 5).

Pretreatment of IA-2ic label with NEM. Pretreatment of WT IA-2ic label with NEM caused a median reduction in binding of 63% (range −35–96%; $P < 0.001$) by the 33 selected patient sera, which was less than that caused by the serine mutation alone (79%, range −27–96%; $P < 0.001$). Pretreatment of C909S with NEM caused little additional reduction in binding by these sera (86%, range −74–98%; $P = 0.001$).

Monoclonal antibody binding. Binding of MAb-A5 to IA-2ic was abolished by the E836K mutation, whereas binding of C909S or C909A labels was reduced by 24 and 6%, respectively, compared with WT. The W799A mutation caused 78% reduction in binding of MAb-A5. In contrast, the C909S mutation increased binding of IA-2ic by the JM-specific MAb-76F4B by 53%. Unexpectedly, the C945S mutation increased binding of IA-2β PTP label by MAb-beta593 and MAb-A9–19 by 600 and 340%, respectively, compared with WT.

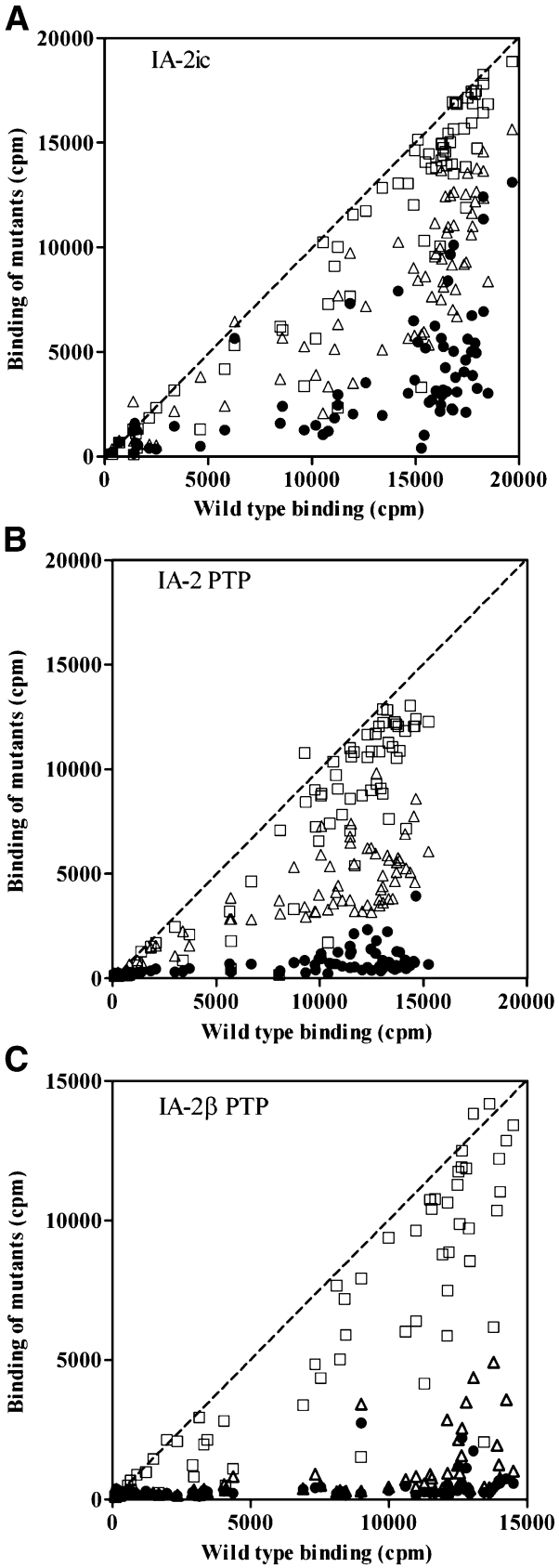


FIG. 4. Binding (cpm) shown by 70 different IA-2A–positive BOX sera to labeled proteins made using IA-2ic (*A*), IA-2 PTP (*B*), and IA-2β PTP (*C*) (open triangles), the IA-2 PTP E836K (E872K in IA2-β) (open squares), and the IA-2 and IA2-β PTP double mutants E836K/C909S and E872K/C945S (filled circles). The dashed line represents equivalent binding with or without the amino acid mutation.

