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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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KEYWORDS

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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.

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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 ³⁵S-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/ml BSA and 0.5%

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

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

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

184 2.4. Proteolytic digestion of IA-2-antibody complexes 185 (“antibody footprinting”)

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

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

207 The influence of monoclonal antibody specificity on
208 proteolytic processing of IA-2 was performed by incubating
209 protein G Sepharose-conjugated antibodies with 20,000 cpm
210 of ³⁵S-methionine-labelled IA-2ic and 10 µg of unlabelled
211 purified recombinant IA-2ic for 2 h at room temperature.
212 Non-bound IA-2 was removed by washing and complexes
213 incubated with trypsin (0.1 mg/ml) for times indicated in
214 the figure legend. Reactions were terminated by addition of
215 phenylmethanesulphonic acid (10 mM final concentration)
216 and non-bound proteolytic fragments removed by washing.

Bound fragments were eluted in 100 mM triethylamine, 217
eluates neutralised with 0.5 M NaH₂PO₄ and analysed by 218
SDS-PAGE and autoradiography. 219

For identification of the antibody-protected peptides by 220
mass spectrometry, bead-bound antibody-antigen complexes 221
were formed by incubating the immobilised antibody with 222
100 µg of purified IA-2 cytoplasmic domain protein for 2 h at 223
room temperature with slow rotation. Unbound antigen was 224
removed by washing with PBS and the complexes equilibrated in 225
chymotrypsin digestion buffer (100 mM Tris, 10 mM CaCl). 226
Activated chymotrypsin was added to the complex at an 227
enzyme:substrate ratio of 1:10 and incubated for 30 mins at 228
30 °C with occasional mixing. Unbound proteolytic fragments 229
were removed by washing with PBS and subsequently with 230
water. Antibody bound fragments were eluted in 100 mM 231
triethylamine pH 11.7. The eluates were vacuum dried and 232
stored at –20 °C prior to mass spectrometry analysis. 233

234 2.5. Mass spectrometry

Samples were analysed by LC-MS/MS on a ProteomeX machine 235
(Thermo Finnigan, Hemel Hempstead, UK). Dried chymotrypsin 236
digests were resuspended in 0.1% formic acid and chromatog- 237
raphy of aliquots of each sample performed on a 100- by 238
0.18-mm BioBasic C18 column (ThermoHypersil-Keystone, 239
Runcorn, UK). Peptides were eluted with aqueous acetonitrile 240
(5 to 65% over 30 min) containing 0.1% formic acid at a flow rate 241
of 2 µl per min. MS/MS data were acquired in data-dependent 242
mode with dynamic exclusion. Spectra were submitted against 243
the IA-2 sequence database using Bioworks v3.1/TurboSEQUEST 244
software (Thermo Electron, Langensfeld, Germany). Proteins 245
were considered to match entries in the database if XCorr 246
values for individual peptides were ≥1.5, ≥2, and ≥2.5 for 247
singly, doubly, and triply charged ions, respectively. 248

249 3. Results

250 3.1. Characterisation of epitopes for juxtamembrane 251 domain monoclonal antibodies

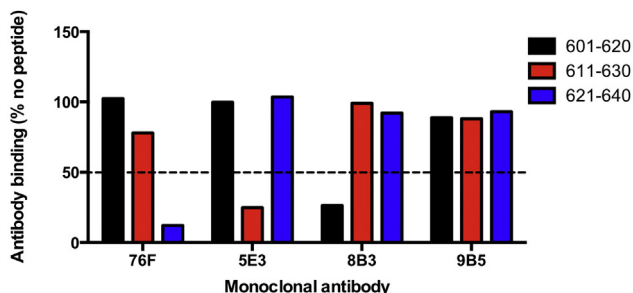
Epitopes for four mouse monoclonal antibodies to IA-2 have 252
been shown by competition studies to overlap with those for 253
autoantibodies in Type 1 diabetic patients' sera [27,28]. All 254
recognise epitopes within the JM domain of the protein. To 255
further define the epitopes for each of the four mouse 256
monoclonal antibodies, the influence of synthetic 20-mer 257
peptides on antibody binding to a chimeric protein representing 258
the 605–693 region of IA-2 fused to the PTP domain of PTP1B 259
was investigated. The four monoclonal antibodies to the JM 260
domain were inhibited differentially by synthetic peptides 261
within the 601–640 region of the protein (Fig. 1A). Binding of 262
antibody 76F was inhibited by the presence of the 621–640 IA-2 263
peptide, but not by peptides 601–620 or 611–630. Antibody 5E3 264
was inhibited only by the 611–630 peptide and 8B3 only by 265
601–620 (Fig. 1A). 9B5 showed no inhibition by any of the 266
peptides. 267

