

**C-terminal clustering of autoantibody and T cell determinants on the structure of GAD65 provide insights into the molecular basis of autoreactivity**

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**Running Title:** C-terminal clustering of epitopes on GAD65

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## ABSTRACT

*Objective:* To gain structural insights into the autoantigenic properties of GAD65 in type 1 diabetes we analyzed experimental epitope mapping data in the context of the recently determined crystal structures of GAD65 and GAD67, to allow “molecular positioning” of epitope sites for B- and T-cell reactivity.

*Research Design And Methods:* Data were assembled from analysis of reported effects of mutagenesis of GAD65 on its reactivity with a panel of 11 human monoclonal antibodies (mAbs), supplemented by use of recombinant Fab to cross-inhibit reactivity with GAD65 by radioimmunoprecipitation of the same mAbs.

*Results:* The C-terminal region on GAD65 was the major autoantigenic site. B-cell epitopes were distributed within two separate clusters around different faces of the C-terminal domain. Inclusion of epitope sites in the PLP- and N-terminal domains was attributed to the juxtaposition of all three domains in the crystal structure. Epitope preferences of different mAb to GAD65 aligned with different clinical expressions of type 1 diabetes. Epitopes for four of five known reactive T cell sequences restricted by HLA DRB1\*0401 were aligned to solvent exposed regions of the GAD65 structure and co-localized within the two B-cell epitope clusters. The continuous C-terminal epitope region of GAD65 was structurally highly flexible, so differing markedly from the equivalent region of GAD67.

*Conclusions:* Structural features could explain the differing antigenicity, and perhaps immunogenicity, of GAD65 versus GAD67. The proximity of B- and T-cell epitopes within the GAD65 structure suggests that antigen-antibody complexes may influence antigen processing by accessory cells and thereby T cell reactivity.

Type 1 diabetes is characterized by autoimmune reactivity to islet cell antigens that include the 65 kDa isoform of GAD (GAD65). Of the two isoforms of GAD, GAD67 and GAD65, only GAD65 is autoantigenic, in diseases that include particularly type 1 diabetes (1,2). Importantly, the specificity of antibodies for particular epitopes on GAD65 rather than their actual levels may be a better indicator of impending or actual destruction of islet  $\beta$ -cells. Thus in genetically prone individuals changes in the focus of autoantibody responses to epitopes of GAD65, i.e. intramolecular epitope shifts during the period of prediabetic insulinitis, herald the onset of overt type 1 diabetes (3).

B-lymphocyte/autoantibody epitopes on GAD65 engage conformational determinants that are widely distributed over the linear sequence of the three domains, N-terminal (amino acids 1-234), pyridoxal-phosphate (PLP)-binding (235-442), and C-terminal (443-585) domains (4). Presently unanswered questions include the precise location of epitope sites and critical binding residues for autoantibody recognition, the differing immune reactivity of the GAD65 versus the GAD67 isoform, and the failure of natural immune tolerance to GAD65 in the first place. These questions prompted the recent crystallographic structure determination of both GAD67 and GAD65 isoforms (5).

We have used the crystal structures for the “molecular positioning” of epitope sites for B- and T-cell reactivity by analysis of effects of mutagenesis, and data from cross-blocking of monoclonal antibody (mAb) reactivity by radioimmunoprecipitation (RIP) with GAD65, using recombinant Fab derived from various mAb to inhibit the reactivity of a panel of mAb. The findings delineate a major autoantigenic region on GAD65 at the C-terminal domain that structurally is highly flexible and in this respect differs markedly from the equivalent region in GAD67. Known

T-cell epitope sequences mostly could be localized to the same region on the structure of GAD65. These findings may explain the unique immunogenicity of GAD65 versus GAD67.

## METHODS

**Monoclonal antibodies to GAD65.** The panel of 11 human mAbs to GAD65 included M2, M3, M4, M5, M6, and DPA, DPB, DPC, DPD from two patients with type 1 diabetes (6,7), and b96.11 and b78 from a non-diabetic patient with autoimmune polyendocrine syndrome type 2 (APS-2) (8). Each of these patients shared the HLA DRB1\*0401 allele associated with a high risk for diabetes. None of the mAbs reacts with GAD67. Data were available from published studies (4, 9-11) on mutagenesis on the putative location of epitopes for these mAbs, shown in **Table 1**.

**Crystal structures of GAD65 and GAD67.** Details of the purification, crystallization and structural determination of human GAD65 and GAD67 have been published previously (5). We used for crystallization an N-terminally truncated form of each isoform (hereinafter referred to as GAD67 and GAD65) that lacked the first 89 and 83 residues respectively; the N-terminal truncation facilitated purification because this region is hydrophobic and highly susceptible to proteolysis, and did not affect enzymatic properties (5) nor reactivity with specific antisera (12-14). The proteins were expressed in *Saccharomyces cerevisiae* as fusions to a C-terminal hexahistidine tag, and purified from the cell lysate by immobilized metal affinity chromatography and size exclusion chromatography in the presence of glutamate and PLP.

**Location of epitopes on the structure of GAD65.** Analysis of structural differences between GAD65 and GAD67, and location of mutations shown to affect mAb binding, were performed with the program Pymol (15). For each mAb to GAD65, the locations of

mutations that either caused reduced reactivity by radioimmunoprecipitation (RIP), or did not affect reactivity, were mapped on the structure of dimeric GAD65; together these data were used to delineate likely epitope regions. Structural alignments were performed with the program MUSTANG (16).

