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

Wiltrud Richter, Yuguang Shi, and Steinunn Bekkeskov*

Departments of Microbiology/Immunology and Medicine and Hormone Research Institute, University of California, San Francisco, CA 94143-0534

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ABSTRACT The γ-aminobutyrate-synthesizing enzyme glutamate decarboxylase (GAD; L-glutamate 1-carboxy-lyase, EC 4.1.1.15) is a major target of autoantibodies associated with both early and late stages of pancreatic β-cell destruction and development of type 1 diabetes. We have used five monoclonal anti-islet-cell antibodies (MICAs 1, 2, 3, 4, and 6) derived from a newly diagnosed diabetic patient to probe the autoimmune epitopes in the enzyme. All the MICAs specifically recognized the smaller GAD protein, GAD₆₅, and did not recognize the nonallelic GAD₂₇ protein. A series of N-terminal, C-terminal, and internal deletion mutants, as well as protein footprinting, were used to identify the target regions in GAD₆₅. Immuno-precipitation revealed two major native epitope areas in the GAD₆₅ molecule. The first, defined by MICAs 1 and 3, is destroyed by deleting 41 amino acids at the C terminus but is also dependent on intact amino acids 244–295. This epitope (or epitopes) may span both middle and C-terminal domains of the protein. The second conformational epitope region, defined by MICAs 4 and 6, is dependent on intact amino acids 245–295 but is not affected by deletion of 110 amino acids at the C terminus and is therefore confined to domain(s) in the middle of the molecule. MICA 2 recognizes a linear epitope close to the C terminus. Thus, the N-terminal domain of GAD₆₅, which differs most significantly from GAD₂₇, does not harbor the MICA epitopes. Rather subtle amino acid differences in the middle and C-terminal domains define the GAD₂₇-specific autoimmune epitopes. Analysis of sera from 10 type 1 diabetic patients suggests that MICAs 1, 3, 4, and 6 represent a common epitope recognition in this disease, whereas the MICA 2 epitope is rare. Furthermore, autoimmune antibodies in some sera are restricted to the MICA 1/3 epitope, suggesting that this epitope may represent a single dominant epitope in the early phases of β-cell autoimmunity.

Glutamate decarboxylase (GAD; EC 4.1.1.15) is encoded by two distinct nonallelic genes, specifying GAD₆₅ and GAD₂₇ (subscripts indicate approximate molecular mass in kilodaltons), that may have developed from a common ancestral gene during vertebrate phylogeny. Thus Drosophila has only one gene encoding GAD, which is similarly homologous to each of the mammalian forms (1). GAD₂₇ and GAD₆₅ are highly diverse in the first 95 amino acids but share significant homology (~78% identity) in the rest of the molecule. Both have a proteolytic hotspot 80–90 amino acids from the N terminus (2, 3), which may represent a domain boundary. The N-terminal region harbors the posttranslational modifications which result in anchoring of GAD₆₅ to the membrane of synaptic vesicles and control the distinct subcellular localization of this protein (3). Drosophila GAD lacks most of the N-terminal amino acid residues, suggesting that this domain evolved to provide GAD₂₇ and GAD₆₅ with their distinct targeting to subcellular compartments, a feature which may have implications for both function and autoantigenicity (3). Some species express both GAD proteins in their pancreatic islets (2). In human islets only GAD₆₅ is expressed (4, 5), in contrast to brain, where both GAD₂₇ and GAD₆₅ are produced (6). GAD₆₅ is a major target of circulating IgG autoantibodies in two human diseases that affect the sites of GAD expression: type 1 diabetes, which is caused by an autoimmune destruction of pancreatic β cells (7), and a rare neurological disorder, stiff-man syndrome (8), in which γ-aminobutyrate-secreting neurons are impaired. GAD-reactive T cells have been demonstrated in type 1 diabetes (9, 10). The GAD₆₅ antibodies seem to be the earliest marker of autoimmune reactions directed to the β cell and are sometimes present up to several years before clinical onset (11, 12). Although β-cell destruction is likely to be T-cell-mediated (13), the autoantibodies present during the early phases of β-cell destruction are important for two reasons. First, it is likely that such antibodies are directed to the same target antigen as pathogenic T cells. Second, the antibodies identify individuals at risk of developing type 1 diabetes.