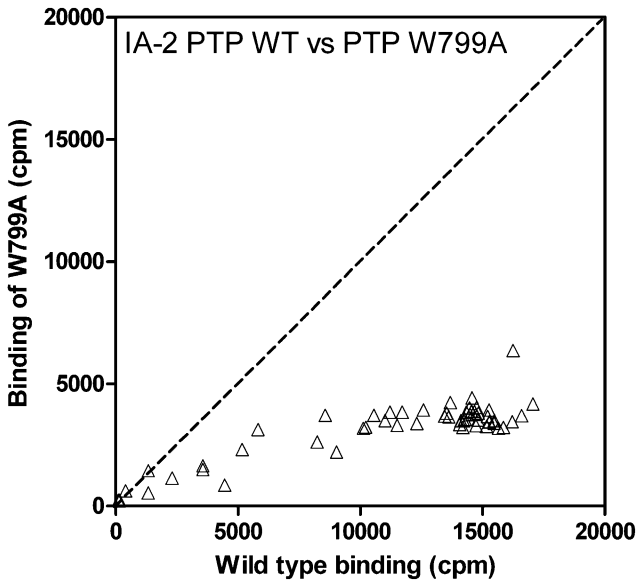
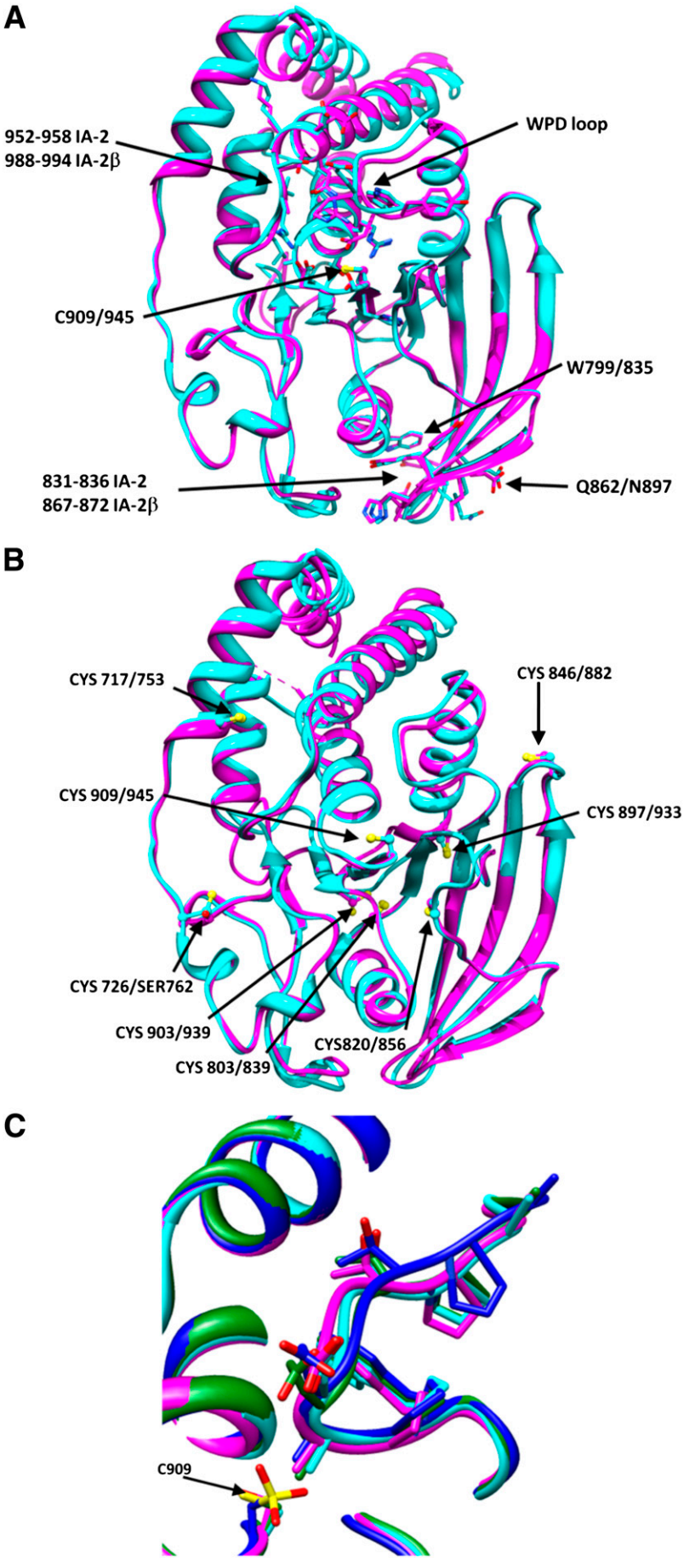