***Inhibition of mAb binding to full length GAD65 using recombinant Fab.*** Tests for co-localization of epitopes by cross-competition were performed using recombinant Fab (rFab) from the mAb b96.11, b78, DPA, DPC, DPD, M2, M3, and M4, prepared as previously described (17). The mAbs were tested at half-maximal binding concentrations, and results shown are from at least two separate experiments. Results were expressed as percent inhibition of full length <sup>35</sup>S-GAD65 bound in the presence of rFab where percent inhibition = 100 – (counts per minute in the presence of mAb/counts per minute in the absence of mAb) x 100. Samples were analysed in triplicate; the average intra-assay coefficient of variation was 5%, and the maximum value was 9%. A negative control rFab D1.3 specific to an irrelevant antigen, hen egg white lysozyme, was included at 5 µg/ml in each assay, and the cut-off for specifying blocking was conservatively set at >20% inhibition.

## RESULTS

***Flexibility in the C-terminal domain distinguishes GAD65.*** The 2.3 Å crystal structures of GAD67 and GAD65 have been reported (5). Both isoforms adopt the same fold and formed obligate dimers from two monomeric units, each comprising the three domains, N-terminal, PLP-binding and C-terminal (**Figures 1A and B**). For each monomer, the N-terminal domain contained two parallel α-helices, helices 1 and 2, that were packed against the N-terminal and PLP-binding domains of the partner monomer. The PLP-binding domain contained nine α-

helices, 3-11, that were surrounded by a seven-stranded mainly parallel β-sheet that adopted the type I PLP-dependent transferase-like fold. The C-terminal domain contained three α-helices, 13-15, and a short buried 4-stranded anti-parallel β-sheet, s3C. This relative orientation of monomers within the dimer for both GAD65 and GAD67 was such that the domains from monomer A formed a contiguous surface patch with the domains of the partner monomer B (**Figure 1A**). In addition, in both cases, the two active sites of the enzyme, located in the center of the PLP-domain at the dimer interface, contained the cofactor PLP and the product GABA (**Figures 1A and 1B**), indicative of the active (holo-) state of the crystallized enzyme. Overall, of 41 non-conservative amino acid differences between the two isoforms, 32 were surface-exposed, and 9 were buried in the region structurally characterized. Differing surface-exposed residues between GAD67 and GAD65 were distributed over the entire structure of the molecule, with no obvious clustering apparent in any region (**Figure 1C**).

There are two key differences between the two structures that have enzymatic and potentially antigenic consequences. Firstly, for GAD67, each of the two active sites was substantially covered by a well-ordered catalytic loop comprising residues 432-443 contributed in *trans* from the partner monomer. Notably, for GAD65, this same catalytic loop, residues 422-433, was highly flexible and thus not visible in the electron density maps. This difference offers a structural rationalization for the contrasting enzymatic characteristics of the GAD isoforms, as well as insight into the requirement of two GAD isoforms in mammals (5). Secondly, the two molecules differ markedly in their flexibility, with GAD65 being the more flexible as indicated by the atomic temperature (B) factors (**Figure 1D**). This difference is particularly

pronounced in the C-terminal domain, in a surface exposed loop (residues 518-520) that was too flexible to be resolved in the crystal structure of GAD65, in marked contrast to the well-defined structure of the corresponding sequence for GAD67.

**Location of contact residues for human mAb on GAD65 based on mutagenesis.** The known sites of point mutations or GAD67/65 sequence exchanges (chimeric molecules) that affected the binding of any of the panel of mAb were mapped onto the structure of GAD65. The location of epitopes or epitope regions in the C-terminal domain, PLP-domain and the N-terminal domain are described below.

**C-terminal domain.** Mutated residues that substantially decreased reactivity with five mAb (M2, M3, M5, DPA and b78) were heavily clustered in the region of GAD65 that surrounded the C-terminal flexible loop, residues 518-520, that differs markedly from the equivalent region of GAD67. Contact residues for the binding of these five mAb were distributed over all four faces of the two autoantigenic C-terminal domains of dimeric GAD65, denoted as the  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  faces (**Figure 2A and 2B**), but two clusters of C-terminal contact residues *cluster 1* and *cluster 2* (ctc1 and ctc2), could be clearly discerned. These were localized predominantly to opposing faces,  $\alpha$  and  $\delta$ , of the C-terminal domain, and could be represented by the epitope regions of two exemplary mAb, M5 and M3 (**Figure 2A and 2B**). Mutated residues in ctc1 that affected binding of mAb M2, M5, and b78 were located in helix 14 (residues 522-540) and the adjacent C-terminal flexible loop in the  $\alpha$  and  $\beta$  faces of the C-terminus of one GAD65 monomer, and close to the catalytic loop of the partner monomer, and mutated residues that affected binding of M3 and DPA were localized to helices 13 and 15. (**Figure 2C and 2D**). Notably, neither point mutation of residues, nor insertions of GAD67 sequences, on the  $\alpha$

face had any effect on the binding of mAb reactive with ctc2 on the opposite  $\delta$  face of the C-terminal region, and vice versa (**Table 1**).

