Two amino acids in glutamic acid decarboxylase act in concert for maintenance of conformational determinants recognised by Type I diabetic autoantibodies

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Abstract

Aims/hypothesis. Glutamic acid decarboxylase 65 is a major autoantigen in Type I (insulin-dependent) diabetes mellitus, autoimmune polyendocrine syndrome and stiff-man syndrome. These disorders are characterised by the presence of multiple autoantibodies to the autoantigen which can be distinguished in a variety of different ways. We have investigated the role of single amino-acid mutations in glutamic acid decarboxylase 65 in distinguishing the binding of serum antibodies and a variety of patient-derived human IgG monoclonal antibodies directed to different determinants of the autoantigen.

Methods. We identified a mutant of glutamic acid decarboxylase 65 that contained four single amino-acid mutations from the wild-type molecule. The role of these mutations was investigated by site-directed mutagenesis. We investigated the binding of patient-derived serum antibodies to glutamic acid decarboxylase 65 to a number of single and double amino-acid mutants using immunoprecipitation with labelled, recombinant antigen. To overcome the heterogeneity of different anti-glutamic acid decarboxylase 65 antibodies present in a patient's serum, the binding of a panel of eleven patient-derived human monoclonal antibodies recognising different determinants on the autoantigen was also studied.

Results. Two replacements in glutamic acid decarboxylase 65 at Asn247Ser and Leu574Pro were identified that preferentially influence the anti-glutamic acid decarboxylase 65 serum antibodies of Type I diabetic patients, without statistically significantly effecting

those recognised in other disorders. Single or double amino-acid replacements Asn247Ser and Leu574Pro in the autoantigen showed differential effects on expression of epitopes recognised by the human monoclonals. The double replacement of Asn247Ser and Leu574Pro in glutamic acid decarboxylase 65 resulted in the loss of binding of all eleven human monoclonal antibodies, irrespective of their epitope recognition. In contrast, single replacement of Leu574Pro statistically significantly reduced the binding of some carboxyl terminal-directed antibodies such as MICA 1, MICA 3 and DP-A without influencing the binding of other monoclonals. Replacement of Asn247Ser did not, however, influence the binding of any patients' serum or human monoclonal antibodies.

Conclusion/interpretation. Two distantly spaced amino acids, Asn247 and Leu574 in glutamic acid decarboxylase 65 were identified that act in concert to greatly influence the conformational structure of the autoantigen and statistically significantly influence the binding of antibodies present in Type I diabetic sera. The single or double amino-acid mutants can be used to distinguish some anti-glutamic acid decarboxylase-65 autoantibodies and could prove useful in distinguishing Type I diabetic from autoimmune polyendocrine syndrome and stiff-man syndrome patients' sera as well as to study changes in antibody patterns during disease progression. [Diabetologia (2000) 43: 881–889]

Keywords Glutamic acid decarboxylase 65, autoimmune polyendocrine syndrome, stiff-man syndrome, human monoclonal antibodies, mutants.

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Abbreviations: APS, Autoimmune polyendocrine syndrome; hmABs, human monoclonal antibodies; mutation A, Trp9Arg; mutation B, Asn247Ser; mutation C, Tyr425Asn; mutation D, Leu574Pro; SMS, stiff-man syndrome.

The enzyme glutamic acid decarboxylase (GAD) is responsible for the synthesis of the major inhibitory neurotransmitter, γ -aminobutyric acid (GABA) [1]. Two related isomers GAD₆₅ and GAD₆₇, encoded by non-allelic genes and sharing an identical exon-intron structure have been described [2–4]. The GAD isoforms are expressed predominantly in the GABA-ergic neurons of the central nervous system and in pancreatic islet beta cells, although there are species differences in their expression in the islet beta cells [1, 5]. At the protein level, GAD₆₅ (of 585 residues) and GAD₆₇ (of 594) residues are highly dissimilar in the amino terminal 100 residues with a 25% homology whereas the remainder of the molecule shows around 75% homology [2, 3].

Glutamic acid decarboxylase is also a major target in autoimmunity, with autoantibodies being present in new-onset Type I (insulin-dependent) diabetes mellitus, autoimmune polyendocrine syndrome (APS) and stiff-man syndrome (SMS) patients, where they serve as important markers of disease activity [6–14]. Autoantibodies to GAD in new-onset Type I diabetic patients are specifically directed to the GAD₆₅; a small proportion of antibodies to the larger isoform GAD₆₇ are also present but these can be blocked with GAD₆₅ indicating there are shared determinants between the molecules [15, 16]. Autoantibodies to GAD₆₅ are present in 70–80% of new-onset Type I diabetic patients but their titre is considerably lower than those in APS and SMS patients [17–20]. There are also other differences between the anti-GAD₆₅ antibodies present in the sera from new-onset Type I diabetic patients and that from patients with APS and SMS. For example, anti-GAD₆₅ antibodies from Type I diabetic patients generally do not react with linear peptide sequences or with denatured antigen and thus are dependent on binding to discontinuous determinants brought together in the three-dimensional folding of the protein [6, 18–24]. Furthermore, antibodies present in APS and SMS sera inhibit the enzymatic activity of GAD₆₅ but the antibodies in Type I diabetic sera do not show this property [25, 26].

