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Relationships between major epitopes of the IA-2 autoantigen in Type 1 diabetes: Implications for determinant spreading

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Abstract Diversification of autoimmunity to islet autoantigens is critical for progression to Type 1 diabetes. B-cells participate in diversification by modifying antigen processing, thereby influencing which peptides are presented to T-cells. In Type 1 diabetes, JM antibodies are associated with T-cell responses to PTP domain peptides. We investigated whether this is the consequence of close structural alignment of JM and PTP domain determinants on IA-2. Fab fragments of IA-2 antibodies with epitopes mapped to the JM domain blocked IA-2 binding of antibodies that recognise epitopes in the IA-2 PTP domain. Peptides from both the JM and PTP domains were protected from degradation during proteolysis of JM antibody:IA-2 complexes and included those representing major T-cell determinants in Type 1 diabetes. The results demonstrate close structural relationships between JM and PTP domain epitopes on IA-2. Stabilisation of PTP domain peptides during proteolysis in JM-specific B-cells may explain determinant spreading in IA-2 autoimmunity.

1. Introduction

The development of Type 1 diabetes is associated with T- and B-cell autoimmunity to multiple islet autoantigens including proinsulin, glutamate decarboxylase, IA-2 and zinc transporter-8 [1]. Studies on the natural history of

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Type 1 diabetes indicate that spreading of autoimmune responses within and between these islet autoantigens is crucial for disease progression, and individuals who maintain a restricted response to single islet antigens have a low risk of developing clinical disease [2–6]. The mechanisms underlying the progressive spreading of autoimmune responses to determinants on islet self proteins are unknown. Studies in animal models of autoimmune disease have implicated B-cells in this process, specifically through their roles as antigen presenting cells [7]. Autoantibody-secreting B-cells are proposed to play a critical role in sustaining T-cell responses to islet antigens by mediating their efficient uptake via the B-cell receptor, facilitating the presentation of peptides derived from antigens to T-cells [8]. Depletion of B-cells impairs T-cell responses to islet antigens, thereby preventing the development of diabetes in animal models and prolonging beta cell function in human Type 1 diabetes [9,10]. There are close links between T- and B-cell responses to islet antigens when these are studied at the epitope level. Thus, both T- and B-cell epitopes are clustered on the structure of islet autoantigens [11–13] and T-cell responses of peripheral blood lymphocytes from diabetic patients to specific IA-2 peptides are associated with the presence of antibodies to epitopes overlapping these peptides [12,13]. Furthermore, the binding of antigen to the B-cell receptor is stable within antigen processing compartments and the formation of such complexes may protect or expose sites at which antigen is cleaved by processing enzymes, leading to the stabilisation of specific peptides for presentation and activation of autoreactive T-cells [14,15]. Such modification of islet antigen processing and presentation may represent one mechanism by which B-cells facilitate determinant spreading in the autoimmune response in Type 1 diabetes.

Studies on autoimmunity to one of the major islet autoantigens in human Type 1 diabetes, IA-2, illustrate the importance of immune diversification in Type 1 diabetes. Antibodies to IA-2 are detected in the majority of patients at the time of diabetes onset and their appearance is strongly predictive of disease progression in non-diabetic subjects [16,17]. Analysis of binding of autoantibodies to deletion mutants of IA-2 has identified several distinct regions of antibody reactivity within the cytoplasmic domain, including at least two linear epitopes between amino acids 621–630 of the juxtamembrane (JM) domain [18,19] and conformational epitopes within the tyrosine phosphatase (PTP) domain, which include a major epitope represented by amino acids within the 831–860 region of the molecule and a second that includes residues 876–880 [12,20–22]. In the early autoimmune response in pre-diabetes, IA-2 antibodies often recognise epitopes in the JM domain of the protein, reactivity then spreads to epitopes in the PTP domain and to the closely related IA-2beta [5]. Recent studies have shown an increase in the prevalence of antibodies to epitopes in the IA-2 PTP domain, concurrent with rising diabetes prevalence [23,24]. Furthermore, diversification of the autoimmune response to multiple epitopes on IA-2 in pre-diabetes increases Type 1 diabetes risk [25], demonstrating that determinant spreading in IA-2 autoimmunity is closely linked to diabetes progression.

We have recently shown that T-cell responses to a peptide representing amino acids 841–860 within the PTP domain of IA-2 are associated not only with PTP domain antibodies, but also more significantly with antibodies to the JM domain [13]. We hypothesised that B-cell receptor binding to the JM domain may facilitate loading of processed peptides in the PTP domain for stimulation of T-cells, potentially as a consequence of these regions being closely aligned on the three dimensional structure of the protein. The aim of this study was to investigate the relationships of antigenic sites within the IA-2 JM and PTP domains by: i.) localising epitopes for monoclonal IA-2 antibodies to the JM and PTP domains by peptide inhibition and site-directed mutagenesis; ii.) investigating possible juxtaposition of the epitopes on IA-2 by cross-competition studies and iii.) determining the influence of JM and PTP domain monoclonal antibodies on peptides generated during proteolytic processing of IA-2:monoclonal antibody complexes.

2. Methods

2.1. Type 1 diabetic patients

Patients with Type 1 diabetes between the ages of 12 and 30 were recruited within 6 months of clinical onset from diabetic clinics in Yorkshire, Durham and King's College Hospital, London, UK, with informed consent and approval from appropriate Ethics Committees. Serum samples from IA-2 antibody-positive patients were selected for characterisation of IA-2 autoantibody epitopes on the basis of strong reactivity to deletion mutants and chimeric constructs representing different regions of the IA-2 molecule [26].