The reactivity of M5, the prototypic mAb for ctc1, depended strongly on a non-conserved surface-exposed residue V<sub>532</sub> located in the C-terminal end of helix 14 of GAD65 (**Figure 2C**) and the binding of M5 required an additional five residues, with two located in the N-terminus of helix 14 (S<sub>524</sub> and S<sub>527</sub>), two flanking the C-terminal unstructured loop (E<sub>517</sub>, E<sub>521</sub>), and one within this loop (E<sub>520</sub>) (**Figure 2C**). Combinations of these residues located in the most structural divergent region between GAD65 and GAD67 (**Figure 2C**) affected the binding of the other four mAb localised to ctc1 (**Table 1**). The important residues in ctc2 that engage mAb M3 and DPA, distributed particularly on the  $\delta$  face, lie close to the PLP-domain, but this epitope region is remote from the catalytic loop. Reactivity of mAb M3 and DPA, as judged by mutations, depended on residues of GAD65 located in helices 13 (N<sub>483</sub>) and 15 (H<sub>568</sub>), noting that mutation of N<sub>483</sub> ablated reactivity only with M3 whereas mutation of H<sub>568</sub> ablated reactivity with both mAb (**Figure 2D**).

**PLP domain.** Epitopes on GAD65 for mAb M4, M6, b96.11, and DPC have been mapped to the PLP-domain by use of GAD65/67 sequence exchanges (**Figure 2E,F**) but information on the exact location of contact sites is limited. However point mutations of GAD65 did decrease reactivity for each of these mAb. Epitope regions for both M4 and b96.11 were localized within residues 308-365, but the single point mutation shown to affect binding of M4 to GAD65 was that of K<sub>358</sub>, a residue that is surface exposed, but this of itself is insufficient data to define an epitope. For mAb b96.11, no single point mutation ablated reactivity, but several mutations that substantially reduced reactivity, including the PLP domain

sequence <sub>305</sub>DER<sub>307</sub> were adjacent to the C-terminal epitope region *ctc2*. Thus, contact residues for b96.11 would lie at the junction of the PLP- and C-terminal domains in the  $\delta/\beta$  face of *ctc2* (**Figure 2D**). For mAb M6, the epitope region was localized within residues 242-282; binding to GAD65 was not affected by mutation of GAD65 E<sub>264</sub>T, but mutation of the equivalent residue of GAD67 T<sub>273</sub>E restored 50% of the reactivity with GAD65 (4). Notably, E<sub>264</sub> lies on the interface of the dimer (**Figure 2E**), within the previously identified <sub>260</sub>PEVKEK<sub>265</sub> region of identity with the P2-C protein of Cocksackie B virus (18), and close to residues 271-285, a major T-cell epitope restricted by HLA DR\*0401 (19) (see below).

For mAb DPC, two distinct epitope regions were revealed by effects of sequence exchange, one involving residues 366-413, and one involving residues 134-242 overlapping the junction of the PLP- and N-terminal domains. Within the crystal structure of GAD65, residues 366-413 in monomer B are positioned directly adjacent to the N-terminal residues 134-242 in the partner monomer A (**Figure 2E**); both sequences contain structural and amino acid differences between GAD65 and GAD67. Also, for mAb DPC, an epitope site was defined by mutagenesis of residues P231 and S234 that are located on a well-defined loop in the PLP-domain that differs structurally from the equivalent region of GAD67 (**Figure 2E,F**). No mutagenesis data exist to identify the residues responsible for binding of mAb DPC within the N-terminal domain.

N-terminal domain. Epitope regions for two mAb, DPD and DPB, have been mapped exclusively to the N-terminal domain, but only by use of GAD65/67 sequence exchanges. For mAb DPD, the epitope region is within residues 96-173 that lie precisely adjacent to the C-terminal end of helix 14 of the same monomer, and include a structural difference between GAD65 and GAD67

created by a deletion of three amino acids in GAD65 (143<sub>Q</sub>---E144 vs 149<sub>EGMEG</sub>153) (**Figure 1C**). For mAb DPB the epitope region is located within residues 1-96 that are lacking in the crystal structure of GAD65.

**Competition by rFabs of mAb binding to GAD65.** Of rFab derived from mAb to GAD65, there are three (M3, DPA, b78) derived from mAb that reacted with epitope regions at the C-terminus, three (b96.11, DPC, M4) with the PLP-domain, and two (DPC, DPD) with the N-terminal domain. When these rFab were tested for their capacity to block the reactivity by RIP between GAD65 and each of the mAb (**Table 2**), each rFab (with one exception) strongly blocked the binding with GAD65 of the corresponding mAb. In general, the mAb that reacted with one or the other of the two distinct C-terminal epitope clusters, *ctc1* and *ctc2*, differed in susceptibility to cross-blocking by the rFab. For example, rFab from mAb M3 and DPA (*ctc2* epitopes,  $\delta$ -face) of the C-terminus cross-blocked, but rFab from mAb b78 (*ctc1* epitope,  $\alpha$ -face) did not. Also, the binding of mAb M3 was blocked by rFab from mAb b96.11, consistent with the localization of the epitope for mAb b96.11 in the  $\beta$  and  $\delta$  faces close to *ctc2*, although rFab from mAb M3 that localizes close to mAb b96.11 did not block binding to GAD65 of mAb b96.11. No other rFab tested blocked the binding of either M3 or DPA.