FIG. 5. Binding (cpm) shown by 58 different IA-2A–positive BOX sera to labeled proteins made using IA-2 PTP WT and IA-2 PTP plasmid with mutation of tryptophan at position 799 to alanine (open triangles). The dashed line represents equivalent binding with or without the amino acid mutation.

Modeling of changes in mutated/modified IA-2 structures and location of epitope regions. Changes to the structure of IA-2ic and IA-2β caused by the amino acid substitutions or modifications were modeled and energy minimized using Discover (Accelrys). This method allows side-chain flexibility and backbone movement toward the end of minimization. These simulations showed that the overall fold is maintained and predicted only small, localized conformational changes when C909 or C945 were mutated to alanine or serine (Fig. 6). C909A caused less disruption to the WPAE loop (RMSD; 0.70 Å) than C909S (RMSD; 0.92 Å). Serine is more hydrophilic than cysteine and may be less likely to adopt the same (inward) side-chain orientation as C909 in the crystal structure. This mutation could therefore destabilize both local and more distant epitopes (Supplementary Fig. 1). Although C945 lies within a deeper pocket in IA-2β, modeling the C945A and C945S mutations showed fold was retained. Alkylation with NEM had little effect on overall conformation; the RMSD between 285 residues of IA-2 PTP and the resulting model was 0.868 Å. Homology modeling of alanine mutations based on templates with just 30–40% sequence identity using the HHPRED server supported the preservation of overall fold after mutation of C909 (Supplementary Table 1).

To identify which cysteines might form disulfide bonds, we determined the proximity of the eight cysteines to each other and the direction of their thiol (SH) side chains in the crystal structure of IA-2 PTP. Only cysteine pairs 803 and 903 or 820 and 909 were nearly within disulfide distance. However, we concluded that disulfide-bond formation at these positions was unlikely, as the SH side chains were pointing in opposite directions in the crystal structure (Fig. 6). This was also true for IA-2β PTP and the core cysteine of all of the 30–40% identity homologs used as HHPRED templates (Supplementary Table 1).

The core cysteines of IA-2 and IA-2β are likely to be activated and may therefore be susceptible to oxidation (14).


A
952-958 IA-2
988-994 IA-2β
WPD loop
C909/945
W799/835
831-836 IA-2
867-872 IA-2β
Q862/N897
B
CYS 717/753
CYS 846/882
CYS 909/945
CYS 897/933
CYS 726/SER762
CYS 903/939
CYS820/856
CYS 803/839
C
C909

To model the effect of oxidation on conformation, a sulfonate was covalently attached to C909 and energy minimized. This resulted in movement around the active site cleft such that the cleft itself may become partially occluded. This would be likely to reduce the affinity of antibodies binding to an epitope located in this region, especially if the cysteine was targeted directly. This change also had the largest effect in disrupting the WPAE loop, with an RMSD of the distance from C909 to the WPAE loop of 1.15 Å (Fig. 6).

In agreement with others (23–25,27), Q862 and E836 seem to be located in one epitope region, whereas the C909 and the WPAE loop area may identify a second epitope region (Fig. 6). Tryptophan 799 is located close to the epitope region identified by E836. Mutation of this residue disrupted binding to both epitope regions, but was predicted to preserve fold (RMSD for 287 residues of 0.148 Å). As previously suggested (23), the W799A mutation may cause an allosteric change in epitope conformation. Because W799 packs closely against the back of a β sheet, reducing the side-chain size from tryptophan to alanine may cause a subtle shift in sheet position (Supplementary Fig. 1). This could have long-range effects on both C909 and E836 epitopes for which orientation relies on the position of this β sheet.