2.2. IA-2 antibodies

Four mouse monoclonal antibodies, 76F, 5E3, 8B3 and 9B5, that recognise epitopes in the JM domain of IA-2 overlapping those for autoantibodies in human Type 1 diabetes [27,28], and three human B cell clones 96/3, M13 and DS329 obtained after EBV-transformation of B lymphocytes from Type 1 diabetic patients [12,28,29] and secreting antibodies to epitopes in the IA-2 PTP domain, were used for epitope characterisation. A polyclonal rabbit antiserum (R2B2; [20]) was also used for epitope studies. Monoclonal antibodies were purified by protein A-sepharose chromatography from tissue culture supernatants of these clones. For antibody competition studies, Fab fragments of the antibodies were prepared by papain digestion, as described [30].

2.3. Analysis of binding of IA-2 antibodies

Antibody binding to radiolabelled IA-2 constructs was analysed by radioligand binding assay, as previously described [12,31]. IA-2 constructs used were the cytoplasmic domain of IA-2 (IA-2ic, residues 605–979), a chimeric construct representing the juxtamembrane domain (JM, residues 605–693) fused to the tyrosine phosphatase (PTP) domain of PTP1B, the IA-2 PTP domain (residues 643–979) and the central region of the IA-2 PTP domain (residues 643–937). IA-2 cDNAs were transcribed and translated in vitro in the presence of ^{35}S-methionine using the TNT Quick Coupled Transcription and Translation System (Promega, Southampton, UK). Radiolabelled protein was incubated with monoclonal antibody or test sera for 16 hours at 4 °C in wash buffer (10 mM HEPES, pH7.4, 150 mM NaCl, 20 mM methionine, 0.5 mg/mml BSA and 0.5%

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Triton X-100). Immune complexes were captured on Protein A-Sepharose and, after washing, the quantity of immunoprecipitated radiolabelled antigen was determined by liquid scintillation counting.

To evaluate their contribution to antibody binding, single amino acids within the IA-2 sequence were substituted for alanine using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Stockport, UK) according to the manufacturer's instructions. Substitutions were verified by sequencing. Mutated constructs were transcribed and translated *in vitro* in the presence of ^{35}S methionine and used in radioligand binding assays as described above. Binding of antibodies to mutated constructs was compared with that to the wild type construct. Single amino acid mutations were considered to have inhibitory effects on antibody binding if binding was reduced by 50% or more.

Relationships between antibody epitopes were investigated by competition studies using Fab fragments of monoclonal antibodies of defined epitope specificity. Monoclonal antibodies or sera from diabetic patients were incubated with ^{35}S-labelled IA-2 cytoplasmic domain (amino acids 605–979) in the presence or absence of 5 μg of Fab fragments of the test antibody for 16 h at 4 °C and radiolabelled protein immunoprecipitated determined as described above. Inhibitory effects on antibody binding of Fab fragments of individual antibodies were tested by analysis of variance.

2.4. Proteolytic digestion of IA-2-antibody complexes ("antibody footprinting")

To generate protein for antibody footprinting, cDNA representing the coding sequence of the cytoplasmic domain of IA-2 (IA-2ic, residues 605–979) was cloned into the pGEX-6P vector to generate a construct encoding an IA-2 fusion protein with an N-terminal glutathione-S-transferase purification tag followed by a PreScission Protease cleavage site. The recombinant protein was expressed in BL21 *E.coli* cells and extracts prepared by lysozyme treatment of bacterial pellets. Recombinant protein in bacterial extracts was captured on Glutathione Sepharose 4b (GE Healthcare) and treated on-column with PreScission Protease to cleave the purification tag and elute the pure IA-2ic protein. The protein was dialysed against phosphate-buffered saline and was >90% pure by SDS-PAGE analysis.

Monoclonal IA-2 antibodies were immobilised by chemical cross-linking to protein G Sepharose. Antibodies were incubated with beads for 1 h at room temperature and cross-linked with dimethylpimelidate in borate buffer [30]. Unreacted sites were blocked with 20 mM ethanolamine for 10 min. Unbound antibody was removed by seqential washes in 100 mM triethylamine pH 11.7, sodium acetate, pH 3.0 and PBS.

The influence of monoclonal antibody specificity on proteolytic processing of IA-2 was performed by incubating protein G Sepharose-conjugated antibodies with 20,000 cpm of ^{35}S-methioine-labelled IA-2ic and 10 μg of unlabelled purified recombinant IA-2ic for 2 h at room temperature. Non-bound IA-2 was removed by washing and complexes incubated with trypsin (0.1 mg/ml) for times indicated in the figure legend. Reactions were terminated by addition of phenylmethanesulphonic acid (10 mM final concentration) and non-bound proteolytic fragments removed by washing. Bound fragments were eluted in 100 mM triethylamine, eluates neutralised with 0.5 M NaH_2PO_4 and analysed by SDS-PAGE and autoradiography.

For identification of the antibody-protected peptides by mass spectrometry, bead-bound antibody-antigen complexes were formed by incubating the immobilised antibody with 100 μg of purified IA-2 cytoplasmic domain protein for 2 h at room temperature with slow rotation. Unbound antigen was removed by washing with PBS and the complexes equilibrated in chymotrypsin digestion buffer (100 mM Tris, 10 mM CaCl). Activated chymotrypsin was added to the complex at an enzyme:substrate ratio of 1:10 and incubated for 30 mins at 30 °C with occasional mixing. Unbound proteolytic fragments were removed by washing with PBS and subsequently with water. Antibody bound fragments were eluted in 100 mM triethylamine pH 11.7. The eluates were vacuum dried and stored at −20 °C prior to mass spectrometry analysis.

2.5. Mass spectrometry

Samples were analysed by LC-MS/MS on a ProteomeX machine (Thermo Finnigan, Hemel Hempstead, UK). Dried chymotrypsin digests were resuspended in 0.1% formic acid and chromatography of aliquots of each sample performed on a 100- by 0.18-mm BioBasic C18 column (ThermoHypersil-Keystone, Runcorn, UK). Peptides were eluted with aqueous acetonitrile (5 to 65% over 30 min) containing 0.1% formic acid at a flow rate of 2 μl per min. MS/MS data were acquired in data-dependent mode with dynamic exclusion. Spectra were submitted against the IA-2 sequence database using Bioworks v3.1/TurboSEQUEST software (Thermo Electron, Langenselbold, Germany). Proteins were considered to match entries in the database if XCorr values for individual peptides were ≥1.5, ≥2, and ≥2.5 for singly, doubly, and triply charged ions, respectively.