The determinants recognised by the anti-GAD₆₅ antibodies present in new-onset Type I diabetic patients have been delineated using chimeras of GAD₆₅ and GAD₆₇ [16, 21, 22]. The antibodies target epitopes in the middle region (residues 240 to 435) and the carboxyl terminal region (residues 451 to 570) of GAD₆₅, termed IDDM-E1 and IDDM-E2, respectively [21, 22]. These and other studies using human monoclonal antibodies (hmABs) to GAD₆₅ from patients indicate that the epitope recognition of GAD₆₅ in new-onset Type I diabetic patients is diverse, with recognition of multiple determinants on the molecule [27–32]. Although the structure of GAD is not known, in a recent study a model was built of the middle (residues 200 to 460) and the carboxyl region (residues 461 to 585) of GAD₆₅ based

on the three-dimensional structures of related decarboxylases and the autoreactive determinants recognised by the hmABs were fine mapped [33]. The model shows that the middle and carboxyl regions form two very separate folding domains where virtually the entire surface of GAD₆₅ is targeted by the autoimmune response [33]. In this study we report on single amino-acid mutagenesis analysis of GAD₆₅ to define the determinants recognised by hmABs to GAD₆₅. The results identify two amino-acid replacements in GAD₆₅ that seem to act together in a concerted manner to ablate the binding of a variety of hmABs directed to different determinants of the molecule.

Materials and methods

Patients' sera and human IgG monoclonal antibodies to GAD₆₅. Sera from new-onset Type I diabetic, APS and SMS patients were obtained after informed consent of the patients and ethical approval from the institution was given. Sera were obtained from new-onset Type I diabetic patients when the patients were first seen in the clinic or within 4 weeks of clinical diagnosis. Patients with APS were diagnosed on the criteria of islet-cell antibody positivity, absence of diabetes and documented presence of two other organ-specific autoantibodies [34]. Stiff-man syndrome was diagnosed according to clinical symptoms and none of these patients had diabetes. Only GAD antibody-positive sera (assay validated in the 2nd International GAD antibody workshop with a sensitivity of 87% and a specificity of 91%) [35] were included in these studies. A total of eleven hmABs to GAD₆₅ were used for immunoprecipitation; these comprised MICA 1, MICA 2, MICA 3, MICA 4, MICA 6, DPA, DP B, DP C, DP D, b78 and b96 [27, 29, 31] and were used as antibody-containing tissue culture supernatants. The b96 antibody used in this study was derived from the B cell line and recognises the C-terminal region of GAD₆₅ [31, 32]; subsequent cloning of the cell line produced the b96.11 clone which recognises the middle region of GAD₆₅ with similar epitope recognition to MICA 4 [33] but this clone was not used in this study.

Cloning of GAD from human islet cells. Total RNA was prepared from 10 000 pelleted and purified human islet cells (from Professor RG Bretzel, Giessen, Germany). We did PCR for GAD₆₅ cDNA with the primers 5'TCTCCGGATCCGATG GCATCTCCGGCTCT3' (BQ0) and 5'GAGCAGAATTCTTATAAATCTTGTTCCAAG-GC3' (BQ1) (containing *Eco*RI and a *Bam* HI sites, respectively) using 2.5 U *Taq* polymerase (Bioline, London, UK) for 30 cycles (40 s at 92°C, 90 s for 55°C and 240 s at 72°C). The faint band at 1.8 kb was purified and re-amplified as above using BQ0/BQ1 primers and two internal primers flanking the unique *Nar*I site in GAD₆₅ (primer A, 5'GGCAGACATGGCTGACATCAAC3' and primer B, 5'TTCTGTTTGGCTTCAAG AAT3') and subcloned into Bluescript (pBS) (Stratagene, Cambridge, UK). Sequencing of the full-length GAD₆₅ cDNA showed that the clone contained eight single base-pair changes from the wild-type sequence (Table 1). Of the base-pair alterations four were silent mutations; the other base-pair changes resulted in the following changes; Trp9Arg, Asn247Ser, Tyr425Asn and Leu574Pro (Table 1). We cloned GAD₆₇ cDNA as described [31].

