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MAPPING OF CONFORMATIONAL AUTOANTIBODY EPITOPES IN ZNT8

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Abstract

Background—Identification of the major humoral epitopes in ZnT8 will expand the range of biomarkers for human type 1 diabetes (T1D), and may provide clues to the mechanisms governing disease progression. Our initial studies suggested that most ZnT8-reactive sera recognize conformational epitopes in the final 100aa region of the molecule. Subsequently we identified residue 325 as a major determinant in 2 epitopes linked to a genetic polymorphism with high minor allele frequency (rs13266634). The goal of the current study was to extend this analysis to identify non-polymorphic epitopes in ZnT8.

Methods—Although the C-terminal domains of human and mouse ZnT8 are ~80% identical, the mouse probe is not precipitated by the majority of human T1D sera. Thus to identify key residues we systematically “humanized” the mouse probe at each position that differs and evaluated the probes in radio-immunoassays.

Results—As previously reported, only alteration of Q>R325 by itself showed any restoration of binding to human sera. However, when clusters of structurally adjacent variant residues were also changed an additional region of antigenicity was revealed that depended on residues R₃₃₂, E₃₃₃, K₃₃₆ and K₃₄₀. Using a panel of 112 sera from recent onset subjects tested with the hC325Q and m-R₃₂₅R₃₃₂E₃₃₃K₃₃₆K₃₄₀ probes, 39.3% of the subjects were ZnT8(Q)A⁺, of which 38.6% (17/44) also recognized the mouse probe.

Conclusions—We conclude that the mR-REKK probe identifies a third major epitope in ZnT8 that may add to the diagnostic utility of measuring autoantibodies to this molecule.

Keywords

Zinc transporter 8; Slc30A8; Autoantibodies; Type 1 Diabetes

Introduction

Autoantibodies are currently the most reliable biomarkers of type 1 diabetes (T1D) in humans (reviewed by [1, 2]). Recently we demonstrated that autoantibodies to zinc transporter 8 (ZnT8A) are present in the blood of approximately 60–80% of patients with T1D at the onset of clinical disease [3]. This result has since been replicated by ourselves and others in different populations [4–7] and it is now generally accepted that the measurement of ZnT8A enhances the predictive power of T1D autoantibody assays. Moreover, analysis of samples obtained prospectively from genetically at risk first-degree relatives followed longitudinally indicate that ZnT8A typically arise late in pre-diabetes, and that together with autoantibodies to IA-2 (IA2A), ZnT8A can identify a sub-population of rapidly progressing individuals who might be the participants of choice for future

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preventative trials [8]. For the other major autoantigens (insulin, GAD65, and IA-2) previous studies have indicated that autoantibodies to the different targets typically arise sequentially [9], and that the risk of progression to clinical disease correlates with the number of autoantibodies rather than individual titers [10]. In addition to the involvement of additional targets (inter-molecular spreading) progression of an immune response may also lead to targeting of new epitopes within the same antigen (intra-molecular spreading). This raises the question of whether or not individuals possessing autoantibodies to 2 distinct epitopes in a single antigen have equivalent risk to those with autoantibodies to single epitopes in 2 distinct proteins. In order to answer this question a knowledge of the major epitopes within each antigen is clearly required. Thus the goal of the current study was to further characterize the major humoral epitopes in ZnT8, with the ultimate aim of determining whether stratification on the basis of individual specificities may further improve the predictive power of the ZnT8A assay.

Research design and Methods

Sera

Sera were obtained after informed consent from patients, relatives and normal volunteers attending the Barbara Davis Center Clinic in accordance with institutionally approved protocols. A rabbit polyclonal serum raised to a recombinant human carboxy-terminal domain (C-term) that cross-reacts with the mouse protein was used as a positive control, and 8–16 sera from previously validated non-diabetic subjects used as negative controls [3, 11].

DNA mutagenesis

Site directed mutagenesis was performed by overlapping PCR using templates encoding human ZnT8 residues 268–369 and mouse ZnT8 residues 267–367 respectively. The products were ligated into the mammalian expression vector pCDNA3.1D-Topo (Invitrogen). The sequences of all inserts were confirmed prior to use.

Radio-immunoassays

RIAs were performed essentially as described previously [3]. In some experiments results are expressed as an index relative to the positive control [11]. Cut-off values were defined as the mean + 4SD of the negative controls for each probe.

Molecular modeling

Models of the C-term of ZnT8 based on the X-ray crystal structure of the bacterial transporter YjiP (PDB:2QFI) were generated using co-ordinates predicted by the PHYRE server (<http://www.sbg.bio.ic.ac.uk/phyre/>) [12].