Most diabetic sera recognize only conformational epitopes in the GAD₆₅ molecule and do not react with the denatured protein on Western blots. In contrast sera from patients with stiff-man syndrome recognize denatured GAD₆₅ (7). Recently, Richter et al. (14) derived a number of Epstein-Barr virus-immortalized B-cell lines from the peripheral blood of newly diagnosed type 1 diabetic patients. Stable IgG-producing monoclonal B-cell lines positive for islet-cell antibodies by indirect immunofluorescence staining of frozen sections of human pancreas were stabilized following repeated single-cell cloning. All the monoclonal anti-islet-cell antibodies (MICAs) were directed to GAD (14). In this study we show that the target epitopes are specific for GAD₆₅ and identify the regions of the molecule that harbor the autoantigenic target epitopes for the monoclonal antibodies. We have furthermore addressed the question whether the MICAs represent an epitope recognition characteristic for autoantibodies to GAD in type 1 diabetes.

MATERIALS AND METHODS

Antisera. Supernatants of human monoclonal B-cell lines containing MICAs 1–6 were prepared as described (14). Mouse mAb GAD1 (15), which recognizes native forms of GAD₆₅ and GAD₂₇, was obtained from the American Type Culture Collection. Mouse monoclonal antibody GAD6 (16) and rabbit polyclonal antibodies 1266 and K2 (2, 3) were described earlier. Serum from a patient with stiff-man syndrome was donated by Vanda Lennon (Mayo Clinic, Rochester, Minn.).

Abbreviations: GAD, glutamate decarboxylase; MICA, monoclonal anti-islet-cell antibody; PAS, protein A-Sepharose.

*To whom reprint requests should be addressed at: Hormone Research Institute, University of California, San Francisco, CA 94143-0534.
ester, MN). Sera from type 1 diabetic patients were obtained from H. J. Aanstoot (University of Rotterdam, The Netherlands) or described earlier (11).

Expression of GAD in Baculovirus Expression Systems. Recombinant baculovirus vectors expressing human GAD$_{65}$ and GAD$_{67}$ were constructed by ligating a 1.8-kb BamHI fragment of a human GAD$_{65}$ cDNA clone into the BamHI site of the baculovirus vector pVL1392 (a gift from D. Morgan, University of California, San Francisco) and a 2.7-kb EcoRI fragment of human GAD$_{67}$ into the EcoRI site of the baculovirus vector pVL1392 (Invitrogen, San Diego). An N-terminal deletion mutant lacking the first 101 amino acids (aa) of rat GAD$_{65}$ was generated by oligonucleotide-directed mutagenesis (17) and inserted into pVL1392 (Y.S. and S.B., unpublished work). GAD cDNAs were a gift from A. Tobin (University of California, Los Angeles). Recombinant viruses were derived and isolated as described (3). Recombinant baculovirus harboring full-length rat GAD$_{65}$ was described earlier (3).

Expression of Wild Type and Mutants of Rat GAD$_{65}$ in COS-7 Cells. Rat GAD$_{65}$ cDNA was subcloned into the Kpn I and Not I sites of the pSV-SPORT vector (BRL) for expression in a COS-7 cell line (American Type Culture Collection). A collection of N-terminal and C-terminal deletion mutants of GAD$_{65}$ was generated by polymerase chain reaction (18) at predetermined sites, using anchored primers (Y.S. and S.B., unpublished work). Expression was analyzed by Western blotting with GAD6 (N-terminal mutants) and a stiff-man-syndrome syndrome (C-terminal mutants). An internal deletion mutant lacking aa 363–422 was generated by using the Nsi I restriction sites in the GAD$_{65}$ cDNA. Similarly, Bgl II restriction sites were used to generate a hybrid molecule containing aa 1–95 from rat GAD$_{67}$ and aa 353–585 from rat GAD$_{65}$.

Immunoprecipitation and Immunoblotting. Expression of cDNAs in COS-7 monkey cells (pSV-SPORT expression vectors) or SF9 insect cells (baculovirus expression vectors), labeling with $[^{35}S]$methionine, harvesting, detergent extraction of cells, immunoprecipitation, SDS/PAGE, and immunoblotting were carried out as described (2, 3, 7) except that incubation with monoclonal antibodies and sera for immunoprecipitation was for 2 hr.