Cross-competition studies provided important evidence that other epitopes that could not be mapped to the C-terminal domain by mutagenesis did in fact lie close to either *ctc1* or *ctc2*. Thus two mAb that reacted with N-terminal epitopes, DPD and DPC, gave reactivity with GAD65 that was strongly blocked by rFab from mAb b78 that engages epitopes in *ctc1*. Indeed, with the exception of rFab from DPD, there was strong mutual cross-competition among all three mAb, suggesting that epitopes for each overlapped, and thus each could be considered as a

component of ctc1 (**Figure 2E,F**). Also consistent with the location of contact residues, the two mAb for which epitopes mapped to the PLP-domain, DPC and b96.11, were not cross-competitive, and the cross-competition that was demonstrable between mAb DPC and b78 (see above) indicates that the epitope for DPC lies close to ctc1 epitopes, whereas the epitope for b96.11 lies close to ctc2 epitopes. The single mAb tested by cross-competition for which an epitope could not be localized to either ctc1 or ctc2 was M4 that maps to the PLP-domain; rFab from M4 blocked the binding to GAD65 of both mAb DPC, DPD and b78 (ctc1) and also of mAb b96.11 (ctc2). The single amino acid K<sub>358</sub> that has been shown by mutagenesis to be required for the binding to GAD65 of M4 lies on the face of the GAD65 dimer between ctc1 and ctc2 (**Figure 2E**).

**T cell epitopes on GAD65.** To examine human T cell epitopes in the context of the crystal structure of GAD65, we selected five particular immunodominant T-cell epitopes of GAD65 identified in several studies (19-21), designated T1-T5, each restricted by HLA DRB1\*0401 (**Table 3**). The C-terminal domain contained three epitopes, T3 (residues 481-495), T4 (residues 511-525) and T5 (residues 551-585), the PLP-domain contained one, T2 (residues 270-286) and the N-terminal domain contained one, T1 (residues 116-130). Positioning of these on the crystal structure of dimeric GAD65, showed that T1, T3, T4, and T5 were closely associated (**Figure 3**). T5 comprised part of helix 15 and the buried C-terminal  $\beta$ -sheet s3C, with its surface-exposed component located between two other epitopes, T1 and T3, indicative of strong interactions with both regions (**Figure 3B,D**). T1 corresponds to helix 1 in the N-terminus, residues 116-130. T3 localizes at the C-terminal end of helix 13 and its adjacent loop, and also is spatially contiguous with the T4 epitope region (residues 511-525) that corresponds to the

highly flexible C-terminal loop for which structure is lacking. Taken together, there are four T cell epitopes that form a contiguous linear patch on the surface of the GAD dimer located within the two major B cell epitope regions, ctc1 and ctc2 and another, T2, with a different location (residues 271-285), close to the <sub>260</sub>PEVKEK<sub>265</sub> sequence of similarity with the Coxsackie B virus protein 2C (18) and mainly buried within the PLP domain.

## DISCUSSION

The analysis of crystal structures of both N-terminally truncated GAD65 and GAD67 has provided a molecular basis for epitope locations, assisted by data derived from several sources: binding assays using a unique panel of human mAb to GAD65; point and sequence exchange mutagenesis of GAD65; and cross-competition experiments with GAD65-specific monoclonal rFab. Notably, we identified a major region of antigenic activity for GAD65 predominantly centered on a flexible and structurally undefined region in the C-terminal domain that differs strikingly between GAD65 and GAD67, and including also conformationally contiguous parts of the PLP and N-terminal domains. Within this region, two independent clusters of B-cell epitopes, ctc1 and ctc2, were positioned on opposing faces of the C-terminal domain as judged by reactivity of mAbs to GAD65 with particular mutants of GAD65, and blocking studies using human rFab. For T cells, four of five previously recognized DRB1\*0401-restricted epitopes on GAD65 formed a contiguous surface-exposed patch between the two B-cell epitope clusters.

The division of the C-terminal epitope region of GAD65 into two epitope clusters, ctc1 and ctc2, on opposing faces ( $\alpha$ ,  $\delta$ ) of the molecule was based on cross-reactivity of mAbs, although boundaries between the two regions were not entirely distinct, since rFab from mAb b78 that engaged residues in ctc1 caused partial inhibition (63%) of the binding

to GAD65 of mAb b96.11 that engaged residues in ctc2, although not *vice versa*. One explanation would be that the epitopes partially overlap, with that for mAb b78 located on the  $\alpha$  and  $\beta$  face of the C-terminal domain, and that for mAb b96.11 located on the  $\delta$  and  $\beta$  faces. The mAb that engaged ctc1 in the present study included M2, M5, b78 and DPD, and published data indicate that additional human mAb (M7, M8, and M9) engage epitopes in ctc1, since these mAbs are cross-competitive, and also block binding of M2 and M5, but not binding of mAb M1 and M3 that engage residues in ctc2 (4). Previous studies have defined epitopes for anti-GAD65 simply according to their location in domains based on the linear sequence, N-terminal, PLP- or C-terminal. This is superseded by the structural data, since some epitopes hitherto assigned to the PLP-domain are either contiguous with, or even part of ctc1 e.g. mAb DPC, or of ctc2 e.g. mAb b96.11, and all mAb for which epitopes apparently include an N-terminal component such as mAb DPD, DPC, M8, M9, engage epitopes in ctc1.