3. Results

3.1. Characterisation of epitopes for juxtamembrane domain monoclonal antibodies

Epitopes for four mouse monoclonal antibodies to IA-2 have been shown by competition studies to overlap with those for autoantibodies in Type 1 diabetic patients' sera [27,28]. All recognise epitopes within the JM domain of the protein. To further define the epitopes for each of the four mouse monoclonal antibodies, the influence of synthetic 20-mer peptides on antibody binding to a chimeric protein representing the 605–693 region of IA-2 fused to the PTP domain of PTP1B was investigated. The four monoclonal antibodies to the JM domain were inhibited differentially by synthetic peptides within the 601–640 region of the protein (Fig. 1A). Binding of antibody 76F was inhibited by the presence of the 621–640 IA-2 peptide, but not by peptides 601–620 or 611–630. Antibody 5E3 was inhibited only by the 611–630 peptide and 8B3 only by 601–620 (Fig. 1A). 9B5 showed no inhibition by any of the peptides.

To identify amino acids on IA-2 that participate in antibody binding, reactivity to IA-2 JM constructs with single amino acid-substitutions were evaluated. The inhibitory effects of substitutions of residues within the 626–629 region on binding of the 76F antibody [18] were confirmed in this study. However,

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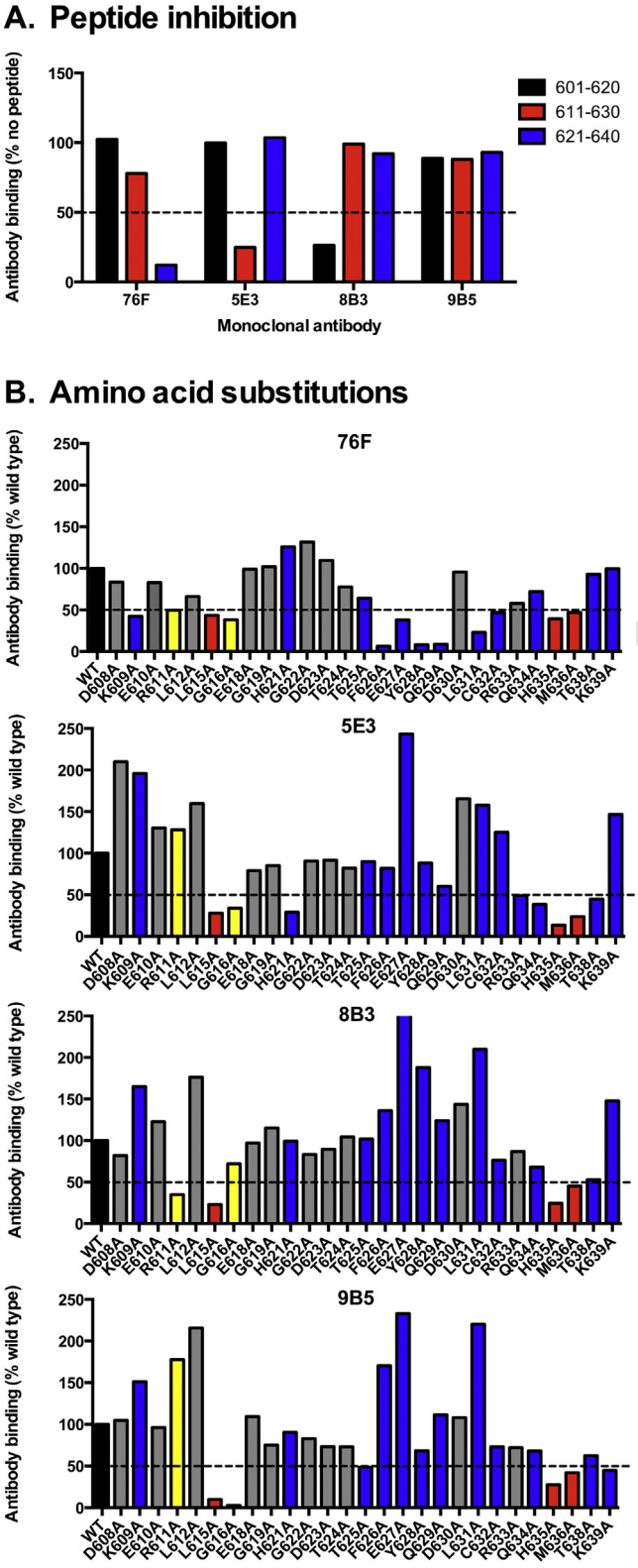
the epitope for this antibody was found to extend beyond this region, as indicated by inhibition by alanine substitutions of amino acids L631, G632, H635 and M636 and of several amino acid substitutions in the region 609–616 (Fig. 1B). Substitution of amino acids between 626 and 629 did not affect binding of the other three mouse monoclonal antibodies, but mutational mapping did show effects common to those seen for 76F. Hence, substitution of amino acids L615, H635 and M636 inhibited binding of all four monoclonal antibodies (marked red in Fig. 1B) and mutation of residues R611 and G616 inhibited at least two antibodies (yellow in Fig. 1). Effects of other amino acid substitutions were clone-specific (blue in Fig. 1B). Some amino acid substitutions enhanced binding of some antibodies, most notably of L612, E627, L631 and K639. The results demonstrate that epitopes for the mouse IA-2 antibodies are represented by two discontinuous regions within the 609–639 region of the IA-2 JM domain with common structural elements for all four JM antibodies.