Table 1. Base-pair alterations in the mutant GAD₆₅ from the sequence of wild-type molecule

bp25, T → C; amino acid (change) Trp → Arg
 bp594, A → C; amino acid (silent) Ser → Ser
 bp740, A → G; amino acid (change) Asn → Ser
 bp1273, T → A; amino acid (change) Tyr → Asn
 bp1410, T → C; amino acid (silent) His → His
 bp1434, A → G; amino acid (silent) Ala → Ala
 bp1515, C → T; amino acid (silent) Cys → Cys
 bp1721, T → C; amino acid (change) Leu → Pro

Four of the base pair changes are silent; the alteration in the amino acids are indicated (underlined). For clarity in the text, the altered amino acids in the mutant, Trp9Arg, Asn247Ser, Tyr425Asn and Leu574Pro have been termed mutations A, B, C and D, respectively and are shown in the schematic diagram

Site-directed mutagenesis. To simplify the mutation nomenclature, the four amino-acid mutations in the mutant GAD₆₅, Trp9Arg, Asn247Ser, Tyr425Asn and Leu574Pro were termed mutations A, B, C and D, respectively (Table 1). Thus, we have denoted the mutant GAD₆₅ with all four mutations mutant GAD₆₅/ABCD. The mutant GAD₆₅ was subcloned into pALTER-1 (Promega, Southampton, UK) and site-directed

mutagenesis was done using the Altered Sites II kit (Promega) according to the manufacturer's instructions. The mutagenic oligonucleotides are shown in Fig. 1 and the presence of the desired mutations confirmed by DNA sequencing. To analyse the role of each of the four alterations in mutant GAD₆₅/ABCD, a number of mutagenesis reactions were done in which one, two or three of the alterations in the mutant were reverted back to the wild-type amino acids. For example, GAD₆₅ containing only the single amino-acid mutation D was generated by reverting amino acids A, B and C in the mutant GAD₆₅/ABCD to wild-type residues. This mutant was termed mutant GAD₆₅/D. Similarly, the double mutant containing only mutation B and mutation D was generated by reverting residues A and C to wild-type residues and termed mutant GAD₆₅/BD.

Antibody analysis. Wild-type GAD₆₅ and all mutant GAD₆₅ cDNAs were subcloned into pGEM4-Z (Promega) and protein produced by transcription translation in vitro in a single-tube reaction using a TNT T7 quick-rabbit reticulocyte lysate system in the presence of ³⁵S-methionine. The size of all translated proteins was confirmed by SDS polyacrylamide gel electrophoresis and autoradiography. The expression of translated products of wild-type GAD₆₅, GAD₆₇ and all mutant GAD₆₅ was monitored using serum from an SMS patient containing high-titre anti-GAD₆₅ antibodies. The conformational stability of the translated products was confirmed by reactivity with a panel of GAD₆₅ antibody-positive sera from Type I diabetic patients. All translated products gave similar reactivity to control antibodies when either used freshly prepared (without freezing) in shorter incubations or after freezing and in overnight incubations with antibodies. Immunoprecipitations were done at 4–7°C in 96-well microtitre plates using MultiScreen filtration plates (Millipore, Watford, UK) as described [36]. Data presented for human sera and hmABs are the mean of duplicate samples. Background binding [binding to 20 normal healthy control sera, GAD antibody negative culture supernatants or protein A Sepharose alone (Amersham-Pharmacia Biotech, Little Chalfont, UK)] was subtracted from the binding of the serum or hmAB-containing culture supernatant.

Fig. 1. Sequences and annealing sites of the mutagenic oligonucleotides used to revert the altered amino acids in mutant GAD₆₅ to those found in the wild-type protein. The sequence is numbered from the first nucleotide of the initiation methionine. Altered amino acids are shown in bold type. New restriction sites created are underscored and the corresponding enzymes *AvaII*, *EcoRV*, *NheI* and *EcoNI* shown below

The binding of the hmABs to GAD₆₅ mutants was considered to be substantially affected if the reactivity represented 5–25% of that precipitated with wild-type GAD₆₅; the antibodies were considered to bind weakly if the reactivity represented 25–40% of wild-type binding. The results of the binding of serum antibodies to mutant GAD₆₅ were analysed by Mann-Whitney U test. We considered *p* values of less than 0.05 significant.

Expression of recombinant GAD₆₅ in insect cells. Recombinant baculovirus was produced using BaculoGold transfection kit with mutant GAD₆₅ cDNAs subcloned into pVL1393 (Becton-Dickinson, Oxford, UK). As control, wild-type GAD₆₅ recombinant baculovirus (from Dr T. Dyrberg) [37] was expressed in parallel Sf9 insect cells. Expression of recombinant GAD₆₅ proteins in Sf9 cells was monitored by SDS polyacrylamide gel electrophoresis and western blotting with rabbit antisera generated to GAD₆₅ peptides (serum 6952 and 7996 to GAD₆₅ peptides 4–22 and 570–585, respectively from Dr Å. Lernmark) [38]. The enzymatic activity of insect-cell mutant GAD₆₅ was measured in homogenates of GAD expressing insect cells by the release of ¹⁴CO₂ from its substrate, ¹⁴C-glutamic acid as described [39] and compared with the activity of wild-type GAD₆₅.