DISCUSSION

We have demonstrated that substituting serine or alanine for the core cysteines at position 909 in IA-2ic and IA-2 PTP or at position 945 in IA-2β PTP caused profound reductions in binding by sera from IA-2A–positive patients with type 1 diabetes. Mutation of glutamate to lysine at position 836 in IA-2ic and IA-2 PTP as well as at the corresponding glutamate residue (872) in IA-2β PTP resulted in smaller reductions in binding. Combined replacement of both the glutamate residue and the core cysteine almost abolished binding of IA-2 PTP and IA-2β PTP by the majority of patient sera, suggesting that these amino acids are critical to the integrity of two distinct major PTP epitope regions.

Mutation of the nine other cysteines in IA-2ic resulted in little or no change in binding by sera from IA-2A–positive patients, suggesting that disulfide-bond formation does not play a critical role in the maintenance of antigenic structure in IA-2ic. Although not ruling out a contribution from disulfide bonding, our findings imply that the abolition of binding to PTP epitopes of IA-2 (28) and IA-2β following alkylation of free SH groups is largely caused by modification of the core cysteine and not disruption of disulfide bonds. The relatively small reduction in binding seen following pretreatment of the C909S IA-2ic label with NEM provides additional indirect evidence to support this mechanism. Previously, it was proposed that cysteines at 717, 803, 846, 897, and 903, which are conserved in IA-2 and IA-2β but not other trypsin-sensitive PTPs, might form disulfide bonds, making both proteins resistant to the action of trypsin (28). However, analysis of IA-2ic expressed in *E. coli* using Ellman's reagent indicated that only two cysteines could form a disulfide bond, because the remaining eight reacted readily and were therefore exposed (29). The crystal structures show the cysteines in close proximity (C820 and C909 or C803 and C903) have incorrectly orientated thiols to favor disulfide-bond formation.

From our mutational analysis, we propose that the core cysteines, C909 in IA-2 and C945 in IA-2β PTP, are central components of the epitope region that incorporates the WPAE loop (25). Despite showing only limited conformational changes following the C909S and C909A mutations, our molecular modeling supports this assertion. Direct antibody access to the core cysteine may be restricted but could be influenced by the conformation of the adjacent loop/helix (residues 952–958 in IA-2 and 988–994 in IA-2β) (Fig. 6). Arginine 954 sits over the core cysteine of IA-2 (7.8 Å apart), and the B-factors for some of the side chains in this area in both IA-2 and IA-2β PTP crystal structures suggest flexibility in this region. The glutamate at position 836 in IA-2 and 872 in IA-2β is localized to a different region of the molecule (Fig. 6) and is an essential component of the second major conformational epitope region in the PTP domain of these molecules (23,24). Monoclonal antibody binding experiments support this conclusion, as binding of label by MAb-A5 was abolished by mutation of E836, but only slightly affected by alanine substitution at C909. The C909 thiol in the crystal structure points toward the interior of the loop, but in solution may spend some of the time exposed on the surface. This would explain how NEM is able to access the C909 thiol. The more hydrophilic serine is likely to favor the more exposed orientation, which could cause greater allosteric perturbation of a distant epitope such as E836. This is consistent with the W799A mutation affecting both epitope regions. The modeling for W799A (Supplementary Fig. 1) suggests that as both epitope regions are linked by a β sheet, they could be influenced by mutations at either end (C909 or W799). This may explain why the C909S mutation caused greater reductions in binding by MAb-A5 than C909A. Some sera still show residual IA-2A binding, despite large reductions caused by mutation of both C909 and E836, suggesting that there are minor epitopes not dependent on these amino acids.

The oxidation state of the core cysteine of IA-2 and IA-2β in vivo is unknown, but it appears that the mature humoral autoimmune response preferentially recognizes the reduced form. Our modeling shows that oxidation of this cysteine to cysteic acid would block access of antibodies to the pocket in which it lies. This may explain why azide and Tween (30) as well as NEM reduce binding of autoantibodies to PTP epitopes of IA-2. Indeed, in this study, we have shown that IA-2A are particularly sensitive to changes at this residue. Maintaining the core cysteine in a reduced state is therefore important for optimum detection of antibodies to PTP epitopes. In contrast, the C909S