To identify amino acids on IA-2 that participate in antibody 268
binding, reactivity to IA-2 JM constructs with single amino 269
acid-substitutions were evaluated. The inhibitory effects of 270
substitutions of residues within the 626–629 region on binding of 271
the 76F antibody [18] were confirmed in this study. However, 272

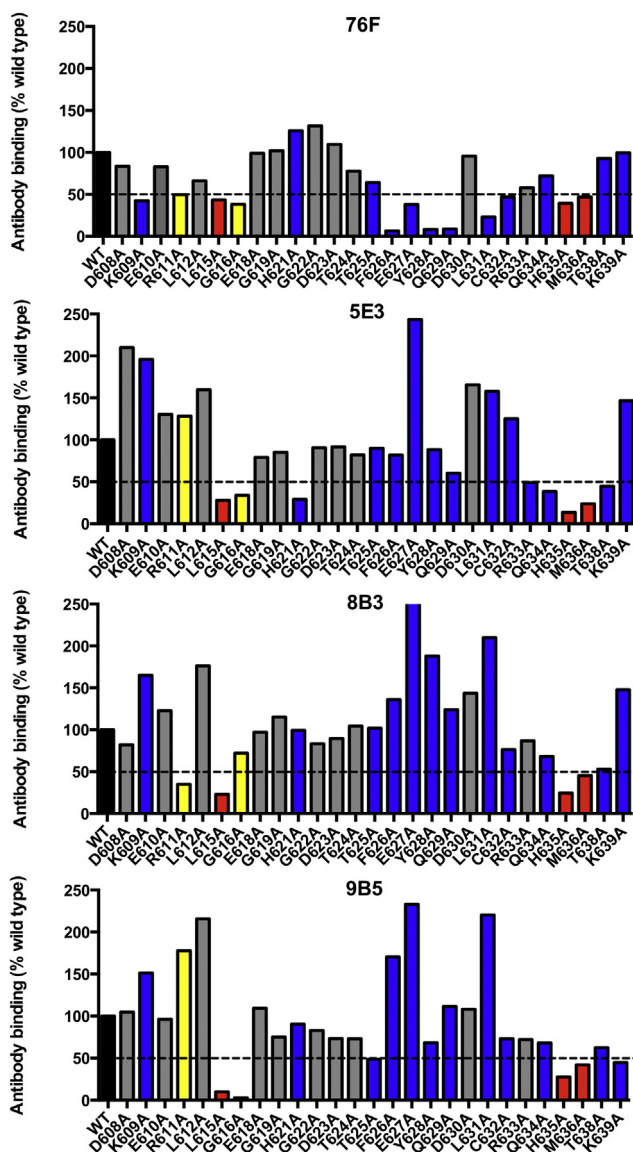
the epitope for this antibody was found to extend beyond this region, as indicated by inhibition by alanine substitutions of amino acids L631, G632, H635 and M636 and of several amino acid substitutions in the region 609–616 (Fig. 1B). Substitution of amino acids between 626 and 629 did not affect binding 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.

A. Peptide inhibition



B. Amino acid substitutions



3.2. Characterisation of epitopes for human auto-antibodies 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 domain (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.)

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

322 3.3. Inhibition of autoantibody binding to IA-2 by 323 Fab fragments of IA-2 monoclonal antibodies

324 To examine relationships between individual defined epitopes
325 in the JM and PTP domains of IA-2, the ability of Fab
326 fragments of PTP and JM domain-reactive monoclonal IA-2
327 antibodies to compete for binding with monoclonal or serum
328 antibodies to IA-2 was investigated. Fab fragments of the
329 PTP domain autoantibody M13 abolished binding to other
330 monoclonal antibodies recognising similar PTP domain epitopes,
331 but had no effect on IA-2 binding of the JM domain-reactive
332 antibody, 76F (Fig. 3A). The rabbit polyclonal antibody to IA-2
333 was also unaffected. Fab fragments of the JM domain antibodies
334 abolished (5E3) or partially inhibited (9B5) IA-2 binding of the
335 JM-reactive 76F antibody. However, Fab fragments of 5E3 and
336 8B3 JM antibodies also partially inhibited IA-2 binding of the
337 monoclonal antibodies M13, 96/3 and DS329 that are reactive to
338 the PTP domain epitope, and of the polyclonal rabbit IA-2
339 antibody. The results indicate that binding of Fab fragments of
340 antibodies to the JM domain are able to impair antibody binding
341 to epitopes within the PTP domain, possibly through steric
342 hindrance or conformational effects.