Results

Cloning of mutant GAD₆₅. We cloned GAD₆₅ from human islet total cDNA by double PCR amplification from total human islet cDNA. Sequencing of both strands showed eight base-pair alterations from the published sequence, with four of these alterations leading to a change in the amino acids Trp9Arg (mutation A), Asn247Ser (mutation B), Tyr425Asn (mutation C) and Leu574Pro (mutation D) [2, 3] (Table 1). This mutant was termed mutant GAD₆₅/ABCD. We investigated whether the mutant GAD₆₅/ABCD arose as a result of incorrect base-pair incorporation by the *Taq* polymerase [40] during the PCR reaction. The sequences around three of the base-pair alterations, nucleotides 740 (mutation B), 1273 (mutation C) and 1721 (mutation D) were amplified using the original stock of human islet cDNA used in the cloning of mutant GAD₆₅. Sequencing of more than ten independent clones of each of the mutants showed a complete concordance of the sequence with wild-type GAD₆₅ giving confirmatory evidence that the alterations in mutant GAD₆₅ derive from incorrect base-pair incorporation during the PCR reaction. Notably three of the amino-acid substitutions, at positions 247 (mutation B), 425 (mutation C) and 574 (mutation D) are also conserved in the related human isoform, GAD₆₇ [2, 3]; furthermore, all four altered residues in the mutant are conserved in the murine and porcine GAD₆₅ [41, 42], suggesting important roles in protein folding or function.

Binding of serum antibodies to mutant GAD₆₅. When binding of GAD₆₅ antibody-positive sera from new-

Fig. 2. Immunoprecipitation analyses of wild-type GAD₆₅ (●), mutant GAD₆₅/ABCD (○) and GAD₆₇ (▲) with GAD₆₅ antibody sera from new-onset Type I diabetic, APS and SMS patients using ³⁵S-methionine-labelled, translated proteins. Binding of individual sera from different patient groups to various GAD proteins was analysed using the Mann-Whitney U test. The bars represent the group mean values for patients sera precipitated with the translated proteins. The binding of Type I diabetic sera to mutant GAD₆₅/ABCD is significantly reduced compared with the reactivity with wild-type GAD₆₅. There was no significant difference in the binding of GAD₆₅-positive APS and SMS patients sera with the mutant and wild-type GAD₆₅.

onset Type I diabetic patients to mutant GAD₆₅/ABCD and wild-type GAD₆₅ was compared, a striking difference was apparent in which substantially lower binding to the mutant was observed for all sera (group mean mutant GAD₆₅ = 1380 cpm, wild-type GAD₆₅ = 6147 cpm) (*p* < 0.0001) (Fig. 2). No such difference between the binding to mutant GAD₆₅/ABCD and wild-type GAD₆₅ was observed, however, in sera from APS patients (group mean mutant GAD₆₅ = 5514 cpm, wild-type GAD₆₅ = 6760 cpm) (*p* > 0.05) or SMS patients (group mean mutant GAD₆₅ = 6538 cpm, wild-type GAD₆₅ = 5904 cpm) (*p* > 0.05). The data indicate that one or more of the amino-acid alterations in mutant GAD₆₅/ABCD results in a statistically significant alteration in the binding of the majority of serum antibodies from Type I diabetic patients. The titres of anti-GAD₆₅ antibodies in APS and SMS patients are considerably higher than those in sera from Type I diabetic patients [17–20]. To exclude the possibility that higher titres of anti-GAD₆₅ antibodies in APS and SMS sera were responsible for the binding to mutant GAD₆₅/ABCD, we diluted APS and SMS sera so that they precipitated similar counts of translated wild-type GAD₆₅ to those precipitated by Type I diabetic sera.

Fig. 3. Immunoprecipitation analyses of GAD₆₅ antibody sera from new-onset Type I diabetic patients with different mutants of GAD₆₅ using ³⁵S-methionine-labelled, translated proteins. The reactivity of GAD₆₅ antibodies in the Type I diabetic sera is restored in the single amino-acid mutants GAD₆₅/ACD and GAD₆₅/ABC indicating that substitution B and substitution D are responsible for the loss of binding with the mutant GAD₆₅/ABCD. The bars represent the group mean values

These diluted sera still reacted with the mutant GAD₆₅/ABCD showing that this effect was not related to antibody titres (not shown). The binding of sera to GAD₆₇ was also assessed. Sera from Type I diabetic patients showed weak binding to GAD₆₇ and no statistically significant correlation was observed to levels of binding to either wild-type GAD₆₅ or mutant GAD₆₅/ABCD. In contrast, sera from APS and SMS patients showed levels of binding to GAD₆₇ similar to wild-type GAD₆₅ (Fig. 2).

