Differential Detection of Rat Islet and Brain Glutamic Acid Decarboxylase (GAD) Isoforms with Sequence-specific Peptide Antibodies

LINSONG LI, JIANJIE JIANG, WILLIAM A. HAGOPIAN, ALLAN E. KARLSEN, MARILYN SKELLY, DENIS G. BASKIN, and ÅKE LERNMARK

Departments of Medicine (LL, JJ, WAH, AEK, DGB, AL), Pathology (MS), and Biological Structure (DGB), University of Washington, and Veterans Affairs Medical Center (DGB), Seattle, Washington.

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We studied the distribution of the Mr 65,000 and Mr 67,000 isoforms of glutamic acid decarboxylase, GAD65 and GAD67, in rat islets and brain by immunocytochemistry. Synthetic peptides representing selected GAD65 or GAD67 sequences were used to produce sequence-specific antibodies, allowing differential immunocytochemical detection of the two isoforms. GAD-specific reactivity of each peptide antiserum was confirmed by ELISA, immunoblotting, and immunoprecipitation. Immunostaining specificity was verified by displacement with either immunizing or irrelevant peptide. Dual immunostaining with GAD isoform-specific antibodies and polyclonal antibodies to glucagon showed that GAD65 was primarily detected in rat pancreatic islet β-cells, whereas α-cells had weak GAD65 staining. In contrast, GAD67 was detected primarily in α-cells. In rat brain, GAD65 and GAD67 were present in neuron cell bodies and processes. These data demonstrate that antibodies raised against the N-terminus of GAD allow differential immunocytochemical identification of GAD67 and GAD65. Differential expression of GAD isoforms within islet α- and β-cells supports the role of GAD65 in autoimmune diabetes and stiff-man syndrome. (J Histochem Cytochem 43:53-59, 1995)

KEY WORDS: Insulin-dependent diabetes, IDDM; Immunostaining; Stiff-man syndrome, SMS; γ-aminobutyric acid, GABA.

Introduction

Glutamic acid decarboxylase (GAD) is the biosynthetic enzyme for γ-aminobutyric acid (GABA), an inhibitory neurotransmitter in the central nervous system (CNS). GABA is also formed outside the CNS, and GAD immunoreactivity is reported in non-neuronal tissues such as testis, ovipuct, ovary, and pancreatic islets (5,7,21,26). GAD exists in at least two isoforms. Whereas GAD67 is a cytoplasmic protein consisting of 594 amino acid residues encoded on human chromosome 2, GAD65 is an amphiphilic and membrane-anchored protein consisting of 385 amino acid residues and encoded on human chromosome 10 (4,13,20). There is 65% amino acid sequence homology between the two isoforms, with the highest diversity located at the N-terminus (3,13).

Insulin-dependent diabetes mellitus (IDDM) and stiff-man syndrome (SMS) are associated with autoimmunity to GAD (2,25). Both GAD isoforms have been detected in islets and cerebellum, but recent studies suggest that GAD65 is the targeted isoform in both IDDM and SMS (10,17), although GAD65 antibody epitopes differ for the two diseases (17,24). Since autoimmunity in IDDM and SMS is directed to only one isoform, we hypothesized that cellular distribution of the two isoforms differs. Furthermore, we propose that this difference is in part responsible for the different immune responses to the two isoforms, and may result in the pattern of observed pathophysiology of the two disorders. Before the demonstration of two distinct GAD isoforms (3,8,13), commonly used antisera to GAD usually reacted with both isoforms. In the present study, we (a) produced a panel of sequence-specific antibodies to both GAD isoforms using synthetic peptides as immunogens, (b) tested their specificity by ELISA, immunoblotting, and immunoprecipitation, and (c) used them to investigate cellular distribution of GAD65 and GAD67 in brain and islets by immunocytochemistry.

Materials and Methods

Peptides and Peptide Antibodies. Peptides were synthesized by the Chemical Synthesis Facility, Howard Hughes Medical Institute, University of Washington (Table 1). Each peptide (5 mg) was conjugated to 5 mg keyhole limpet hemocyanin (KLH) with N-maleimido-benzoyl-N-hydroxy-
**Table 1. Characteristics of sequence-specific peptide antibodies**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Isoform</th>
<th>Sequence</th>
<th>ELISA (titers)</th>
<th>Immunoprecipitation</th>
<th>Immunoblotting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GAD65</td>
<td>GAD67</td>
<td>GAD65</td>
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<tr>
<td>A 7309</td>
<td>GAD65</td>
<td>4–22</td>
<td>10⁴</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>B 7134</td>
<td>GAD65</td>
<td>73–91</td>
<td>10⁴</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>C 7641</td>
<td>GAD65</td>
<td>250–269</td>
<td>10⁴</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>D 5565</td>
<td>GAD65</td>
<td>390–403</td>
<td>10⁴</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>E 7647</td>
<td>GAD65</td>
<td>250–269</td>
<td>10⁴</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>F 11616</td>
<td>GAD67</td>
<td>2–19</td>
<td>10⁴</td>
<td>−</td>
<td>−</td>
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</tbody>
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* The E antiserum, 7647, is reactive with GAD67, since it has the same C-terminal sequence (position 578-594).