3.2. Characterisation of epitopes for human autoantibodies to the central PTP domain of IA-2

We have previously localised the epitopes for three human monoclonal IA-2 autoantibodies isolated from Type 1 diabetic patients (96/3, M13 and DS329) to the 831–860 region of the protein [12,20]. To further define the epitopes for these antibodies, substitutions of those amino acids within the region 826–862 located on the surface of the crystal structure of IA-2 [32] were introduced into a truncated IA-2 PTP domain construct (residues 643–937) and inhibitory effects of each substitution on binding of the three monoclonal antibodies were investigated.

Alanine substitution of amino acids L831, V834, E836, L839, K857, N858 and V859, that are clustered on the surface of IA-2 in the structural model, inhibited binding to all three monoclonal antibodies (red in Fig. 2A, 2B). Further inhibition of binding was observed in two of the three monoclonal antibodies (yellow in Fig. 2A, 2B) following mutation of residues H833 (M13 and DS329) and Q862 (M13 and 96/3). Binding to M13 was additionally inhibited by the substitution of amino acids E827 and Q860. A polyclonal rabbit anti-serum to IA-2 (R2B2) was unaffected by any of the mutations (Fig. 2A).

The effects of these mutations were also assessed in thirteen patient sera positive for antibodies to the central region.

Figure 1 Mapping of epitopes for mouse monoclonal antibodies to the IA-2 JM domain by peptide blocking and site-directed mutagenesis. A: Effect of synthetic peptides representing IA-2 residues 601–620 (black), 611–630 (red) or 621–640 (blue) on binding of four mouse monoclonal antibodies to a radiolabelled construct representing the IA-2 JM doman (amino acids 605–693) fused to the PTP domain of PTP-1B. Data are expressed as % of antibody binding to the construct in the absence of peptide and substitutions reducing binding by 50% or more were considered inhibitory. B: Influence of single amino acid substitutions on binding of four mouse monoclonal antibodies to the radiolabelled JM construct. Data are expressed as % of antibody binding to the wild-type JM construct and bars representing each amino acid are colour coded according to whether the substitution inhibits binding of one (blue), two or three (yellow) or all four (red) monoclonal antibodies by >50% (dashed lines). Grey bars indicate amino acids where substitutions had no inhibitory effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Substituted residues that inhibited binding to monoclonal antibodies were also found to inhibit binding to antibodies in Type 1 diabetic patients' sera, indicating a common area of antibody recognition. Mutation of amino acids L831, V834, E836, L839, K857, N858 and V859 inhibited binding to the IA-2 construct in at least 11/13 samples (Fig. 2C).

3.3. Inhibition of autoantibody binding to IA-2 by Fab fragments of IA-2 monoclonal antibodies

To examine relationships between individual defined epitopes in the JM and PTP domains of IA-2, the ability of Fab fragments of PTP and JM domain-reactive monoclonal IA-2 antibodies to compete for binding with monoclonal or serum antibodies to IA-2 was investigated. Fab fragments of the PTP domain autoantibody M13 abolished binding to other monoclonal antibodies recognising similar PTP domain epitopes, but had no effect on IA-2 binding of the JM domain-reactive antibody, 76F (Fig. 3A). The rabbit polyclonal antibody to IA-2 was also unaffected. Fab fragments of the JM domain antibodies abolished (5E3) or partially inhibited (9B5) IA-2 binding of the JM-reactive 76F antibody. However, Fab fragments of 5E3 and 8B3 JM antibodies also partially inhibited IA-2 binding of the monoclonal antibodies M13, 96/3 and DS329 that are reactive to the PTP domain epitope, and of the polyclonal rabbit IA-2 antibody. The results indicate that binding of Fab fragments of antibodies to the JM domain are able to impair antibody binding to epitopes within the PTP domain, possibly through steric hindrance or conformational effects.

Inhibitory effects of Fab fragments of monoclonal antibodies were also investigated using serum antibodies from IA-2 antibody-positive Type 1 diabetic patients categorised according to antibody reactivity to the IA-2 JM domain only (Fig. 3B), to both JM and PTP domains (Fig. 3C) or to the PTP domain only (Fig. 3D). Fab fragments of the JM domain reactive antibodies abolished (5E3) or partially inhibited (8B3, 9B5) binding of antibodies from patients with reactivity restricted to the IA-2 JM domain, whereas M13 Fab fragments had no effect (Fig. 3B). Fab fragments of the JM domain antibodies inhibited IA-2 binding of autoantibodies from patients positive for both JM and PTP domain antibodies (Fig. 3C), but also those negative for JM antibodies (Fig. 3D). The ability of Fab fragments of JM domain-reactive antibodies to inhibit binding of antibodies to PTP domain epitopes points to structural interactions between these two regions of autoantibody reactivity.

3.4. Characterisation of antibody epitopes by antibody footprinting

Antibody footprinting is a technique by which structural interactions between antibody and antigen are investigated by limited digestion of antibody:antigen complexes with proteases or hydroxyl radicals [33]. Antibody binding protects regions close to the antibody epitope from cleavage and identification of the protected regions defines the antibody "footprint". In this study, antibody footprinting was used to compare and identify antibody-protected IA-2 proteolytic fragments using monoclonal antibodies directed to epitopes localised within the JM or PTP domains of the protein.

Initial studies used SDS-PAGE and autoradiography to characterise radiolabelled proteolytic products generated after trypsin digestion of complexes of bead-conjugated monoclonal antibodies with ^{35}S-methionine-labelled IA-2ic. Time course studies demonstrated clear differences in the dominant tryptic digestion products eluted from bead-conjugated 5E3 (JM domain epitope) and M13 (PTP domain epitope) antibodies, with predominant bands at Mr 3500 and 7000 for 5E3 and at Mr 11,000 and 23,000 for M13 (Fig. 4). However, despite the differences in epitope recognition, common bands were also eluted from both antibodies, in particular, a trypsin product of 9000 Mr (Fig. 4).