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

359 3.4. Characterisation of antibody epitopes by antibody 360 footprinting

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

371 Initial studies used SDS-PAGE and autoradiography to
372 characterise radiolabelled proteolytic products generated

373 after trypsin digestion of complexes of bead-conjugated
374 monoclonal antibodies with ³⁵S-methionine-labelled IA-2ic. 374
375 Time course studies demonstrated clear differences in the
376 dominant tryptic digestion products eluted from bead-
377 conjugated 5E3 (JM domain epitope) and M13 (PTP domain
378 epitope) antibodies, with predominant bands at Mr 3500 and
379 7000 for 5E3 and at Mr 11,000 and 23,000 for M13 (Fig. 4).
380 However, despite the differences in epitope recognition,
381 common bands were also eluted from both antibodies, in
382 particular, a trypsin product of 9000 Mr (Fig. 4). 382

383 To identify the regions protected by the JM and PTP
384 domain monoclonal antibodies, similar experiments were
385 performed using purified recombinant IA-2ic as antigen,
386 digesting antibody:IA-2ic complexes with chymotrypsin which,
387 being a more frequent cutter than trypsin, provides better
388 resolution of antibody-protected regions of the protein. 388
389 Chymotrypsin digestion products eluted from bead-conjugated
390 antibodies were identified by LC-MS/MS. A total of 39 distinct
391 peptides were identified in the eluates, and the percent
392 recovery of each of these peptides relative to the total number
393 of peptides identified is shown in Table 1. Several of the
394 peptides could be clustered according to the presence of a
395 common core sequence (bold font in Table 1) with varying
396 length extensions at the C- or N-terminus. Peptides containing
397 the core motif AALGPEGAHGTTF representing amino acids
398 613–626 of IA-2 were highly represented in eluates from the
399 JM epitope-reactive 5E3 antibody (21.4%), but almost absent
400 from the M13 eluates (0.2%; $p < 0.0001$, Fisher's exact test with
401 Bonferroni correction). These peptides include residues L615,
402 G616 and H621 that were identified as part of the 5E3 epitope in
403 the mutagenesis studies above. However, the majority of
404 peptides eluted from the 5E3 antibody were derived from the
405 PTP domain, with peptides containing the sequences SHTIADFW
406 (788–795, 21%), KNVQTQETRTL (857–867, 8.4%), TAVAEVNAIL
407 (964–974, 21%) and NRMAKGVKEIDIAATL (927–942, 14.5%)
408 being highly represented (Table 1). These latter peptides were
409 also detected in eluates from the PTP domain-reactive M13
410 antibody. Peptides with the core sequences INASPIIHDPRMPAY
411 (765–780, 32.7%) and SWPAEGTPASTRPL (874–887, 18.1%) were
412 detected in eluates from the M13 antibody, but found at low
413 abundance in eluates from 5E3 (2.7% and 1.5%, respectively;
414 $p < 0.0001$).

415 4. Discussion

416 Studies on the appearance of autoantibodies to islet
417 antigens in early life [2,5], together with assessment of the
418 risk of development of Type 1 diabetes by detection of single
419 and multiple islet autoantibody specificities [16,34], have
420 emphasised the importance of determinant spreading for
421 progression from autoimmunity to disease. A key role for
422 B-cells in promoting determinant spreading has been
423 demonstrated in animal models of autoimmune disease
424 [35], probably through alterations in uptake, processing
425 and presentation of relevant antigens. We now demonstrate
426 a close structural relationship between determinants in two
427 distinct domains of a major autoantigen in Type 1 diabetes that,
428 together with previous observations, point to an important role
429 for B-cells secreting antibodies to the JM domain of IA-2 in the
430 diversification of the immune response in human Type 1
431 diabetes. Thus: i.) antibodies to the JM domain appear early