succinimide as previously described (1,15,23). Coupled peptides (250 μg/ml) were separately emulsified in an equal volume of complete Freund's adjuvant (Sigma; St Louis, MO) and injected intradermally at multiple locations as described (6,13) in separate New Zealand rabbits (R&R Rabbitry; Stanwood, WA). All rabbits were boosted after 15 days with 250 μg KLH-coupled peptide emulsified in incomplete Freund's adjuvant, then bled 4 days later. Each rabbit was subsequently boosted every 10 days and bled 4 days later until a high-titer antiserum was produced.

**ELISA Screening for Sequence-Specific Antibodies.** The synthesized peptides (10 μg/ml) or recombinant GAD65 or GAD67 (3 μg/ml in Tris-HCl buffer, pH 9.1) from the detergent phase of BHK cells (10,13) were used (overnight, 25°C. 100 pl well) to coat bead surfaces on an ELISA beaded lid (Becton Dickinson; Oxnard, CA). The beads were blocked for 60 min at 37°C in 5% (w/v) non-fat dry milk in PBS containing 0.05% Tween-20 (blocking buffer). Sequence-specific antiserum or pre-immune rabbit serum was serially diluted in blocking buffer, added to the 96-well plate, and incubated with the bead lid for 60 min at 25°C. After rinsing in PBS, goat anti-rabbit IgG conjugated with peroxidase (Vector; Burlingame, CA) diluted 1:1,000 in PBS was added for 30 min. After the final wash, 100 μl/well tetramethylbenzidine substrate (1 mg/ml in 0.1 M sodium acetate, pH 5.0) was added for 10 min at 25°C. The reaction was stopped by adding 50 μl of 1 M sulfuric acid. Antibody binding was measured by optical density at 450 nm using a ThermoMax microplate reader (Molecular Devices; Menlo Park, CA).

**Immunoblotting.** Total protein (20 μg/lane) extracted from BHK cells expressing recombinant GAD65 or GAD67 (10,13) was subjected to gel electrophoresis in the presence of sodium dodecyl sulfate (16) on 7% polyacrylamide gels (SDS-PAGE). After electrophoretic transfer (27), the nitrocellulose filters were cut into 5-mm strips for immunoblotting. The strips were first blocked for 60 min in blocking buffer. Strips were then incubated for another 60 min at 25°C with peptide antibodies or pre-immune rabbit serum, all at 1:100 dilution. After washing, strips were incubated for 60 min at 25°C with biotinylated goat anti-rabbit IgG (Vector), washed again, and incubated for 60 min at 25°C with streptavidin-peroxidase complex. Strips were developed with 0.01% diaminobenzidine substrate (Sigma).

**Immunoprecipitation.** The detergent phases of radiolabeled BHK cells expressing recombinant GAD65 or GAD67 (10,13) were separately preincubated at 4°C for 12 hr with normal rabbit serum not subsequently used in the assay. Immunoglobulin was cleared with excess protein A-Sepharose (Zymed; San Francisco, CA). The final supernatant was diluted in 10 mM Hepes buffer (pH 7.4) containing 0.25% BSA, 0.1 mM phenylmethylsulfonylfluoride (Sigma), 0.1% aprotinin (NOVO; Bagsvaerd, Denmark), and 150 mM NaCl. Recombinant antigen extract (5 × 10⁵ cpm in 350 μl per tube) was separately incubated for 12 hr at 4°C with 10 μl of each peptide antiserum or pre-immune serum. Immune complexes were precipi-
Rat Resource, Tissue Preparation, and Immunostaining. The rat tissue was from a diabetes-resistant (DR) diabetes-prone (DP) BB rat line (18,19) maintained under specific pathogen-free (SPF) conditions at the University of Washington. Under the SPF condition, 100% of our diabetes-prone (DP) rats develop diabetes by 6 to 150 days of age, whereas DR rats remain diabetes-free throughout life (12,19). DR rats aged 160-200 days were used in this study. Rats were anesthetized and perfusion-fixed in PBS supplemented with 2% sodium nitrate saline and 4% paraformaldehyde (pH 7.4). Pancreas and brain were removed and fixed overnight at 4°C in 4% paraformaldehyde in PBS (pH 7.4). After rinsing with PBS, tissues were blocked for endogenous peroxidase, incubated for 1 hr at 25°C in preimmune or peptide antiserum. diluted 1:200-1:5000 in PBS containing 1% BSA. In experiments to test specificity, the peptide antiserum was supplemented with 250 μg/ml of either the immunizing or an irrelevant peptide. The sections were then rinsed, incubated in swine anti-rabbit serum (PAP kit system; Dako, Glostrup, Denmark) for 30 min, incubated for 30 min with rabbit PAP (Dako), and finally developed with Dako AEC substrate. Sections were counterstained with hematoxylin and mounted under coverslips with Vectashield mounting medium (Vector).