Protein Footprinting. Immune complexes were prepared with $[^{35}S]$methionine-labeled extracts of human GAD$_{65}$ from SF9 cells and either 500 μl of MIA supernatant or 10 μl of GAD6 or GAD1 ascites (19). They were then isolated by binding to protein A-Sepharose (PAS) and washed as described above. To stabilize the immune complexes, anti-human IgG antibody (heavy- and light-chain-specific Fab mixture; Jackson ImmunoResearch) was incubated with the antigen–antibody–PAS complex for 45 min at 4°C before incubation with proteases. Incubations with proteases were for 30 min on ice (chymotrypsin and trypsin) or 1 hr at 37°C (chymotrypsin). Protease treatment was stopped by washing the PAS-bound complexes in 10 mM Hepes–NaOH, pH 7.4/10 mM benzamidine/150 mM NaCl/0.5 mM $[^{35}S]$methionine/0.01% bovine serum albumin/5 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/0.5% Triton X-114 and was followed by SDS/PAGE analysis and fluorography.

RESULTS

All MICA3s Specifically Recognize Native GAD$_{65}$ but Not GAD$_{67}$ in Immunoprecipitation Experiments. GAD$_{65}$ is identical to the 64-kDa autoantigen in type 1 diabetes, and previous analyses of GAD antibodies using islet-cell lysates have focused on this form of GAD (11, 12, 20, 21). Some sera may also recognize GAD$_{67}$, but analysis of antibodies to this protein in human sera is still hampered by background problems (unpublished results). We first addressed the ques-

tion whether the set of MICA3s derived from a type 1 diabetic patient (MICA1–6) recognize human GAD$_{65}$ and/or GAD$_{67}$ proteins expressed in SF9 insect cells or in COS-7 cells under native conditions. MICA3s 1, 2, 3, 4, and 6 recognized GAD$_{65}$ but not GAD$_{67}$ in immunoprecipitation experiments under native conditions using PAS (Fig. 1). MICA5, the only MICA of subclass IgG3, did not bind PAS. MICA 5, however, showed specific binding to GAD$_{65}$ and did not recognize GAD$_{67}$ in immunoprecipitates isolated with protein G-Sepharose (results not shown). Since MICA5 bound only weakly to protein G-Sepharose and seemed to recognize an epitope similar to that of MICA 2 in preliminary experiments, it was not included in further analysis. The absence of reactivity with GAD$_{67}$ suggests that the MICA epitopes are defined by amino acids which differ between GAD$_{65}$ and GAD$_{67}$. Serum of the type 1 diabetic patient from whom MICA3s 1–6 were derived was also strongly positive for antibodies recognizing human GAD$_{65}$ but showed only a weak reactivity with human GAD$_{67}$ (results not shown), consistent with the specificity of these monoclonal antibodies.

Only MICA 2 Recognizes a Linear Epitope in GAD$_{65}$. In contrast to stiff-man-syndrome sera, which recognize denatured GAD$_{65}$ on Western blots, sera from most type 1 diabetic patients only recognize conformational epitopes in the GAD molecule (7). When we analyzed the reactivity of the individual MICA3s for denatured GAD$_{65}$ and GAD$_{67}$, only MICA2 recognized denatured GAD$_{65}$ on Western blots. Furthermore, when we tested sera of the type 1 diabetic patient from whom MICA3s 1–6 were derived, it weakly stained denatured GAD$_{65}$ but not GAD$_{67}$ on Western blots (results not shown).

The results above show that MICA1, 3, 4, and 6 recognize only nonlinear or conformational epitopes, whereas MICA2 recognizes a linear epitope that is specific for GAD$_{65}$. Thus GAD$_{65}$ harbors linear as well as nonlinear or conformational autoimmune epitopes that distinguish it from GAD$_{67}$.

Protein Footprinting Reveals Two to Four Distinct Epitopes. The similarities and differences in epitope recognition by the MICA3s were analyzed by protein footprinting (19). These analyses revealed two major different footprinting patterns which distinguished MICA3s 1–3 from MICA3s 4 and 6. Within the first group (MICA3s 1 and 3) displayed identical patterns which were similar to yet distinct from that of MICA2. Further, in the second group (MICA3s 4 and 6) displayed only minor differences (Fig. 2).
Both MICA 4 and MICA 6 more effectively protected the full-length GAD65 molecule and a 55-kDa fragment lacking the N terminus than did MICAs 1–3. Thus, in contrast to MICAs 4 and 6, no full-length GAD65 or 55-kDa fragment was detected in immunocomplexes with MICAs 1–3 after prolonged chymotrypsin incubation (Fig. 2, compare lanes 10 and 11 with lanes 7 and 9). These results suggest that MICAs 4 and 6 may bind areas of the molecule closer to the N terminus and therefore protect this part of the molecule better than MICAs 1–3.