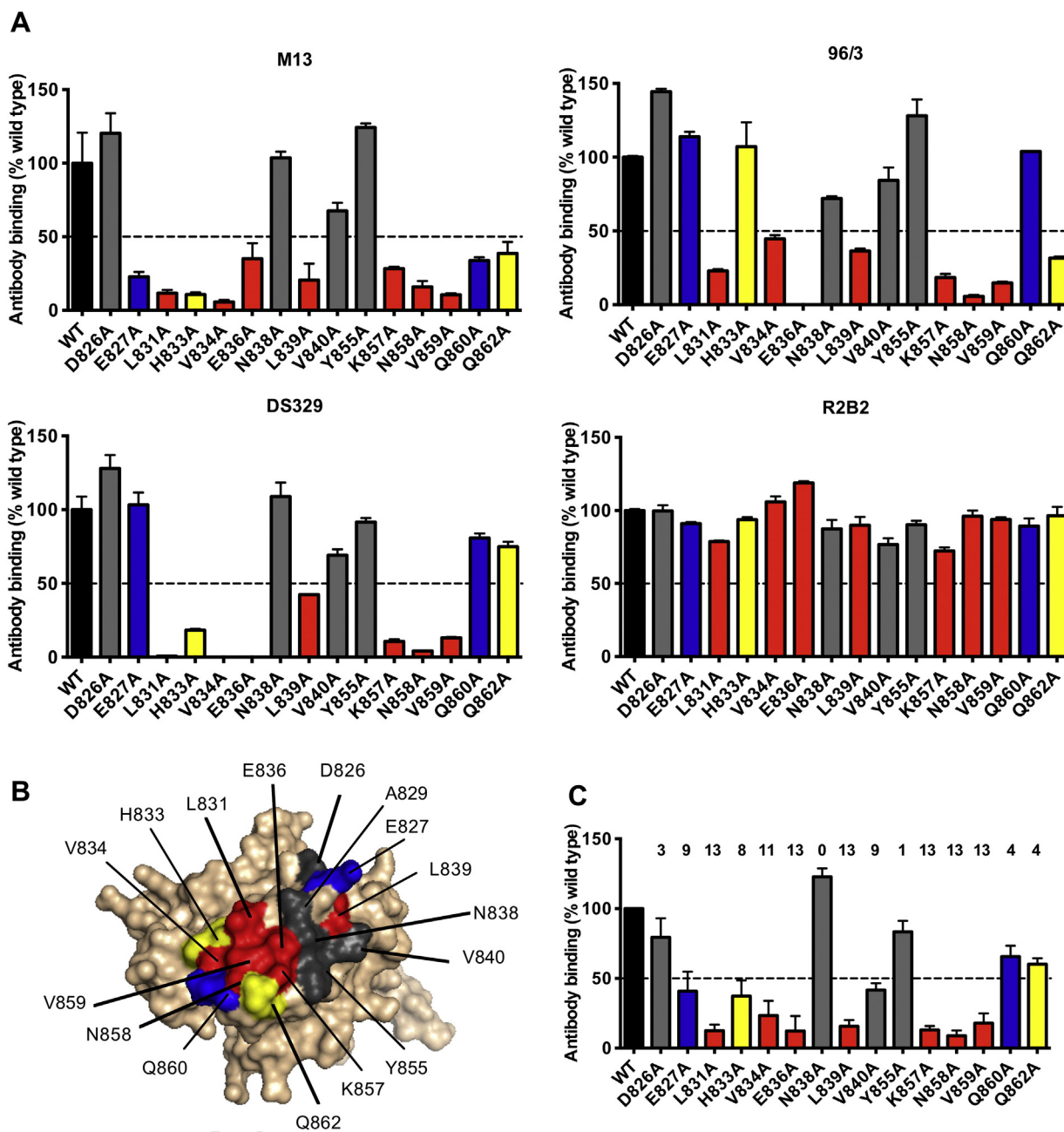


Figure 2 Influence of single amino acid substitutions on binding of human monoclonal antibodies and patients' sera to IA-2. **A:** Influence of single amino acid substitutions on binding of three human monoclonal antibodies or a rabbit polyclonal antiserum to a radiolabelled construct representing amino 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 \pm 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.)