Double staining was performed with Zymed Histostain-DS broad-spectrum kit. Deparaffinized, rehydrated slides were blocked for endogenous peroxidase, rinsed, blocked with serum blocking solution, incubated for 1 hr in swine anti-glucagon serum (Linco Research; St Louis, MO), washed, incubated for 10 min in biotinylated universal secondary antibody, rinsed, incubated for 10 min in streptavidin–alkaline phosphatase (AP), rinsed, and developed with AP substrate chromogen. After color development, slides were rinsed in distilled water and blocked for 30 min in double-staining enhancer. The second antibody cycle included sequential incubations in GAD antibodies (diluted 1:100), biotinylated universal second antibody, and streptavidin–peroxidase. Finally, slides were developed with peroxidase substrate chromogen (AEC). As a negative control, either the primary or the second antibody was omitted. This showed that there is no cross-over from the second cycle to the first cycle. No counterstain was used. After washing, slides were mounted with Vectashield mounting medium.

Results

High-titer Isoform-specific Antibodies Recognize GAD by Immunoblotting and Immunoprecipitation

Antibody Titer. Analysis of the individual antisera by ELISA against the immunizing peptides showed that the end-point titer for each corresponding antiserum was 1:10,000 and even more for the C-terminal GAD65 antibody 7647 (Table 1). ELISA using recombinant GAD65 or GAD67 instead of peptides, however, gave a much greater range of intensities (Figure 1). Of the six sequence-specific antibodies, antibody 7647 directed to the common C-terminus of GAD recognizes both isoforms with the highest titer (Figure 1). Antibodies 11616, specific for the N-terminus of GAD65, and antibody 11616, specific for the N-terminus of GAD67, both gave strong signals at 1:1000 dilution when tested with their respective antigens.
Antibody Specificity. As indicated in Table 1, antibody 7309 and antibody 11616, directed to the N-terminus of GAD65 and GAD67, respectively, can differentially detect their respective GAD isoforms by both immunoblotting and immunoprecipitation (Figure 2, Lane A and Lane F, respectively). Antibody 7641, raised against GAD65250-269, also specifically recognizes only GAD65 (Figure 2, Lane C). The 40 K band in Lanes A and C (Figure 2A, upper part) could not be confirmed by immunoprecipitation or by immunoblotting with antibody 7647, and it is unclear whether or not this band represents a component of GAD65. Antibody 7647 against the common C-terminus of GAD65 and GAD67 recognizes both isoforms of GAD (Figure 2, Lane E). Antibody 7134, raised against GAD6573-91, immunoprecipitated GAD65 only (Figure 2B, Lane B), but showed some reactivity with GAD67 on the immunoblots (Figure 2A lower panel, Lane B). Antibody 5565 was raised against GAD65390-403, which contains the binding site of GAD co-factor pyridoxal phosphate and has one amino acid substitution of Pro in GAD65 for Leu in GAD67. Specific immunoprecipitation of GAD65 (Figure 2B, Lane D) was observed, but antibody 5565 did not show any staining in the immunoblotting assay (Figure 2A, Lane D). Results for all sequence-specific antibodies are summarized in Table 1.