While MICAs 1, 3, 4, and 6 still displayed a complex footprinting pattern following prolonged incubations with chymotrypsin (Fig. 2, lanes 7–12), only one band of 14 kDa was protected by MICA 2 under those conditions (Fig. 2, lane 8).

In sum, the complex footprinting patterns of MICAs 1, 3, 4, and 6 are consistent with a nonlinear epitope recognition, whereas the 14-kDa single fragment protected by MICA 2 is consistent with a linear epitope recognition by this monoclonal antibody. Further, the footprinting results suggest that MICAs 4 and 6 may recognize areas more toward the N terminus than those recognized by MICAs 1–3.

Distinct Patterns of Recognition of Deletion Mutants by MICAs. To localize the domains recognized by the MICAs, we analyzed their binding to a number of N-terminal as well as C-terminal deletion mutants of rat GAD65 expressed in COS-7 cells (Fig. 3). The sizes of the expressed GAD65 fragments, their location in the amino acid sequence, and their reactivity with the various MICAs are summarized in Fig. 3C. The first N-terminal deletion mutant which was recognized differently from the full-length protein was GAD65N33 (Δ1–295). This mutant was not recognized by MICAs 1, 4, and 6 and was either very weakly positive or negative with MICA 3 (Fig. 3A and C). MICA 2 reacted equally well with GAD65N33 and the full-length molecule on Western blots. However, MICA 2 reacted only weakly with this form in immunoprecipitation experiments (Fig. 3A and C), suggesting that the linear epitope recognized by this monoclonal antibody is sequestered in the folded truncated protein.

Analysis of C-terminal deletion mutants showed that the removal of 41 aa at the C terminus abolished the binding of MICAs 1–3 (Fig. 3B and C). However, MICAs 4 and 6 recognized both this mutant (GAD65C61, Δ545–585) and the C-terminal deletion mutant GAD65C53 (Δ476–585), which lacks an additional 69 aa at the C terminus (Fig. 3B and C). MICA 4 showed a stronger binding to both mutants than MICA 6. None of the MICAs showed reactivity to the C-terminal deletion mutant GAD65C41 (Δ376–585), which is missing an additional 100 aa at the C terminus (Fig. 3C).

None of the MICAs except MICA 2 recognized a deletion mutant, GAD65Δ59, lacking aa 363–422, which harbor the C-terminal deletion.

**Fig. 2.** Analysis of MICA binding sites by protein footprinting. Complexes between the MICAs or human IgG (control) and [35S]methionine-labeled human GAD65 from Sf9 cells were isolated and subjected to limited proteolysis as indicated. The GAD65 fragments remaining complexed with IgG were then analyzed by SDS/PAGE and fluorography. Sizes of molecular mass markers are shown in kilodaltons.

**Fig. 3.** Analysis of reactivity of MICAs with GAD65 mutants. (A) Immunoprecipitation of N-terminal deletion mutants of rat GAD65, expressed in COS-7 cells, with MICAs, GAD6, and human IgG (control). (B) Immunoprecipitation of C-terminal deletion mutants of rat GAD65, expressed in COS-7 cells, with MICAs, GAD1, GAD6, and human IgG (control). (C) Summary of the characteristics of antibody binding to rat GAD65 mutants expressed in COS-7 cells. GAD1 (15), which recognizes a conformational epitope in both GAD65 and GAD6, is distinct from all the other monoclonal antibodies by recognizing only the intact GAD65 molecule. GAD6, which is specific for GAD65 and recognizes a linear epitope (2, 3), has a recognition pattern similar to that of MICA 2, except that GAD6 recognizes the mutants equally well in native and denaturing conditions. MICA 1, 3, 4, and 6 and GAD 1 were positive only by immunoprecipitation. MICA 2 and GAD 6 were positive by both immunoprecipitation and Western blotting. *, Positive by Western blotting, weakly positive by immunoprecipitation.
pyridoxal phosphate binding site of the enzyme or a hybrid molecule containing aa 1–95 from GAD67 linked to the last 233 aa of GAD65 (Fig. 3C). It can be concluded that amino acids within the last 41 residues of GAD65 are a significant part of the linear epitope for MICA 2.