Results and Discussion

Our initial studies of humoral autoimmunity to human ZnT8 suggested that the majority of ZnT8A⁺ sera react primarily with the ~100aa C-term, and that few if any of these responses could be blocked with linear peptides, suggesting that the epitopes are predominantly conformational [3]. Subsequently we identified 2 epitopes in this domain that were “restricted” by a non-synonymous SNP with high minor allele frequency (rs13266634), with approximately 30% of all ZnT8A⁺ individuals exclusively recognizing either the 325Arg or 325Trp probes respectively [11, 13]. These latter studies took advantage of our observation that despite being ~80% identical (Figure 1A), probes encoding the mouse C-term were not recognized by the majority of human ZnT8A⁺ sera, allowing us to use gain and loss of function mutants to begin to map these epitopes [13]. We therefore took a similar approach to begin to map the “unrestricted” epitopes in the human C-term.

Initially a series of chimeras between the human and mouse proteins fused at regions of high sequence conservation were analyzed. Substitution of the first 36 residues of the human protein with the equivalent domain from mouse ZnT8 had only a minimal effect on reactivity, suggesting that the 6 variant residues in this region do not contribute significantly to the major epitope(s). However, replacement of the next 17 residues abolished reactivity. This was surprising since there are only 2 variant residues in this region (Figure 1A) neither of which alone could restore activity to the mouse probe (data not shown). Although no crystal structure of the ZnT8 C term currently exists we and others have shown that it can be modeled by molecular threading based on the coordinates of the *E. coli* family member Yiiip [11, 14]. The results of this analysis are shown in Figure 1B. Interestingly, modeling of the m267-321/h322-369 probe using the same template revealed that the chimera was predicted to exhibit subtle differences in folding throughout the molecule, consistent with our conclusion that the majority of epitopes are conformational rather than linear in nature.

Our ongoing studies of epitopes in the PTP domain of IA-2 [15] have suggested that considerable insight can be gained by identifying patches of charged residues on the surface of the molecule. Intriguingly the structural modeling also predicted that several of the variant residues are clustered in manner that could form a potential epitope (Figure 1B). We therefore examined the effect of substituting these residues either singly or in combination. Initially we screened the various mutants using a single serum (BT) that exclusively recognizes unrestricted epitope(s) in ZnT8. As previously observed, no single substitution restored activity (Fig. 2A). However, introduction of the 4 charged residues R₃₃₂, E₃₃₃, K₃₃₆, and K₃₄₀, but not the related cluster V₃₂₉, R₃₃₂, E₃₃₃, and K₃₃₆, as a group completely restored binding. The generality of this result was confirmed by comparing the reactivity of a series of 112 sera from newly diabetic subjects to the human C term(325Q) and mouse R₃₂₅R₃₃₂E₃₃₃K₃₃₆K₃₄₀ probes (Fig. 2B). This showed that 39.3% of the subjects were ZnT8(Q)A⁺, of which 38.6% (17/44) also recognized the mouse probe. Thirteen of fifteen (86.7%) of the subjects whose sera precipitated the mouse probe but not hC(325Q), also recognized the hC(325R) probe (Figure 2C), suggesting that they recognized one of the “restricted” epitopes. We conclude that residues R₃₃₂, E₃₃₃, K₃₃₆, and K₃₄₀ contribute to a major epitope in the human ZnT8 C term, but that additional “unrestricted” epitopes also exist that can be identified on the basis of the differential behavior of the human C term(325Q) and mouse R₃₂₅R₃₃₂E₃₃₃K₃₃₆K₃₄₀ probes.

Although we expect that our results will be generally applicable, the relatively small sample size and unique demographics of our patient population suggests that additional work is required before such a conclusion is fully justified. We are therefore actively seeking to address this potential weakness in our study by recruiting additional subjects of more diverse backgrounds. Similarly, the utility of assays that combine the various probes on the stratification of diabetes risk is currently under investigation.

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Figure 1. Location of amino acids that potentially contribute to an “un-restricted” epitope in ZnT8

A. Alignment between the C terminal domains of mouse and human ZnT8. Variant residues are shown in red with the polymorphic residue 325 highlighted. B. Residues 268–357 of human ZnT8(R325) were modeled based on the X-ray crystal structure of *E. coli* Yip (PDB: 2QFI). Residues that vary between human and mouse are shown in red (contributing) or blue (non-contributing). Two conserved serines that might also contribute to the epitope are shown in orange

Figure 2. Analysis of “humanized” mouse ZnT8 probes

The C terminal domain of mouse ZnT8 was “humanized” by site directed mutagenesis of codons encoding selected variant residues (Figure 1A). **A.** RIAs were conducted with a representative “unrestricted” serum (BT) that precipitates all polymorphic variants of the human ZnT8 C-term. Numbers refer to the position of the residue in the human sequence. **B.** RIAs were conducted with 112 sera from recent onset subjects using the hCQ and mR-REKK probes. The dotted lines indicate the cut-offs for the two probes. **C.** RIAs were conducted with 112 sera from recent onset subjects using the hCR and mR-REKK probes. The dotted lines indicate the cut-offs for the two probes. Sera positive for mR-REKK but negative for hCQ are indicated by the red triangles.