To identify the regions protected by the JM and PTP domain monoclonal antibodies, similar experiments were performed using purified recombinant IA-2ic as antigen, digesting antibody:IA-2ic complexes with chymotrypsin which, being a more frequent cutter than trypsin, provides better resolution of antibody-protected regions of the protein. Chymotrypsin digestion products eluted from bead-conjugated antibodies were identified by LC-MS/MS. A total of 39 distinct peptides were identified in the eluates, and the percent recovery of each of these peptides relative to the total number of peptides identified is shown in Table 1. Several of the peptides could be clustered according to the presence of a common core sequence (bold font in Table 1) with varying length extensions at the C- or N-terminus. Peptides containing the core motif AALGPEGAHGTTF representing amino acids 613–626 of IA-2 were highly represented in eluates from the JM epitope-reactive 5E3 antibody (21.4%), but almost absent from the M13 eluates (0.2%; $p < 0.0001$, Fisher's exact test with Bonferroni correction). These peptides include residues L615, G616 and H621 that were identified as part of the 5E3 epitope in the mutagenesis studies above. However, the majority of peptides eluted from the 5E3 antibody were derived from the PTP domain, with peptides containing the sequences SHTIADFW (788–795, 21%), KNVQTQETRTL (857–867, 8.4%), TAVAEEVNAIL (964–974, 21%) and NRMAKGVKEIDIAATL (927–942, 14.5%) being highly represented (Table 1). These latter peptides were also detected in eluates from the PTP domain-reactive M13 antibody. Peptides with the core sequences INASPIIEHDPRMPAY (765–780, 32.7%) and SWPAEGTPASTRPL (874–887, 18.1%) were detected in eluates from the M13 antibody, but found at low abundance in eluates from 5E3 (2.7% and 1.5%, respectively; $p < 0.0001$).

4. Discussion

Studies on the appearance of autoantibodies to islet antigens in early life [2,5], together with assessment of the risk of development of Type 1 diabetes by detection of single and multiple islet autoantibody specificities [16,34], have emphasised the importance of determinant spreading for progression from autoimmunity to disease. A key role for B-cells in promoting determinant spreading has been demonstrated in animal models of autoimmune disease [35], probably through alterations in uptake, processing and presentation of relevant antigens. We now demonstrate a close structural relationship between determinants in two distinct domains of a major autoantigen in Type 1 diabetes that, together with previous observations, point to an important role for B-cells secreting antibodies to the JM domain of IA-2 in the diversification of the immune response in human Type 1 diabetes. Thus: i.) antibodies to the JM domain appear early

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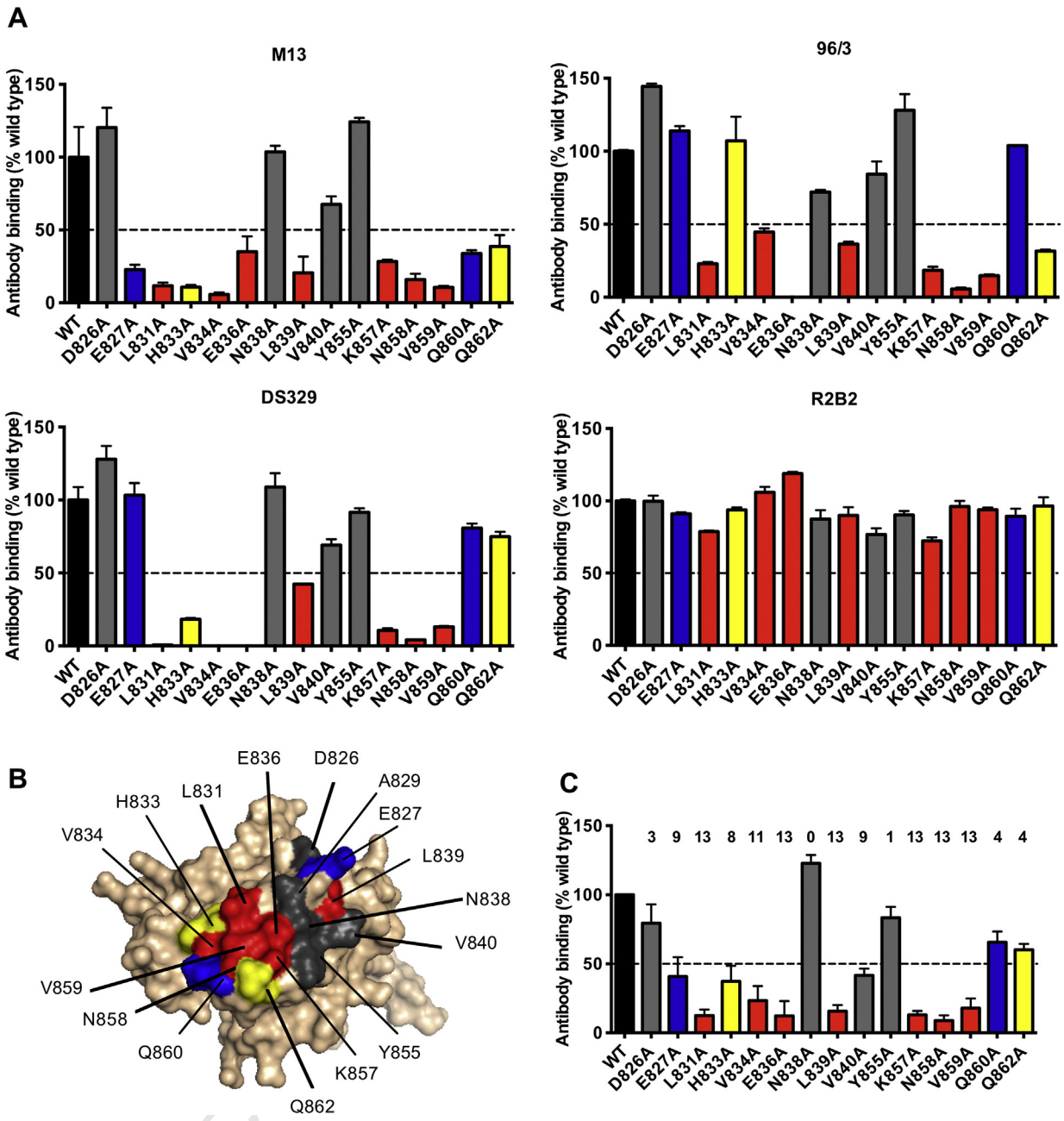