Effect of reversion of single amino-acid replacements of mutant GAD₆₅/ABCD to wild-type residue. To assess the amino acid(s) in mutant GAD₆₅/ABCD responsible for the loss of binding of GAD₆₅ antibodies in Type I diabetic sera, each of the four mutant amino acids was individually reverted back to wild-type residue, creating four new mutants termed mutant GAD₆₅/BCD, GAD₆₅/ACD, GAD₆₅/ABD and GAD₆₅/ABC. Translation products from all mutants were found to co-migrate with wild-type GAD₆₅ in SDS polyacrylamide gel electrophoresis (not shown). Binding of sera from Type I diabetic patients showed higher levels of binding to mutant GAD₆₅/ACD (mean = 5000 cpm, $p < 0.0001$) and mutant GAD₆₅/ABC (mean = 5483, $p < 0.0001$) compared with mutant GAD₆₅/ABCD (Fig. 3). Binding to mutant

GAD₆₅/BCD (mean = 1571, $p > 0.05$) and mutant GAD₆₅/ABD (mean = 1210, $p > 0.05$) was, however, similar to mutant GAD₆₅/ABCD (Fig. 3). The higher levels of antibody binding from Type I diabetic sera observed when either residues B or D were reverted to wild-type residues suggests that mutations B and D were responsible for the loss of antibody reactivity to mutant GAD₆₅/ABCD; in contrast, mutations A or C have minimal influence on antibody binding in the mutant GAD₆₅/ABCD.

To focus on the critical roles of mutations B and D in antibody binding from Type I diabetic sera, new mutants were generated which contained only these mutations either singly or in combination, i.e. mutant GAD₆₅/B, GAD₆₅/D, GAD₆₅/BD and were used in immunoprecipitation experiments with Type I diabetic sera. The binding of sera to mutant GAD₆₅/BD was similar to that observed to mutant GAD₆₅/ABCD whereas binding to mutant GAD₆₅/B and GAD₆₅/D was statistically significantly higher, confirming the importance of mutations in residues B or D for loss of antibody binding (data not shown).

Binding of hmABs to single and double amino-acid mutants of GAD₆₅. As the GAD₆₅ autoantibodies in Type I diabetic sera are heterogeneous and recognise a variety of conformational-dependent determinants on GAD₆₅, we used hmABs to GAD₆₅ to determine the role of mutations B and D in maintaining the integrity of conformational-dependent regions in GAD₆₅. A panel of 11 hmABs, where all (except MICA 2) are dependent on recognition of different discontinuous determinants on GAD₆₅, were used [27–33, 43]. None of the hmABs react with GAD₆₇ [28, 29, 31]. The binding of the hmABs with the mutant GAD₆₅/ABCD was either abolished (DP A, DP B, DP D and MICA 3) (< 10% binding of wild-type GAD₆₅ binding) or statistically significantly reduced (MICA 1, MICA 2, MICA 4, MICA 6, DP C, b78 and b96) (10–25% binding of wild-type GAD₆₅). There was similar reduced binding to the double amino-acid mutant, GAD₆₅/BD with all the hmABs (Fig. 4). Analysis of the binding to the single amino-acid mutants, GAD₆₅/B and GAD₆₅/D showed two patterns of binding. All the hmABs show reactivity with the mutant GAD₆₅/B and thus are not influenced by the single amino-acid mutation B in GAD₆₅. In contrast, the binding to the other single amino-acid mutant, GAD₆₅/D with MICA 1 and DP A was statistically significantly effected; MICA 3 showed reduced reactivity with this mutant. Notably, all these three hmABs are dependent on C-terminal epitopes of GAD₆₅ [33]. The binding of the remaining hmABs with the mutant GAD₆₅/D, including other C-terminal-directed hmABs such as MICA 2 and b78, was not significantly effected (> 60% binding of wild-type GAD₆₅) (Fig. 4). In summary, all eleven hmABs to GAD₆₅ show a loss of binding to mutant

Fig. 4A-K. Immunoprecipitation analyses of eleven hmABs (**A** MICA 1, **B** DP A, **C** MICA 2, **D** MICA 4, **E** MICA 6, **F** DP B, **G** DP C, **H** DP D, **I** b78, **J** b96, **K** MICA 3) with mutants of GAD₆₅ using ³⁵S-methionine-labelled, translated proteins. The results are expressed as percentage values of wild-type GAD₆₅ precipitated in the same experiment. The GAD mutants used are as follows: GAD₆₅/ABCD (□); GAD₆₅/B (▨); GAD₆₅/D (▩) and GAD₆₅/BD (▧). The experiments have been repeated at least three times with similar results. In particular, the hmABs MICA 1, MICA 3 and DP-A which are influenced by mutation D, the reduction in percentage binding to mutant D compared with wild-type GAD₆₅ binding was between 6–24%, 38–39% and 11–26%, respectively. None of the hmABs react with GAD₆₇ run in a parallel experiment (not shown)

GAD₆₅/BD. Mutation B on its own in mutant GAD₆₅/B did not statistically significantly effect the binding of any of the hmABs but mutation D in mutant GAD₆₅/D resulted in the loss of binding of a small number of hmABs. Therefore, the simultaneous presence of mutations B and D together in mutant GAD₆₅/BD was necessary to disrupt the binding of conformationally dependent hmABs to GAD₆₅.

Expression of recombinant mutant GAD₆₅. The mutants, GAD₆₅/ABCD, GAD₆₅/BD and GAD₆₅/D were expressed as recombinant proteins with baculovirus in insect cells. All mutant GAD₆₅ recombinant proteins co-migrated in SDS polyacrylamide gels with the control, wild-type GAD₆₅ expressed in insect cells (not shown). None of the mutants showed decarboxylase enzymatic activity; replacement of residues A, B, C and D in the mutant with those present in wild-type GAD₆₅ led to restoration of decarboxylase activity, which matched the control GAD₆₅ expressed in insect cells [37] (not shown).