Although different mAb from a single anti-GAD positive patient could be reactive with either ctc1 or ctc2 epitopes (**Table 1**), published data suggest that a response biased towards one or other of the two epitope regions aligns with differing clinical expressions of type 1 diabetes. Thus, anti-GAD65 reactive with ctc1, as defined by blocking by rFab from mAb b78, DPD and DPC, was associated with enzyme inhibitory antibodies, whether from patients with Stiff Person Syndrome (22), or a subset of ketosis-prone type 1 diabetes with a higher  $\beta$ -cell functional reserve and a more benign clinical course (23). Also, anti-GAD65 reactive with ctc1/N-terminal epitopes was associated with slowly progressive type 1 diabetes, i.e. latent autoimmune diabetes of adults (LADA) (24,25). In contrast, anti-GAD65 reactive with ctc2 epitopes as judged by inhibition by

rFab from mAbs b96.11 and DPA was associated with high risk HLA-DQ alleles and rapidly progressive diabetes. (3). Thus our data now structurally establish the epitope preference of autoantibodies to GAD65 that may dictate differing clinical expressions of type 1 diabetes.

The identification of an immunodominant B-cell epitope region in the C-terminal region of GAD65 conforms with known features of the normal antibody response. Immunodominant regions of antigens comprise sites on the antigenic molecule that are strongly recognized by most immunized individuals although there are differences among individuals that reflect the summated reactivity of multiple B cell clones that contribute to the response. Affinity maturation of an on-going antibody response is associated with both "epitope focusing" to provide for selection of higher affinity antibodies, and also "epitope spreading" to enable the immune response to encompass wider regions of the antigenic molecule. In type 1 diabetes specifically, there is evidence that the autoantibody response may first involve the C-terminal domain, with later spreading to the PLP- and N-terminal domains (3,26).

Notably, 4 of 5 major T-cell epitopes restricted by the high risk HLA allele DRB1\*0401 could be localized on the structure of GAD65 within the same region as the immunodominant B-cell epitope regions ctc1 and ctc2, and the fifth epitope was close to E264 that defines the M6 epitope in the PLP-domain. Moreover, as CD8<sup>+</sup> T cells, restricted by HLA class I appear to be more directly relevant to the effector stage of pathogenesis, it is of interest that the Class II CD4<sup>+</sup> T cell epitope (T1, residues 115-130) overlaps a major Class I CD8<sup>+</sup> T cell epitope (residues 113-124) restricted by HLA A2 (27). The finding that T-cell epitopes often occur close to B-cell epitopes, for both extrinsic and autoantigens (28), directs

attention to the role of B-cells in modulating T-cell responses (29-33). In antigen-presenting cells, antigen-antibody complexes can remain intact after the internalization and fragmentation by proteases along the antigen-processing pathway (32,33), thus protecting residues from proteolysis and modulating presentation of peptides and ensuing T-cell responses. Studies on B-cell modulation of the T cell response to GAD65 indicate that B-cell clones producing mAb DPA and DPD, (but not DPC), can present the T1 (residues 115-130), T2 (residues 270-283) and T5 (residues 556-575) peptide epitope sequences to DRB1\*0401-restricted T-cell clones (30).

An enduring question for autoimmunity is why only a limited proportion, say 2-3% (35), of all human proteins become selected as autoantigens and why only limited sites on such molecules serve as autoepitopes. Among possible explanations structural characteristics are cited (35), albeit in the absence of knowledge of the actual protein structure of most autoantigenic molecules. This can now be examined in the case of GAD in the light of the contrasting antigenicity of the two isomers, GAD65 and GAD67, together with the substantial information derived from mutants of GAD65 based on sequence exchanges or single point mutations, and the reactivity of such mutants with human mAbs exclusively to GAD65. We have already commented on distinctive features of epitope distribution on GAD65, notably the clear association of epitopes with regions of increased flexibility of protein structure of GAD65 versus GAD67 (5). Structural features associated with antigenicity for other proteins include high flexibility, loops and protrusion from the antigen surface, and charged amino acids (36-39), all features of

the C-terminal domain of GAD65. Comparably, structural flexibility was described for the immunodominant epitope of the human autoantigen thyroid peroxidase (40), although not for epitope regions on the crystal structure of the type 1 diabetes autoantigen IA-2 (41). Finally, if the engagement of epitopes of ctc2 were to be predictive of progression of individuals at risk to overt type 1 diabetes, knowledge of the clustering of epitopes for anti-GAD65 at the C-terminus and the existence of two separate C-terminal epitope clusters, could allow the development of assay systems to exploit this. Also, if GAD65-based immunotherapies were to appear promising in retarding progression in at risk individuals to overt type 1 diabetes, consideration could be given to vaccine constructs based on epitopes associated with disease progression.

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**TABLE 1.** Panel of 11 human mAb to GAD65 derived from two patients (i, ii) with type 1 diabetes and one with autoimmune polyendocrine syndrome type 2 (APS-2) with corresponding epitope data.

<b>mAb</b>	<b>Source†</b>	<b>Epitope region‡</b>	<b>Mutants of GAD65 that affect reactivity</b>
<b>C-terminus</b>			
M3	Type 1 diabetes i	483-499, 556-585	N483A, H568Q
DPA	Type 1 diabetes ii	483-499, 556-585	H568Q
M2	Type 1 diabetes i	514-528***	E517P, E520P
M5	Type 1 diabetes i	512-540	E517P, E520P, E521Q, S524E, S527H, V532K
b78	APS-2	532-540, 514-528***	V532K, S524A/R525A/L526A*
<b>N-terminus</b>			
DPD	Type 1 diabetes ii	96-173	No data
DPB	Type 1 diabetes ii	1-102	No data
<b>PLP-domain</b>			
b96.11	APS-2	308-365	R357A/K376A**, F344A** D305A/E306A/R307A** K498A/P499A/Q500A**
M4	Type 1 diabetes i	308-365	K358N
M6	Type 1 diabetes i	242-282	E264§
<b>PLP and N-terminus</b>			
DPC	Type 1 diabetes ii	134-242, 366-413	P231S/S234D

† Derivation of mAbs were, for type 1 diabetes i, Richter *et al* (6); type 1 diabetes ii Madec *et al* (7); APS-2, Tremble *et al* (8)

‡ From data on epitope regions on linear sequence of GAD65, and GAD65 mutants according to Schwartz *et al.* (4),

\* O'Connor *et al.*(10), \*\* Fenalti *et al.*(11), \*\*\* Powers *et al.*(9).