FIG. 6. *A*: General overview ribbon diagram of the overlaid structures of IA-2 PTP (cyan) residues 687–976 and IA-2β (magenta) residues 724–1012 showing the side chains of amino acids important for antibody binding. The position of C909 in IA-2 and C945 in IA-2β is indicated to show its proximity to the WPAE epitope. Residues 831–836 and 867–872 indicate the position of epitopes affected by the glutamate-to-lysine mutations. Residues 952–958 and 988–994 form a ridge separating two clefts, one of which holds the core cysteine. The positions of tryptophan 799 and glutamine 862 are also shown. *B* shows the positions of the cysteine residues in IA-2 PTP (cyan) and IA-2β (magenta). *C* shows the effects of mutating C909 in WT (green) to alanine (magenta), serine (cyan) and oxidizing the cysteine by adding sulphonate (blue). All minimized models were superimposed in InsightII using C-α positions for the whole proteins. RMSDs were calculated comparing all non–hydrogen atom positions in the loops of the mutated C909 models with WT.

mutation in IA-2ic caused increased binding by the small number of JM-specific patient sera as well as a 53% rise in binding by MAb-76F4B, an antibody specific to the JM region. Similar increases were observed following NEM pretreatment (data not shown). These findings suggest that binding to linear epitopes in the JM region, often seen as part of the early IA-2A response, may be enhanced by conformational changes in the core cysteine epitope. Introduction of the C945S mutation unexpectedly increased binding of IA-2β PTP by MAb-beta593 and MAb-A9–19, suggesting that these antibodies recognize a minor epitope that is unmasked by mutating the core cysteine: a mechanism similar to that induced by antibody binding (24).

This study used antigens produced by in vitro labeling, a standard technique for measuring IA-2A, but these proteins have not been completely characterized. The mutant antigens could have aggregated and precipitated, although the effects of the mutations varied widely between sera, indicating that the majority of the labeled protein was likely to be appropriately folded and available for antibody binding. We also found no evidence that the mutations altered the integrity of the labels produced (Supplementary Fig. 2). Even though binding to the double mutants was often very low, the pattern of binding remained consistent with epitope-specific effects. This was evident particularly for IA-2ic, because binding to the JM epitopes was unchanged, or even enhanced, by changes in the PTP region. Our modeling showed no gross fold changes caused by substitution of the core cysteine with serine or alanine. This was corroborated using WT and C909A models produced by HHPRED from suboptimal templates (Supplementary Table 1). These models are necessarily biased toward conformations in the crystal structures and may vary slightly from those in solution. Ideally, our in silico findings need to be confirmed by experimental studies using purified proteins, which was not possible with our expression system. Further, as the crystal structure of the complete intracellular domain of IA-2 has not been resolved, we were unable to model the influence of PTP modifications on the JM region.

Our study used sera from IA-2A–positive patients at diagnosis, and our findings are therefore representative of a mature humoral response. The epitope regions recognized by these sera do not take account of any epitope spreading or changes that may have occurred prior to disease onset (15). However, the critical importance of the core cysteine to IA-2β antibody binding suggests that it is central to an epitope associated with a high risk of disease (37). T-cell epitopes have been identified throughout the cytoplasmic domain, but many appear to cluster within the region 795–889 (16). Although B-cell epitopes flank or reside within the T-cell epitope regions (26), the core cysteine does not form part of T-cell epitopes described so far. Development of IA-2A, however, has been suggested to coincide with a critical switch in disease progression (38). Identifying the determinants of the IA-2A response will help to clarify their importance to disease pathogenesis, particularly as IA-2A prevalence in patients may be increasing in parallel with the rising incidence of type 1 diabetes (39). Our study has shown that in most patients at disease onset, >90% of antibodies binding to the PTP domain of IA-2 recognize just two epitope regions. Greater understanding of the relationship between the humoral and T-cell responses to IA-2 may therefore hold the key to preventing disease progression in a high proportion of individuals currently destined to develop type 1 diabetes.

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K.T.E. and A.J.K.W. designed the study, researched data, and wrote the manuscript. I.G. researched data and reviewed the manuscript. D.K.S. performed the structural modeling. D.K.S., V.L., and P.J.B. researched data, contributed to the discussion, and reviewed and edited the manuscript. P.J.B. coordinated the BOX study. A.J.K.W. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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