Discussion

Our study reports on a fortuitous finding on a mutant GAD₆₅ that arose as a result of random mutations during PCR cloning of the molecule. The mutant GAD₆₅ arose as a result of fortuitous incorrect base incorporations due to the use of *Taq* polymerase [40]. There are differences between the antibody specificities to GAD₆₅ present in new-onset Type I diabetic patients and those in APS and SMS patients, with the former antibodies recognising predominantly conformational-dependent epitopes on GAD₆₅. [6, 13, 18–26]. The preferential loss of binding of anti-

bodies from new-onset Type I diabetic sera to the mutant GAD₆₅ suggests that the mutations probably effect the surface conformation and hence the three-dimensional folding of the molecule. The replacement of two residues present in the mutant GAD₆₅, Asn247 (residues B) and Leu574 (residue D) was necessary to restore the binding of the autoantibodies of Type I diabetic sera, indicating the crucial nature of these two amino acids in maintaining the structural integrity of GAD₆₅.

It was remarkable that two single amino-acid replacements in GAD₆₅ were able to discriminate the multiple antibody specificities to GAD₆₅ present in sera from Type I diabetic patients from those of APS and SMS sera. The preferential binding of GAD₆₅ antibodies in APS and SMS sera with the mutant GAD₆₅ was not related to the lower antibody titres generally found in Type I diabetic sera [17–20]. To simplify the complex patterns of serum anti-GAD₆₅ antibody specificities, the role of the two replacements was clarified using hmABs to GAD₆₅. The finding that all eleven hmABs used in this study, which recognise different determinants localised to the N-region, C-region and the middle region of GAD₆₅ [28–33], loose their binding to the double mutant Asn247Ser and Leu574Pro suggests that the two replacements have a considerable effect on the overall surface conformation of the entire molecule.

The single amino-acid replacements at residues 247 (position B) or 574 (position D) of GAD₆₅ was found to influence the binding of the hmABs differentially. Whereas the replacement of residue 247 was found to be silent in terms of reactivity of the hmABs, the replacement of residue 574 influenced the binding of some hmABs from the panel, including MICA 1, MICA 3 and DP-A. All these three hmABs are directed to the C-terminal region of GAD₆₅ [33], but notably other C-terminal-directed hmABs such as MICA 2 and b78 [30–33], were not affected in their reactivity to the single amino-acid replacement in the mutant GAD₆₅. Recent mapping of the determinants recognised by MICA 1, MICA 3 and DP-A on GAD₆₅ indicate that they recognise three distinct, but similar, determinants which are dependent on residues 483–499 and residues 556–585 [33], whereas the determinants recognised by MICA 2 and b78 reside in distinct residues 512–540 and 514–570, respectively [32, 43]. It is likely that residue 574 forms a contact amino acid for MICA 1, MICA 3 and/or DP-A or it has an effect on the conformation that is localised to the extreme C-terminal region of the molecule without affecting the nearby regions of amino acids 512–540 recognised by MICA 2 and b78. Other single amino acid substitutions in GAD₆₅ have been described but their effects appear to be limited to influencing the binding of some hmABs [33], as described above for residue 574. A three-dimensional model of the middle and the C-terminal

domain of the GAD₆₅ dimer has been constructed based on structure modelling from other decarboxylases whose structure is known [33]. The model indicates that the middle and the C-terminal regions form two very separate folding domains in the GAD₆₅ model. Notably, the two mutations identified in this study that lead to destabilisation of the GAD₆₅ structure reside in predicted helices (helix I and helix T) which are buried in the two domains [33], raising the possibility that the simultaneous disruption of both the folded domains leads to total destabilisation of the GAD₆₅ structure whereas single replacement of these residues either has minimal (residue 247) or limited (residue 574) local conformational effects on the molecule.

The concerted effect of two amino acids Asn-247 and Leu-574 in GAD₆₅ is essential for maintenance of the conformational determinants recognised by anti-GAD₆₅ autoantibodies directed to a variety of discontinuous epitopes, as well as the maintainance of functional enzymatic activity of the molecule. The replacements of these two residues could be useful in discriminating autoimmune sera from Type I diabetic patients from those of APS and SMS by immunoprecipitation assay. In conjunction with GAD₆₇ precipitation, ambiguous sera could also be confirmed with the use of the two mutants. It is also possible that some changes in epitope patterns of anti-GAD₆₅ antibodies during progression to disease activity in Type I diabetes could be monitored with the two mutants [44].