**Analysis of Sera from Type 1 Diabetic Patients.** All the MICAs were derived from a single newly diagnosed diabetic individual. Since this patient’s serum showed reactivity with GAD65 on Western blots, a feature which is rare among sera from type 1 diabetic individuals, it was of importance to establish whether the immune recognition pattern of the MICAs is shared by other type 1 diabetic individuals. Sera from young newly diagnosed type 1 diabetic patients (D1–9), ages 4½–26 years (6 female, 3 male), and a prediabetic individual (P1), age 11 years (female), were analyzed for their binding to the N-terminal and C-terminal deletion mutants (Fig. 4). Two of the sera (D6 and D7) reacted very weakly with the full-length molecule and were therefore not suitable for epitope analysis. The remaining prediabetic/diabetic sera, which were strongly positive for GAD65 antibodies, displayed similar reactivity toward the N-terminal deletion mutants. Thus, deletion of approximately one-third (Δ1–194) of the GAD65 molecule from the N terminus did not result in a detectable decrease in immunoreactivity with the prediabetic/diabetic sera, and deletion of an additional 50 aa at the N terminus (GAD65N39) resulted in only a slightly decreased reactivity with some of the sera (Fig. 4). In contrast, further deletion of 51 aa (GAD65N33) abolished recognition by all of the sera (results not shown). The sera proved distinguishable in the analysis of the mutant lacking 41 aa at the C terminus (GAD65C61). Thus, four sera—including the serum from the prediabetic individual sampled 32 months before clinical onset of disease, as well as sera from three newly diagnosed patients (an 11-year-old girl (D3), a 4½-year-old boy (D4), and a 26-year-old man (D5)), all of which were strongly positive for GAD65 antibodies—showed either very weak or no reactivity with the GAD65C61 mutant (Fig. 4). The remainder of the sera were still strongly positive for this truncated protein (Fig. 4). The results are consistent with the data obtained with the MICAs and suggest that humoral epitopes associated with type 1 diabetes are concentrated in the middle and C-terminal domains of GAD65. Our data are in agreement with a previous study, which showed that antibodies to an ~50-kDa tryptic fragment of GAD65 correlate completely with antibodies to the full-length GAD65 molecule in type 1 diabetic patients (22, 23). The tryptic fragment lacks the N-terminal 80–90 aa but contains the C terminus (3). None of the 11 patient sera recognized GAD65 on Western blots and thus did not contain autoantibodies with a linear epitope recognition such as MICA 2 (results not shown).

**DISCUSSION**

The **N-Terminal Region Is Not the Site of Autoimmune Epitopes in GAD65.** GAD67 and GAD65 are highly diverse in the first 95 aa but share significant homology in the rest of the molecule. However, none of the GAD65-specific epitopes recognized by the MICAs were localized in the first 244 aa at the N terminus. Thus, the epitopes of the MICAs are concentrated in areas of the molecule that are significantly distant from the N-terminal membrane-anchoring domain. The last 110 aa at the C terminus do not contribute to the MICA 4/6 epitope(s), which spans residues in the middle of the molecule. In contrast, deletion of 41 aa at the C terminus abolishes the MICA 1/3 epitope. Furthermore, deletion of aa 245–295 abolishes all the conformational epitopes. Since deletions in one part of a protein molecule can significantly alter the conformation in distant parts of the molecule, it is not clear whether residues among aa 245–295 as well as 545–585 are part of the MICA 1/3 epitope or whether they are important for the conformation of the MICA 1/3 epitope, without having a direct involvement in binding. Indirect evidence, however, supports the explanation that the C-terminal region is the major site of the MICA 1/3 epitope. The protein footprinting patterns of MICAs 1 and 3 are very similar to that of MICA 2, which recognizes an epitope in this region, but clearly distinct from those of MICAs 4 and 6, which recognize epitopes or epitopes in the middle of the molecule. Furthermore, deletion of aa 245–295 (GAD65N33 mutant) seems to result in major conformational changes in the C-terminal region, as suggested by the weak binding of MICA 2 to this mutant, whereas deletion of aa 545–585 and 476–585 in the C terminus does not seem to affect the middle region of the GAD65 molecule, where the MICA 4 and 6 epitopes remain intact. Finally, MICA 3 did bind weakly to the GAD65N33 mutant, whereas no binding to the C-terminal deletion mutants was detected. Thus deletion of aa 244–295 is likely to be affecting the conformation of the C-terminal region, whereas deletion of the C-terminal amino acids appears to affect the MICA 1/3 epitope directly.