§ Binding not affected by mutation of GAD65 E264T, but mutation of GAD67 T273E conferred 50% reactivity (4)

**TABLE 2.** Cross-competition of binding of human mAb to GAD65 by rFab, data are shown as percent blocking of binding by rFab at the highest concentration of rFab used

<b>mAb</b>	<b>M3</b>	<b>DPA</b>	<b>b78</b>	<b>DPD</b>	<b>DPC</b>	<b>b96.11</b>	<b>M4</b>
<b>Km</b>	†	†	<b>1 x 10<sup>-10</sup></b>	<b>0.8 x10<sup>-8</sup></b>	<b>4.7 x10<sup>-9</sup></b>	<b>3.4 x10<sup>-9</sup></b>	
<b>rFab</b>							
<b>M3</b>	<b>47</b>	<b>75</b>	19	36	nd	13	nd
<b>DPA</b>	<b>45</b>	<b>65</b>	0	0	8	15	4
<b>b78</b>	20	0	<b>84</b>	<b>75</b>	<b>77</b>	<b>63</b>	24
<b>DPD</b> ‡	23	24	<b>49</b>	21	32	nd	0
<b>DPC</b>	8	0	<b>71</b>	<b>85</b>	<b>92</b>	23	0
<b>b96.11</b>	<b>64</b>	17	17	0	36	<b>93</b>	nt
<b>M4</b>	0	36	<b>76</b>	<b>66</b>	<b>75</b>	<b>86</b>	<b>73</b>

† Not determined

‡ rFab from DPD blocked GAD65 binding of mAb DPD by only 21%, and did not block the reactivity of several other mAb from which the corresponding rFab strongly blocked the binding of DPD with GAD65

**TABLE 3.** Reported antigenic T-cell peptides on GAD65 restricted by HLA DR\*0401

Epitope	Position	T cell epitopes†	Reactive peptides
T1	116-130	<b>MNILLQYVV</b> KSFDRS VD---N--R-T----	115-127‡ 116-130§
T2	271-286	<b>PRLIAFTSEH</b> SHFSL -K-VL----Q--Y-L	274-286‡ 266-280§ 271-285§ 276-290§
T3	481-495	<b>LYNIIKNREG</b> YEMVF --AK-----EF----	481-495§
T4	511-525	<b>PSRLTLEDNE</b> ERMSR Q---GVP-SPQ-REK	511-525§
T5	551-585	<b>GDKVNF</b> <b>FRMVIS</b> <b>NPAA</b> ---A---N----- THQDIDFLIEEIERLGQDL --QS-----	551-565§ 556-570§ 566-580§ 553-572   555-567‡,    569-585   554-570‡

† Sequence of T cell epitopes in GAD65 and below the corresponding sequence of GAD67 showing differences; boldface sequences mark reported core immunogenic sequences, alternate immunogenic sequences are underlined.

‡ Wicker LS et al (22),

§ Patel SD et al (20)

|| Nepom GT et al (21)

## FIGURE LEGENDS

**Figure 1. A.** The crystal structure of dimeric GAD65 showing assembly of N-terminal, PLP- and C-terminal domains of monomers A and B. The catalytic loop (red lines, residues 431-441) and the C-terminal loop (red dotted lines, residues 518-520) are highly mobile and thus were not visible in the structure, and the cofactor PLP and the product GABA are shown in red and yellow spheres respectively. **B.** The monomeric structure of GAD65 within the asymmetric unit of the crystal with secondary structure elements of GAD65 monomer B labeled accordingly. Also shown are the  $\alpha$ -helices in the N-terminal, PLP and C-terminal domains colored according to 1A, the PLP and C-terminal  $\beta$ -sheets colored in red and yellow respectively, the catalytic loop and the C-terminal mobile loops represented by red solid and dotted lines respectively, and the  ${}_{260}\text{PEVKEK}_{265}$  region of sequence identity with P2C of Coxsackie B virus. **C.** Molecular surface of GAD65 dimer colored according to sequence conservation between GAD67 and GAD65, with green representing conserved residue and wheat non-conserved residues. Mutations that decreased mAb binding are outlined (black dotted lines), and a box indicates the location of a three amino acid deletion in GAD65 (see Results). **D.** Molecular surfaces of GAD65 and GAD67 colored according to flexibility/mobility as measured by atomic temperature (B) factors. The flexibility of the GAD isoforms is represented as a gradient, blue (low, ordered) to red (high, mobile). Residues 518-520 that are too mobile to be visualized in the crystal structure are represented as red dotted lines. GAD65 residues 434-437 (red) are a flexible sequence flanking the C-terminal end of the unstructured catalytic loop.