Figure 2 Influence of single amino acid substitutions on binding of human monoclonal antibodies and patients' sera to IA-2. A: Influence of single amino acid substitutions on binding of three human monoclonal antibodies or a rabbit polyclonal antiserum to a radiolabelled construct representing amino acids 643–937. Data are expressed as % of antibody binding to the wild-type IA-2 construct (n = 3). Substitutions that reduced binding by 50% or more (dashed line) were considered inhibitory and bars representing each amino acid are colour coded according to whether the substitution inhibits binding of one (blue), two (yellow) or all three (red) monoclonal antibodies. Grey bars indicate amino acids where mutations had no inhibitory effect. B: Influence of single amino acid substitutions on binding of recent onset Type 1 diabetic patients' sera to the same IA-2 construct as in A. Data for each amino acid substitution are expressed as % of antibody binding to the wild-type construct (mean ± SEM, n = 13). Numbers above bars indicate the number of individual patient sera from the panel of 13 in which the mutation inhibited binding by more than 50%. Bars are colour coded as in A. C: Localisation of substituted amino acids on a model of the surface of IA-2 tyrosine phosphatase domain. The colour coding of individual amino acids are as in A. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the IA-2 autoimmune response and precede spreading to epitopes in the IA-2 PTP domain and to the related autoantigen, IA-2beta [5]; ii.) the presence of autoantibodies to the IA-2 JM domain in Type 1 diabetic patients is associated with T-cell responses to a peptide in the PTP domain that itself overlaps a major autoantibody epitope [13]; iii.) as shown in this study,

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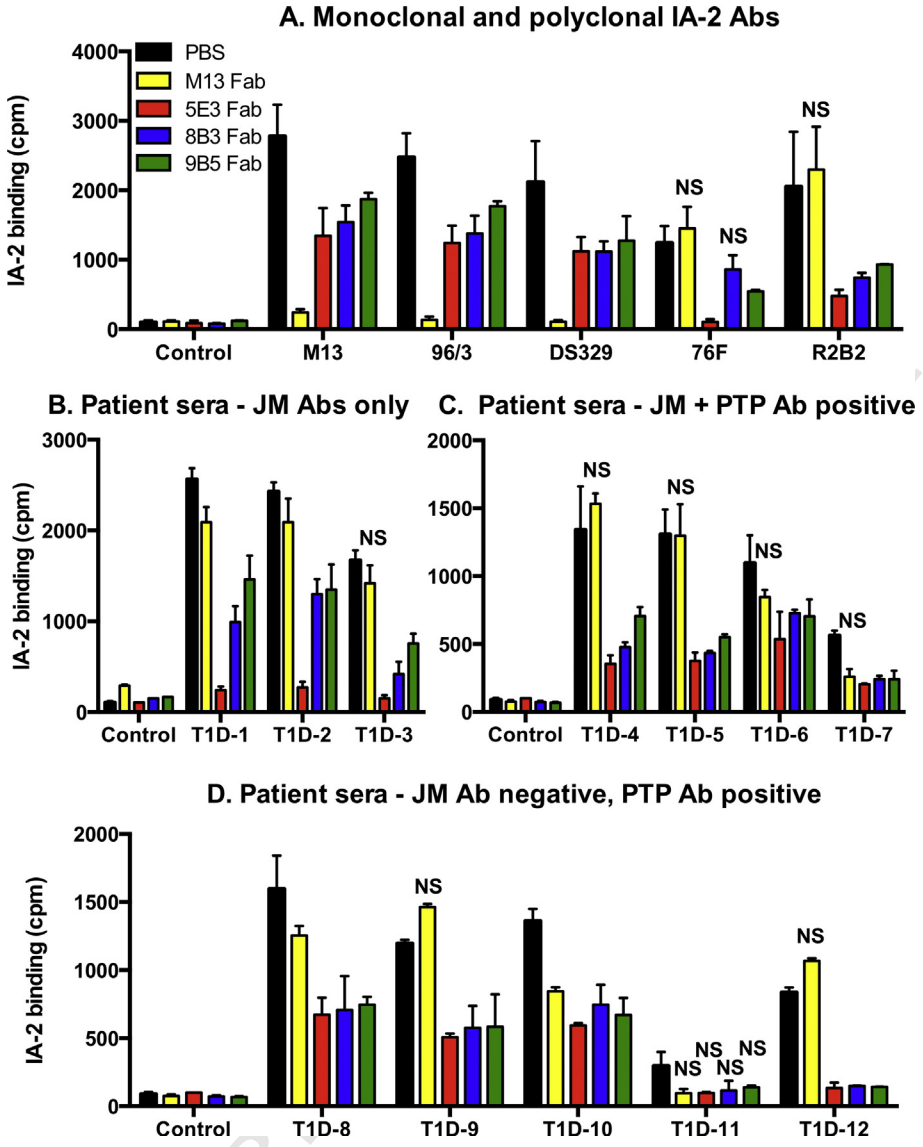