432 in the IA-2 autoimmune response and precede spreading to
 433 epitopes in the IA-2 PTP domain and to the related autoantigen,
 434 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,

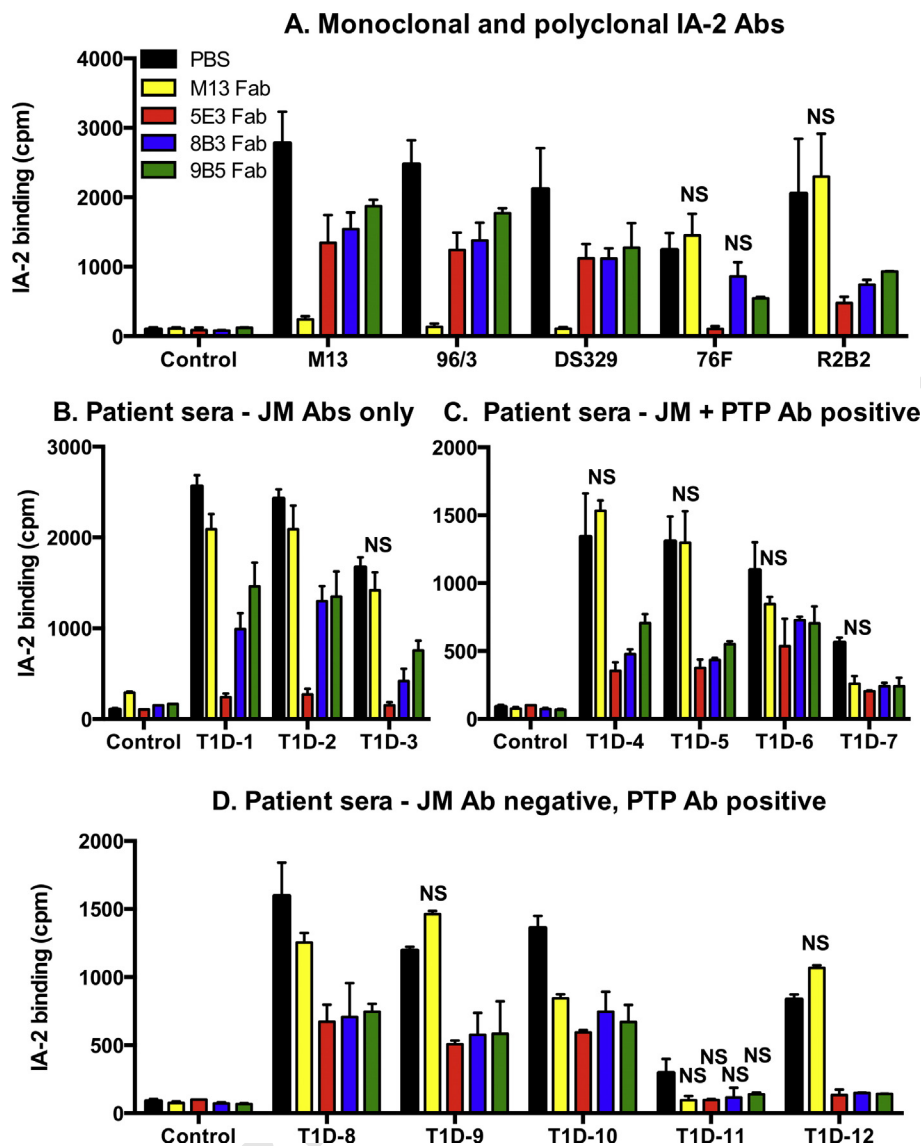


Figure 3 Inhibitory effects of Fab fragments of monoclonal IA-2 antibodies on binding of antibodies to IA-2. The ability of Fab fragments of monoclonal antibodies M13 (yellow bars), 5E3 (red bars), 8B3 (blue bars) or 9B5 (green bars) to compete for binding of monoclonal IA-2 antibodies 76F, M13, 96/3 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 Dunnett'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.)