**Figure 2. A, B.** Views of the surface structure of dimeric GAD65 with monomers A and B in dark blue and cyan respectively showing two C-terminal clusters of epitopes (ctc1, ctc2) on opposing faces of the C-terminal domain. Epitopes for mAb M5 and M3 represent ctc1 and ctc2 respectively. In B the molecule is rotated  $180^\circ$  along the vertical -axis, and different faces of the C-terminal domain are shown as  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$  faces. The M5 epitope resides primarily on the  $\alpha$  and  $\beta$  faces and the M3 epitope on the  $\delta$  and  $\gamma$  faces, with broad epitope locations mapped using GAD67/65 sequence exchanges shown in yellow and single contact sites for mAb binding in red. The disordered catalytic loop and the C-terminal flexible loop are represented by red dotted lines

**C,D** Superposition of the GAD65 (dark blue) and GAD67 (green) C-terminal domains in the ctc1 and ctc2 regions, with broad epitope locations mapped using GAD67/65 sequence exchanges (514-528, and 532-540) in yellow, single contact sites for mAb binding shown as red sticks, and corresponding side chains of residues in the non-antigenic structure of GAD67 as green sticks. Notably, residues critical for binding of mAb M8 and M9 (R536 and Y540) in  $\alpha$ -helix 14 (C), identified by point mutations (9), are contiguous to N-terminal domain residues (131-140) that also are required for binding of mAb M8 and M9 according to epitope mapping data using GAD67/65 chimeras (residues 96-242 and 532-540 in the N- and C-terminus of GAD65 respectively) (9).

**E.F.** Views of the surface of GAD65 showing epitope regions within the PLP-domain mapped using GAD67/65 sequence exchanges (yellow) and contact sites for mAbs by point mutations (red). **E.** The epitope recognized by mAb DPC lies within the PLP-domain of monomer B and the N-terminal domain of monomer A. Single contact sites for two other mAb, M6 and M4, that can be mapped to the PLP-domain are shown, with evident proximity of E<sub>264</sub> to the catalytic loop (solid red line). **F.** The epitope region of mAb DPD lies within the N-terminal domain, residues 96-173, as mapped using chimeric GAD67/65 molecules but with no point

mutation data available. Epitope regions for mAb DPC and DPD overlap in the N-terminus of GAD65, and lie close to residues in the C-terminal domain that affect binding of mAb b78, consistent with the strong cross-inhibition obtained using the three rFab.

**Figure 3. A-D.** Sites of established of autoantibody (**A**) and T-cell determinants (**B-D**) on the structure of dimeric GAD65. **A.** Three views of the surface structure of GAD65 showing B cell epitope regions ctc1 and ctc2, and single contact sites in the C-terminus for mAb binding in red, and site in the PLP-domain in yellow. **B.** Three views of the surface structure of GAD65 showing T cell epitope sequences in orange (T1), green (T3) red (T4) and yellow (T5). These T cell sequences are mostly surface exposed, and form a contiguous patch on the surface of GAD65 in the same region as most B-cell epitopes.

**C.** A cartoon representation of the GAD65 structure showing the single T cell epitope sequence (T2) in the PLP-domain located on the dimer interface, and not surface exposed, but close to E<sub>264</sub> that is a component of the epitope for mAb M6.