Figure 3 Inhibitory effects of Fab fragments of monoclonal IA-2 antibodies on binding of antibodies to IA-2. The ability of Fab fragments of monoclonal antibodies M13 (yellow bars), 5E3 (red bars), 8B3 (blue bars) or 9B5 (green bars) to compete for binding of monoclonal IA-2 antibodies 76F, M13, 96/3 or DS329 (panel A; n = 4), polyclonal rabbit IA-2 antiserum R2B2 (A) or serum antibodies from 12 recent onset diabetic patients (panels B–D) to radiolabelled construct representing amino acids 605–979 was tested. Diabetic patients were categorised according to the presence of antibodies only to the JM domain of IA-2 (panel B), to both JM and PTP domain epitopes (panel C), or only to PTP domain epitopes (panel D). The significance of effects of each Fab fragment on antibody binding compared to that seen with phosphate buffered saline (PBS, black bars) was analysed by two way analysis of variance with Dunnet's correction for multiple comparisons. Significant inhibition ($p < 0.05$) of antibody binding by each Fab was observed except where indicated on figure (NS: not significant). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

monoclonal antibodies to the JM domain block binding of autoantibodies to the same PTP domain epitope, suggesting juxtaposition of the two epitopes (Fig. 3); and iv.) these JM domain antibodies protect and stabilise PTP domain peptides containing major T-cell determinants during proteolysis of antibody:antigen complexes (Fig. 4, Table 1). If similar antibody-mediated stabilisation of PTP domain peptides occurs within processing compartments of JM-specific B-cells, then presentation of those PTP domain peptides to T-cells would be promoted, providing a mechanism underlying the association of JM antibodies with T-cell responses to PTP domain peptides in Type 1 diabetes [13] and for the spreading of autoimmunity from JM to PTP domain determinants as disease develops.

The study of determinant spreading at the B-cell level requires a detailed understanding of the structures of dominant autoantibody epitopes, most easily acquired through the study of cloned antibodies. Although human mononoclonal

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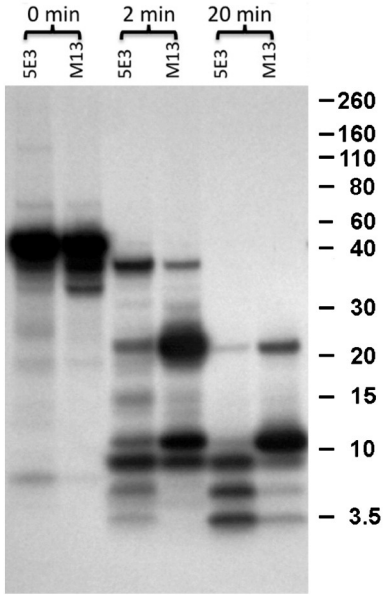

Figure 4 Radiolabelled fragments generated after trypsin treatment of complexes of ^{35}S-methionine-labelled IA-2 with monoclonal antibodies to JM and central PTP domain epitopes. ^{35}S-methionine-labelled IA-2 constructs representing amino acids 605–979 were incubated with monoclonal IA-2 antibodies to either JM (5E3) or PTP (M13) domain epitopes cross-linked to protein G Sepharose and complexes treated with trypsin (0.1 mg/ml) for the times incubated on the figure. Non-bound IA-2 fragments were washed away and polypeptide fragments remaining bound to antibody eluted and analysed by SDS-PAGE and autoradiography. The figure illustrates major IA-2 tryptic fragments eluted from each antibody. The migration of protein standards of relative molecular mass ($M_r \times 10^{-3}$) indicated is shown.

autoantibodies to IA-2 JM domain epitopes from Type 1 diabetic patients have been reported [29], transformed B-cells secreting these JM autoantibodies were unstable and are no longer available for study (J Endl, personal communication). To our knowledge, the only IA-2-specific B-cell clones from diabetic patients that are currently available secrete antibodies to overlapping PTP domain epitopes within the region 827–862 [12 and this study]. Analysis of amino acid substitutions affecting binding of three human monoclonal antibodies to the PTP domain suggest a core region of antibody binding represented by amino acids 831, 834, 836, 839, 857, 858 and 859, with individual B-cell clones showing different involvement of residues peripheral to this common core (Fig. 2B). Analysis of the effects of amino acid substitutions on binding of serum antibodies from individual Type 1 diabetic patients demonstrated that the pattern of reactivity to this region is typical of B-cell responses in Type 1 diabetes generally, consistent with it being a major target of autoantibody reactivity in disease. The protein footprint of the M13 human monoclonal PTP domain autoantibody included peptides with core regions 836–845 and 857–867 which encompass the amino acids implicated in the autoantibody epitope (Table 1) and are included within major T-cell determinants [12,13,36]. However, the antibody also stabilised other PTP domain peptides extending beyond the epitope, including those containing regions 765–780, 788–795, 874–887 and 964–974 (Table 1). Peptides from the JM domain were rarely detected. Analysis of the crystal structure of the IA-2 PTP domain shows the 765–780 region to be buried in the molecule beneath the proposed epitope region [32]. The 874–887 region includes peptides immediately adjacent to those harbouring the antibody epitope, but lies on the opposite face of the protein to the epitope region in the 3-dimensional structure [22,32]. The 874–887 motif includes the 876–880 sequence of amino acids, substitutions of which have been shown previously to inhibit IA-2 autoantibody binding and that may form part of a distinct PTP domain epitope [22,37].