438 monoclonal antibodies to the JM domain block binding of
 439 autoantibodies to the same PTP domain epitope, suggesting
 440 juxtaposition of the two epitopes (Fig. 3); and iv.) these JM
 441 domain antibodies protect and stabilise PTP domain peptides
 442 containing major T-cell determinants during proteolysis of
 443 antibody:antigen complexes (Fig. 4, Table 1). If similar
 444 antibody-mediated stabilisation of PTP domain peptides occurs
 445 within processing compartments of JM-specific B-cells, then
 446 presentation of those PTP domain peptides to T-cells would

be promoted, providing a mechanism underlying the associa- 447
 tion of JM antibodies with T-cell responses to PTP domain 448
 peptides in Type 1 diabetes [13] and for the spreading of 449
 autoimmunity from JM to PTP domain determinants as disease 450
 develops. 451

The study of determinant spreading at the B-cell level 452
 requires a detailed understanding of the structures of dominant 453
 autoantibody epitopes, most easily acquired through the 454
 study of cloned antibodies. Although human monoclonal 455

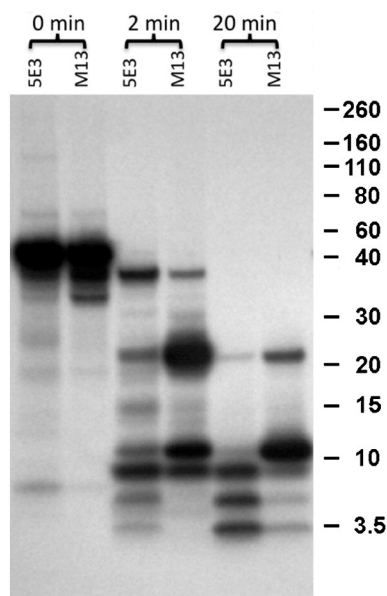


Figure 4 Radiolabelled fragments generated after trypsin treatment of complexes of ^{35}S -methionine-labelled IA-2 with monoclonal antibodies to JM and central PTP domain epitopes. ^{35}S -methionine-labelled IA-2 constructs representing amino acids 605–979 were incubated with monoclonal IA-2 antibodies 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.

456 autoantibodies to IA-2 JM domain epitopes from Type 1 diabetic
 457 patients have been reported [29], transformed B-cells secreting
 458 these JM autoantibodies were unstable and are no longer
 459 available for study (J Endl, personal communication). To our
 460 knowledge, the only IA-2-specific B-cell clones from diabetic
 461 patients that are currently available secrete antibodies to
 462 overlapping PTP domain epitopes within the region 827–862 [12
 463 and this study]. Analysis of amino acid substitutions affecting
 464 binding of three human monoclonal antibodies to the PTP
 465 domain suggest a core region of antibody binding represented
 466 by amino acids 831, 834, 836, 839, 857, 858 and 859, with
 467 individual B-cell clones showing different involvement of
 468 residues peripheral to this common core (Fig. 2B). Analysis of
 469 the effects of amino acid substitutions on binding of serum
 470 antibodies from individual Type 1 diabetic patients demon-
 471 strated that the pattern of reactivity to this region is typical of
 472 B-cell responses in Type 1 diabetes generally, consistent with it
 473 being a major target of autoantibody reactivity in disease. The
 474 protein footprint of the M13 human monoclonal PTP domain
 475 autoantibody included peptides with core regions 836–845 and
 476 857–867 which encompass the amino acids implicated in the
 477 autoantibody epitope (Table 1) and are included within major
 478 T-cell determinants [12,13,36]. However, the antibody also
 479 stabilised other PTP domain peptides extending beyond the

480 epitope, including those containing regions 765–780, 788–795,
 481 874–887 and 964–974 (Table 1). Peptides from the JM domain
 482 were rarely detected. Analysis of the crystal structure of the
 483 IA-2 PTP domain shows the 765–780 region to be buried in the
 484 molecule beneath the proposed epitope region [32]. The
 485 874–887 region includes peptides immediately adjacent to
 486 those harbouring the antibody epitope, but lies on the opposite
 487 face of the protein to the epitope region in the 3-dimensional
 488 structure [22,32]. The 874–887 motif includes the 876–880
 489 sequence of amino acids, substitutions of which have been
 490 shown previously to inhibit IA-2 autoantibody binding and that
 491 may form part of a distinct PTP domain epitope [22,37].