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References

1. Erlander MG, Tobin AJ (1991) The structural and functional heterogeneity of glutamic acid decarboxylase: a review. *Neurochem Res* 16: 215–226
2. Karlens AE, Hagopian WA, Grubin CE et al. (1991) Cloning and primary sequence of a human islet isoform of glutamic acid decarboxylase from chromosome 10. *Proc Natl Acad Sci USA* 88: 8337–8341
3. Bu D, Erlander MG, Hitz BC et al. (1992) Two human glutamate decarboxylases, 65 kDa GAD and 67 kDa GAD, are each encoded by a single gene. *Proc Natl Acad Sci USA* 89: 2115–2119
4. Bu D, AJ Tobin (1994) The exon-intron organisation of the genes (GAD1 and GAD2) encoding two human glutamate decarboxylases (GAD67 and GAD65) suggests that they derive from a common ancestral GAD. *Genomics* 21: 222–228
5. Kim J, Richter W, Aanstoot HJ et al. (1993) Differential expression of GAD65 and GAD67 in human, rat, and mouse pancreatic islets. *Diabetes* 42: 1799–1803

6. Baekkeskov S, Aanstoot HJ, Christgau S et al. (1990) Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 347: 151–156
7. Christie MR (1996) Antibodies to glutamic acid decarboxylase in the prediction of insulin dependent diabetes. In: Palmer J (ed) *Diabetes; Prediction, Prevention and Genetic Counselling*. John Wiley and Sons, Chichester, pp 77–96
8. Lernmark Å (1996) Glutamic acid decarboxylase – gene to antigen to disease. *J Intern Med* 240: 259–277
9. Solimena M, De Camilli P (1991) Autoimmunity to glutamic acid decarboxylase (GAD) in Stiff Man Syndrome and insulin dependent diabetes mellitus. *Trends Neurosci* 14: 452–457
10. Atkinson MA, Kaufman DL, Newman D, Tobin AJ, MacLaren NK (1993) Islet cell cytoplasmic autoantibody reactivity to glutamate decarboxylase in insulin-dependent diabetes. *J Clin Invest* 91: 350–356
11. Kaufman DL, Erlander MG, Clare-Salzler M, Atkinson MA, MacLaren NK, Tobin AJ (1992) Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* 89: 283–292
12. Seissler J, Amann J, Mauch L et al. (1993) Prevalence of autoantibodies to the 65- and 67-kD isoforms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* 92: 1394–1399
13. Tuomi T, Bjorsen P, Falorni A et al. (1996) Antibodies to glutamic acid decarboxylase and insulin-dependent diabetes in patients with autoimmune polyendocrine syndrome type I. *J Clin Endocrinol Metab* 81: 1488–1494
14. Davenport C, Radford PM, Al-Burhari T, Lai M, Bottazzo GF, Todd I (1998) Heterogeneity in the occurrence of a subset of autoantibodies to glutamic acid decarboxylase in autoimmune polyendocrine patients with islet cell antibodies. *Clin Exp Immunol* 111: 497–505
15. Hagopian WA, Michelsen B, Karlens AE et al. (1993) Autoantibodies in IDDM primarily recognize the 65,000-M(r) rather than the 67,000-M(r) isoform of glutamic acid decarboxylase. *Diabetes* 42: 631–636
16. Falorni A, Ackefors M, Carlberg C et al. (1996) Diagnostic sensitivity of immunodominant epitopes of glutamic acid decarboxylase (GAD65) autoantibodies in childhood IDDM. *Diabetologia* 39: 1091–1098
17. Christie MR, Genovese S, Cassidy D et al. (1994) Antibodies to islet 37k-antigen, but not to glutamate decarboxylase, discriminate rapid progression to insulin-dependent diabetes mellitus in endocrine autoimmunity. *Diabetes* 43: 1254–1259
18. Solimena M, Folli F, Aparisi R, Pozza G, De Camilli P (1990) Autoantibodies to GABA-ergic neurons and pancreatic beta cells in stiff-man syndrome. *New Engl J Med* 322: 1555–1560
19. Seissler J, Beig S, Yassin N et al. (1994) Association between antibodies to the MR 67, 000 isoform of glutamate decarboxylase (GAD) and type 1 (insulin-dependent) diabetes mellitus with coexisting autoimmune polyendocrine syndrome type II. *Autoimmunity* 19: 231–238
20. Kim J, Namchuk M, Bugawan T et al. (1994) Higher autoantibody levels and recognition of a linear NH₂-terminal epitope in the autoantigen GAD65 distinguish GAD65 distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. *J Exp Med* 180: 595–606
21. Ujihara N, Daw K, Gianani R, Boel E, Yu L, Powers AC (1994) Identification of glutamic acid decarboxylase autoantibody heterogeneity and epitope regions in type I diabetes. *Diabetes* 43: 968–975
22. Daw K, Ujihara N, Atkinson M, Powers AC (1996) Glutamic acid decarboxylase autoantibodies in stiff-man syndrome and insulin-dependent diabetes mellitus exhibit similarities and differences in epitope recognition. *J Immunol* 156: 818–825
23. Butler MH, Solimena M, Dirks R, Hayday A, De Camilli P (1993) Identification of a dominant epitope of glutamic acid decarboxylase (GAD-65) recognized by autoantibodies in stiff-man syndrome. *J Exp Med* 178: 2097–2106
24. Li L, Hagopian WA, Brashear R, Daniels T, Lernmark Å (1993) Identification of a dominant epitope of glutamic acid decarboxylase (GAD-65) recognized by autoantibodies in stiff-man syndrome. *J Immunol* 152: 930–934
25. Bjork E, Velloso LA, Kampe O, Karlsson A (1994) GAD autoantibodies in IDDM, Stiff-man syndrome, and Autoimmune Polyendocrine Syndrome Type I recognize different epitopes. *Diabetes* 43: 161–165
26. Dinkel K, Meinck HM, Jury KM, Karges W, Richter W (1998) Inhibition of gamma-aminobutyric acid synthesis by glutamic acid decarboxylase autoantibodies in stiff-man syndrome. *Ann Neurol* 44: 194–201
27. Richter W, Endl J, Eiermann TH et al. (1992) Human monoclonal islet cell antibodies from a patient with insulin-dependent diabetes mellitus reveal glutamate decarboxylase as the target antigen. *Proc Natl Acad Sci USA* 89: 8467–8471
28. Richter W, Shi Y, Baekkeskov S (1993) Autoreactive epitopes defined by diabetes-associated human monoclonal antibodies are localized in the middle and C-terminal domains of the smaller form of glutamate decarboxylase. *Proc Natl Acad Sci USA* 90: 2832–2836
29. Madec AM, Rousset F, Ho S et al. (1996) Four IgG anti-islet human monoclonal antibodies isolated from a type 1 diabetes patient recognize distinct epitopes of glutamic acid decarboxylase 65 and are somatically mutated. *J Immunol* 156: 3541–3549
30. Syren K, Lindsay L, Stoehrer B et al. (1996) Immune reactivity of diabetes-associated human monoclonal autoantibodies defines multiple epitopes and detects two domain boundaries in glutamate decarboxylase. *J Immunol* 157: 5208–5214
31. Tremble J, Morgenthaler NG, Vlug A et al. (1997) Human B cells secreting immunoglobulin G to glutamic acid decarboxylase-65 from a non-diabetic patient with multiple autoantibodies and Graves disease: a comparison with those present in Type I diabetes. *J Clin Endocrinol Metab* 82: 2664–2670
32. Powers AC, Bavik K, Tremble J, Daw K, Scherbaum WA, Banga JP (1999) Comparative analysis of epitope recognition of glutamic acid decarboxylase by autoantibodies from different autoimmune disorders. *Clin Exp Immunol* 118: 349–356
33. Schwartz H, Chandonia JM, Kash S et al. (1999) High-resolution autoreactive epitope mapping and structural modeling of the 65 kDa form of human glutamic acid decarboxylase. *J Mol Biol* 287: 983–999
34. Maes M, Eisenbarth GS (1999) The polyglandular Autoimmune syndromes. In: Volpe R (ed) *Autoimmune Endocrinopathies*. Humana Press, New Jersey, pp 349–363
35. Schmidli RS, Colman PG, Bonifacio E (1995) Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. The Second International GADAB Workshop. *Diabetes* 44: 636–640
36. Hatfield ECI, Hawkes CJ, Payton MA, Christie MR (1997) Cross reactivity between IA-2 and phogrin/IA-2 β in binding of autoantibodies in IDDM. *Diabetologia* 40: 1327–1333