Although no monoclonal IA-2 JM domain autoantibodies derived from Type 1 diabetic patients are currently available for study, there is good evidence that antibodies cloned from IA-2-immunised mice show very similar JM epitope specificities to those appearing in the human disease [28,38]. Studies to localise the epitopes of mouse monoclonal antibodies to the JM domain show that synthetic peptides known to inhibit serum antibodies from Type 1 diabetic patients (601–620, 611–630 and 621–640 [18]) also inhibit binding of three of the mouse antibodies (Fig 1A). Site-directed mutagenesis indicated that amino acids 615, 635 and 636 represent key residues for antigen binding to all four monoclonal antibodies, with differing contributions of amino acids within the 608–638 region of IA-2 to binding of individual antibodies. For the 76F antibody, substitutions affecting binding included amino acids 626–629 which form part of the "JM2" and "JM3" epitopes described by the Bonifacio group [18,19] and, for 5E3, residue 621 which contributes to a "JM1" epitope [19]. Consistent with the mutagenesis data, the protein footprint of the 5E3 antibody included JM-localised peptides with a 613–626 core, that were poorly represented in the M13 footprint, strongly supporting this region as part of the 5E3 antibody epitope. However, peptides within the PTP domain containing regions 788–795, 857–867, 927–942 and 964–974 were also highly represented in eluates from the 5E3 antibody, again indicative of antibody-mediated protection from proteolysis of peptides outside of the immediate epitope region. Fab fragments of 5E3 and other JM domain antibodies were more effective than those of PTP domain antibodies at blocking binding of serum antibodies to epitopes in both the JM and PTP domain. These strong inhibitory effects of JM-targetted antibodies on binding of antibodies to the PTP domains is suggestive of close structural relationships between the two epitopes and juxtaposition of the two epitopes may explain the stabilisation of PTP-derived peptides by the JM domain antibody.

The results of this study point to close structural relationships between two major regions targetted by autoantibodies in Type 1 diabetes that may have implications for the diversification of IA-2 autoimmunity in Type 1 diabetes. Confirmation that these *in vitro* observations have pathophysiological relevance requires analyses of the influence of B-cell epitope specificity on peptides generated within cellular processing compartments. Our identification of antibody epitopes, and core regions of IA-2 protected by JM and PTP domain antibodies, will facilitate studies to fully understand the natural history of spreading of B- and T-cell responses to determinants during the early stages of IA-2 autoimmunity. Such studies would identify B- or T-cell responses to determinants most closely linked to disease progression that would represent effective targets for immunotherapy.

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Table 1 Proportion of total number of peptides detected (%).

Peptide	Mass (Da)	Sequence	5E3	M13
606–612	1474.73	GPLGSMQQDKERL	0.5	0
606–626	2783.34	GPLGSMQQDKERL**AALGPEGAHGDTTF**	2.3	0
606–626	2532.18	GSMQQDKERL**AALGPEGAHGDTTF**	0.5	0
613–626	1343.62	**AALGPEGAHGDTTF**	18.6	0.2
643–661	2030.99	NRAEGPPEPSRVSSVSSQF	0	0.4
747–753	852.50	DHARIKL	0.9	0.4
754–764	1254.60	KVESSPSRSDY	1.4	0.4
765–780	1823.91	**INASPIIEHDPRMPAY**	0.9	22.2
765–787	2504.30	**INASPIIEHDPRMPAY**IATQGPL	1.8	10.5
781–795	1656.84	IATQGPL**SHTIADFW**	0.5	5.5
788–795	976.45	**SHTIADFW**	20.0	9.1
788–799	1520.70	**SHTIADFW**QMVW	0.5	0.2
800–831	3526.65	ESGCTVIVMLTPLVEDGVKQCDRYWPDEGASL	0	0.4
800–832	3689.72	ESGCTVIVMLTPLVEDGVKQCDRYWPDEGASLY	1.8	0.7
833–839	873.44	HVYEVNL	0	0.2
833–845	1624.81	HVY**EVNLVSEHIW**	0.9	0.4
836–845	1225.62	**EVNLVSEHIW**	0.5	0.2
836–849	1719.77	**EVNLVSEHIW**CEDF	0	0.2
855–867	1593.86	YL**KNVQTQETRTL**	4.1	4.5
855–870	1970.03	YL**KNVQTQETRTLT**QF	0.5	0.5
856–867	1430.80	**LKNVQTQETRTL**	2.3	1.3
856–870	1806.97	**LKNVQTQETRTL**TQF	0	0.4
857–867	1317.71	**KNVQTQETRTL**	1.4	0.5
857–870	1693.89	**KNVQTQETRTL**TQF	0	0.4
871–887	1866.95	HFL**SWPAEGTPASTRPL**	0.5	0.4
873–887	1582.82	**LSWPAEGTPASTRPL**	0.5	7.5
873–888	1695.91	**LSWPAEGTPASTRPL**L	0	0.2
873–890	1958.00	**LSWPAEGTPASTRPL**LDF	0.5	0.2
874–887	1469.74	**SWPAEGTPASTRPL**	0	9.6
874–888	1582.82	**SWPAEGTPASTRPL**L	0	0.2
920–926	832.48	ILIDMVL	1.4	0.4
927–942	1729.96	NRMAKGVKEIDIAATL	14.5	8.7
943–952	1206.63	EHVRDQRPGL	1.8	2.9
943–961	2343.20	EHVRDQRPGLVRSKDQFEF	0.5	1.1
953–959	879.46	VRSKDQF	0	0.4
953–961	1155.58	VRSKDQFEF	0.5	1.5
960–974	1589.84	EFAL**TAVAEEVNAIL**	0.5	0.4
962–974	1313.73	AL**TAVAEEVNAIL**	20.0	8.2
964–974	1129.61	**TAVAEEVNAIL**	0.5	0.2

Peptides generated after chymotrypsin treatment of complexes of IA-2 with monoclonal antibodies to JM and central region epitopes. Purified recombinant IA-2 representing amino acids 605–979 were incubated with monoclonal IA-2 antibodies to either JM (5E3) or PTP (M13) domain epitopes cross-linked to protein A Sepharose and complexes treated with chymotrypsin (0.1 mg/ml) for 30 min. After washing, peptides remaining bound to beads were eluted with triethylamine buffer, pH 11.7, dried and analysed by LC-MS/MS. Groups of peptides were identified with common core sequence (bold text), and the representation of each peptide as a percentage of the total number of peptides detected are presented.

Conflict of interest

The author(s) declare that there are no conflicts of interest.

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