**D.** Cartoon representation of the structure of GAD65 showing T-cell epitope sequences T1, T3, T4, T5

FIGURE 1

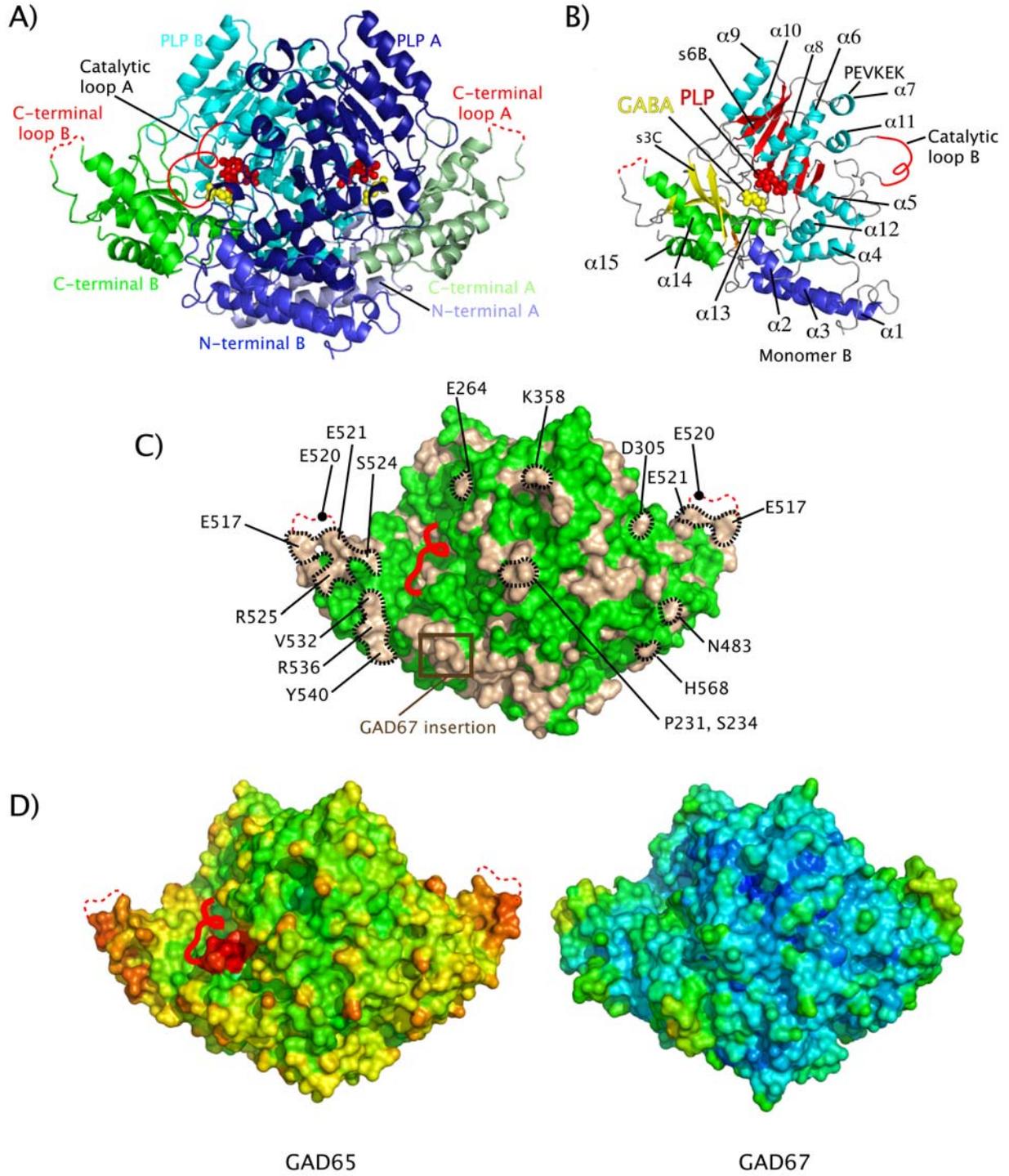
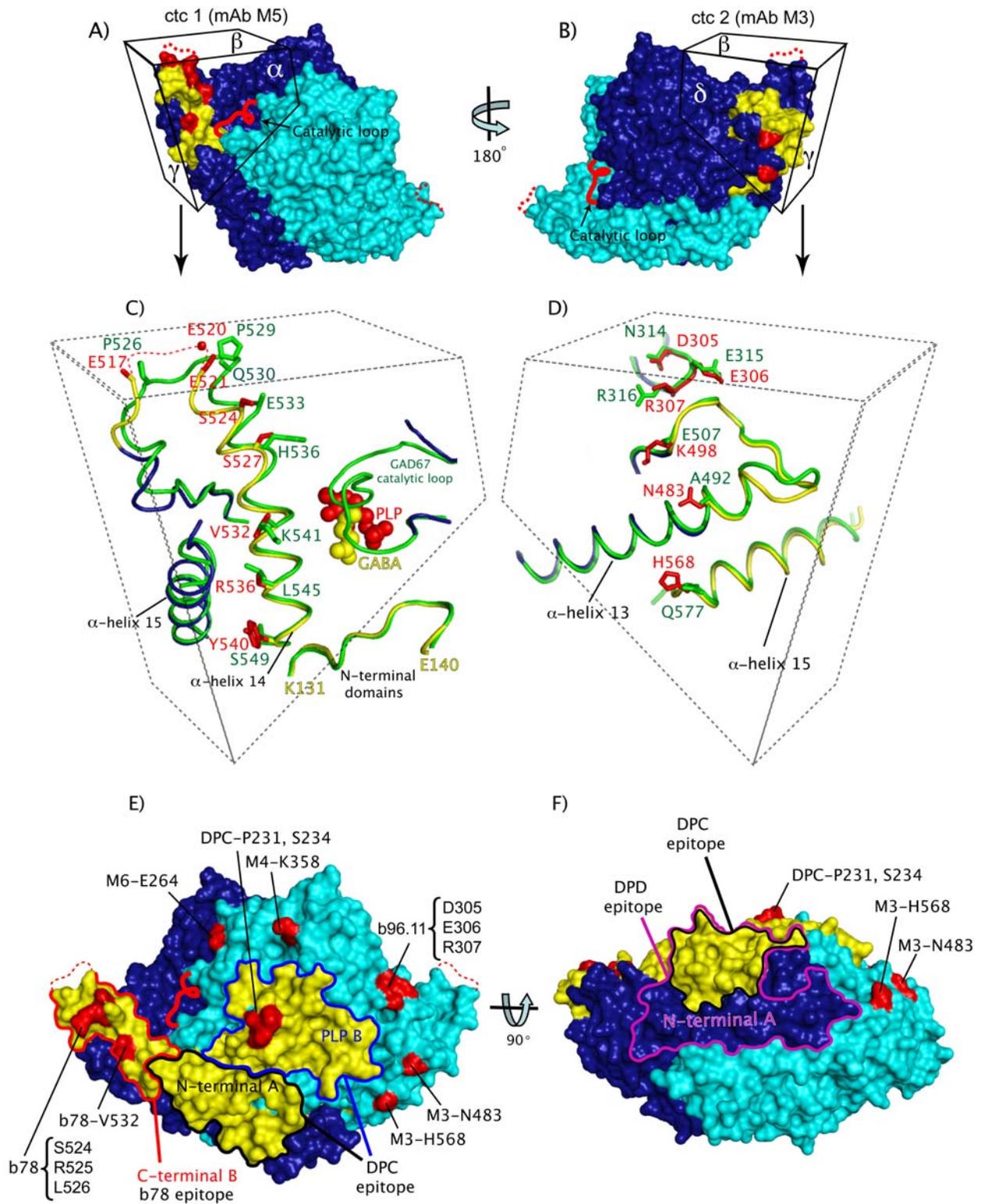


FIGURE 2



**FIGURE 3**