The **MICA 2 Epitope.** MICA 2 was the only MICA which recognized the GAD65 molecule on Western blots. This reactivity represents a rare case of recognition of linear epitopes by autoantibodies from type 1 diabetic patients. This linear epitope recognition by MICA 2 was confined to the C-terminal domain, which also seems to be important for the conformational epitope of MICAs 1 and 3. Consequently, the protein footprint of MICA 2 was highly similar to those of MICAs 1 and 3. A significant part of the linear MICA 2 epitope is localized between aa 545 and 585—i.e., close to the C terminus. We have also identified and characterized a linear epitope that is recognized by 20 of 21 (95%) sera of stiff-man-syndrome patients and shown that it is clearly distinct from that of MICA 2 (unpublished results). This clear difference in epitope recognition between the two diseases suggests that the humoral autoimmunity to GAD is not only an epiphenomenon of the autoimmune process but related to the pathogenic mechanisms which result in either stiff-man syndrome or type 1 diabetes.
Does the MICA 1/3 Epitope Represent a Single Dominant Early Epitope Recognized by Diabetic Autoantibodies? In autoimmune diseases, both humoral and cellular autoimmunity is often directed to a single dominant epitope in the early phases but may spread to other regions in the autoantigen with increased duration of autoimmune responses (24-26). GAD$_5$ autoantibodies have been detected during early phases of pancreatic $\beta$-cell destruction, which is often several years before the majority of the $\beta$ cells have vanished and the clinical symptoms develop (11, 12). It can be suggested that the epitope recognition of GAD$_5$ autoantibodies at the clinical onset of diabetes may be more diverse than during the primary autoimmune response, where it may be limited to a single dominant epitope. The newly diagnosed type 1 diabetic patient from whom the MICAs were derived was 32 years of age, which is older than the average age at clinical onset, in puberty. It is therefore conceivable that the GAD$_5$ epitopes defined by the MICAs represent both early and late humoral responses. Analysis of nine newly diagnosed diabetic patients and one child 32 months before clinical onset of disease suggests that the MICAs may in fact represent both common and unusual epitope recognition in type 1 diabetes. Most importantly, four sera, including the serum from the prediabetic child, displayed a pattern which is consistent with the MICA 1/3 epitope being the dominant epitope recognized by the sera and the MICA 4/6 specificity either being absent or playing a minor role. Other sera, however, were clearly not restricted to the MICA 1/3 specificity and displayed a pattern suggesting that they harbored antibodies directed to the areas of the MICA 4/6 epitope(s). Finally, MICA 2 epitope recognition was not detected in any of the patient sera except the MICA donor, in agreement with earlier results demonstrating that recognition of linear epitopes in GAD$_5$ is very rare among type 1 diabetic patients (7). One possible hypothesis is that the GAD$_5$ epitope recognition is HLA-restricted. It is of note, however, that sera from a pair of monozygotic twins included in this study (D1 and D5, HLA-DR3.7) showed significant differences in their epitope restriction. Thus, whereas the MICA 1/3 specificity predominated in serum D5, this was not the case for serum D1. An alternative hypothesis, which we currently favor, is that the MICA 1/3 epitope represents the immunodominant humoral epitope in early responses to GAD$_5$ and that the other epitopes represent later (MICA 4/6) and very late (MICA 2) stages of epitope spreading in the molecule. Longitudinal analysis of serum samples from prediabetic individuals will test this hypothesis. There is some evidence suggesting epitope spreading in the GAD molecule. Thus Christie et al. (22, 23) have described antibodies to a 37-kDa tryptic fragment, which do not always correlate with antibodies to the full-length GAD molecule and may develop late in $\beta$-cell destruction.

The results of this study will contribute to the development of assays for GAD$_5$ autoantibodies in type 1 diabetes for prediction and diagnosis. First, they demonstrate the importance of intact conformation of the middle and C-terminal parts of the molecule. Further, the data indicate that the N-terminal region, with its lipid modifications, is dispensable and can either be deleted to improve the solubility of the protein or be used as a site for immobilization and other modifications without affecting the humoral autoantigenic epitopes in type 1 diabetes. Lipid modification of peptides and proteins can, however, play a major role in T-cell responses (27, 28), and the N-terminal region of GAD$_5$ may still harbor important T-cell epitopes.

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