37. Moody AJ, Hejnaes KR, Marshall MO et al. (1995) Isolation by anion exchange of immunologically and enzymatically active human islet glutamic acid decarboxylase 65 overexpressed in Sf9 insect cells. *Diabetologia* 38: 14–23
38. Li L, Jiang J, Hagopian WA et al. (1995) Differential detection of rat islet and brain glutamic acid decarboxylase (GAD) isoforms with sequence-specific peptide antibodies. *J Histochem Cytochem* 43: 53–59
39. Christie MR, Brown TJ, Cassidy D (1992) Binding of antibodies in sera from Type I diabetic patients to glutamate decarboxylase from rat tissue. Evidence for antigenic and non-antigenic forms of the enzyme. *Diabetologia* 35: 380–384
40. Tindall KR, Kunkel TA (1988) Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* 27: 6008–6013
41. Lee DS, Tian J, Phan T, Kaufman DL (1993) Cloning and sequence analysis of a murine cDNA encoding glutamate decarboxylase (GAD65). *Biochim Biophys Acta* 1216: 157–160
42. Suzuki R, Asami N, Amann E, Wagatsuma M (1995) Sequences of two porcine glutamic acid decarboxylases (65- and 67-kDa GAD). *Gene* 152: 257–260
43. Richter W, Northemann W, Muller M, Boehm BO (1996) Mapping of an autoreactive epitope with glutamate decarboxylase using a diabetes-associated human monoclonal autoantibody and an epitope cDNA library. *Hybridoma* 15: 103–108
44. Bingley PJ, Christie MR, Bonifacio E et al. (1994) Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. *Diabetes* 43: 1304–1310