492 Although no monoclonal IA-2 JM domain autoantibodies
 493 derived from Type 1 diabetic patients are currently available
 494 for study, there is good evidence that antibodies cloned
 495 from IA-2-immunised mice show very similar JM epitope
 496 specificities to those appearing in the human disease [28,38].
 497 Studies to localise the epitopes of mouse monoclonal antibodies
 498 to the JM domain show that synthetic peptides known to inhibit
 499 serum antibodies from Type 1 diabetic patients (601–620,
 500 611–630 and 621–640 [18]) also inhibit binding of three of the
 501 mouse antibodies (Fig 1A). Site-directed mutagenesis indicated
 502 that amino acids 615, 635 and 636 represent key residues for
 503 antigen binding to all four monoclonal antibodies, with differing
 504 contributions of amino acids within the 608–638 region of IA-2
 505 to binding of individual antibodies. For the 76F antibody,
 506 substitutions affecting binding included amino acids 626–629
 507 which form part of the “JM2” and “JM3” epitopes described by
 508 the Bonifacio group [18,19] and, for 5E3, residue 621 which
 509 contributes to a “JM1” epitope [19]. Consistent with the
 510 mutagenesis data, the protein footprint of the 5E3 antibody
 511 included JM-localised peptides with a 613–626 core, that were
 512 poorly represented in the M13 footprint, strongly supporting this
 513 region as part of the 5E3 antibody epitope. However, peptides
 514 within the PTP domain containing regions 788–795, 857–867,
 515 927–942 and 964–974 were also highly represented in eluates
 516 from the 5E3 antibody, again indicative of antibody-mediated
 517 protection from proteolysis of peptides outside of the immedi-
 518 ate epitope region. Fab fragments of 5E3 and other JM domain
 519 antibodies were more effective than those of PTP domain
 520 antibodies at blocking binding of serum antibodies to epitopes in
 521 both the JM and PTP domain. These strong inhibitory effects of
 522 JM-targetted antibodies on binding of antibodies to the PTP
 523 domains is suggestive of close structural relationships between
 524 the two epitopes and juxtaposition of the two epitopes may
 525 explain the stabilisation of PTP-derived peptides by the JM
 526 domain antibody.

527 The results of this study point to close structural relation-
 528 ships between two major regions targetted by autoantibodies in
 529 Type 1 diabetes that may have implications for the diversifica-
 530 tion of IA-2 autoimmunity in Type 1 diabetes. Confirmation that
 531 these *in vitro* observations have pathophysiological relevance
 532 requires analyses of the influence of B-cell epitope specificity
 533 on peptides generated within cellular processing compart-
 534 ments. Our identification of antibody epitopes, and core regions
 535 of IA-2 protected by JM and PTP domain antibodies, will
 536 facilitate studies to fully understand the natural history of
 537 spreading of B- and T-cell responses to determinants during the
 538 early stages of IA-2 autoimmunity. Such studies would identify
 539 B- or T-cell responses to determinants most closely linked to
 540 disease progression that would represent effective targets for
 541 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	GPLGSMQQDKERLAALGPEGAHGDTTF	2.3	0
606–626	2532.18	GSMQQDKERLAALGPEGAHGDTTF	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	INASPIIEHDPMPAY	0.9	22.2
765–787	2504.30	INASPIIEHDPMPAYIATQGPL	1.8	10.5
781–795	1656.84	IATQGPLSHTIADFW	0.5	5.5
788–795	976.45	SHTIADFW	20.0	9.1
788–799	1520.70	SHTIADFWMVW	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	HVYEVNLVSEHIW	0.9	0.4
836–845	1225.62	EVNLVSEHIW	0.5	0.2
836–849	1719.77	EVNLVSEHIWCEDF	0	0.2
855–867	1593.86	YLKNVQTQETRTL	4.1	4.5
855–870	1970.03	YLKNVQTQETRTLQF	0.5	0.5
856–867	1430.80	LKNVQTQETRTL	2.3	1.3
856–870	1806.97	LKNVQTQETRTLQF	0	0.4
857–867	1317.71	KNVQTQETRTL	1.4	0.5
857–870	1693.89	KNVQTQETRTLQF	0	0.4
871–887	1866.95	HFLSWPAEGTPASTRPL	0.5	0.4
873–887	1582.82	LSWPAEGTPASTRPL	0.5	7.5
873–888	1695.91	LSWPAEGTPASTRPLL	0	0.2
873–890	1958.00	LSWPAEGTPASTRPLLDLF	0.5	0.2
874–887	1469.74	SWPAEGTPASTRPL	0	9.6
874–888	1582.82	SWPAEGTPASTRPLL	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	EHVRDQRPLVRSKDQFEF	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	EFALTAVAEEVNAIL	0.5	0.4
962–974	1313.73	ALTAVAEEVNAIL	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.

542 Conflict of interest

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

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