Immunocytochemistry with Peptide Displacement
All sequence-specific antibodies except for antibody 5565 detected
GAD in paraffin-embedded rat tissues by immunochemical staining (data not shown). Antibodies 7309 (Table 1, A), 11616 (Table 1, F), and 7647 (Table 1, E), were superior in staining intensity. The two N-terminal end antibodies 7309 and 11616 were therefore chosen to study the differential distribution of GAD65 (Figure 3) and GAD67 (Figure 4), respectively. Displacement tests demonstrated that the antibody 7309 staining (Figures 3A, 3C, and 3E) was blocked by immunizing peptide GAD652-19 (Figures 3B, 3D, and 3F) but not by GAD672-19 (data not shown) in both rat islets (Figure 3A) and rat brain (Figures 3C and 3E). Antibody 11616 staining was also (Figures 4A, 4C, and 4E) blocked by immunizing peptide GAD672-19 in rat brain (Figures 4D and 4F) and showed greatly reduced staining in rat islets (Figure 4B). However, antibody 11616 was not blocked by GAD652-22 in any tissue (data not shown). Irrelevant peptides (0.25–1 mg/ml) did not affect the immunostaining of any of the above antisera (data not shown).

Differential Distribution of GAD Isoforms
Both GAD isoforms were detected in GABAergic neurons of rat cerebellum (Figures 3C and 4C) and hippocampus (Figures 3E and 4E). In cerebellum, GAD65 staining was prominent in cell bodies...
and processes (Figure 3C), as was GAD67 staining in cell bodies (Figure 4C). In rat pancreatic islets, GAD65 staining was detected in most islet cells (Figure 3A), whereas GAD67 was detected primarily in peripheral islet cells (Figure 4A). Double staining with our sequence-specific GAD antibodies along with a glucagon antiserum confirmed that GAD65 was detected in centrally located islet cells (Figure 5A). The N-terminal GAD67 antiserum 11616 reacted with peripheral islet cells, including α-cells, as revealed by both double staining with the glucagon antiserum (Figure 5C) and GAD67 11616 antiserum alone (Figure 5D).

Discussion

Glutamic acid decarboxylase is an autoantigen associated with both insulin-dependent diabetes mellitus (IDDM) and stiff-man syndrome (SMS) (2,25). Even though GAD65 and GAD67 isoforms show 65% amino acid identity (3,13), humoral autoimmunity in both diseases is usually restricted to GAD65 (10,17,28). To address whether differences in GAD isoform distribution lead to this autoimmune response or result in different pathological manifestations of this autoimmunity, we have studied GAD isoform distribution in rat pancreas islets and rat brain by immunohistochemistry using GAD isoform-specific antibodies. Our data support a previous study with other antisera (22), showing that rat GAD65 but not GAD67 is primarily localized in islet β-cells.

Autoimmune killing of islet cells in IDDM is specific for β-cells (9). This raises two possibilities regarding GAD autoimmunity. First, specificity of the autoimmune response may be determined by autoactivity to GAD65, resulting in β-cell but not α-cell killing. Second, β-cells could be destroyed by a specific immune response to another antigen or process, but since they contain GAD65, a secondary response only to this isoform is generated. Because it cannot be excluded that a response to GAD65 generated from the latter process then targets β-cells, a combination of the above two possibilities may operate.

In a previous study (22), we reported limited GAD67 expression in β-cells. The 11616 antiserum here reported did not reveal staining of islet β-cells. In contrast, it showed reactivity to peripheral islet cells that double stain with a glucagon antiserum, suggesting co-localization with α-cells. Since GAD67 expression is sensitive to culture conditions (22), lack of α-cell GAD67 in the previous study may result from differences in rat strain, colony environment, tissue preparation, or non-specific reaction. Wistar rats (22) may differ from the inbred DR BB rats (19) used in our experiments. Taken together, the two studies suggest that, when induced, rat islet GAD67 expression occurs primarily in the α-cells.

In rat brain, GAD65 was detected in cerebellar cell bodies and processes. The GAD67 antiserum appeared to stain cell bodies most prominently, in agreement with the subcellular distribution reported previously (4). Furthermore, it is consistent with the effectiveness of GAD65 autoantibodies in SMS in interrupting GABA-based neurotransmission from synapses located along neuron processes. However, 5-μm sectioning may completely miss primary dendrites. A more detailed study using antisera to specific neuron processes along with our GAD antisera should be useful to analyze...
GAD isoform distribution in specific neuronal subcellular compartments.

Knowledge of islet GAD isoform expression is important in understanding experiments investigating IDDM autoimmunity. For example, since only GAD65 is expressed in human islets (14) and since the humoral immune response is primarily to this isoform (10,28), studies using GAD67 to demonstrate humoral GAD immunity in humans (11) must be interpreted with caution. Use of peptides based on selected GAD65 and GAD67 sequences has been very effective in creating isoform-specific antibodies, which are useful in analyzing GAD isoform distribution. Given the markedly different distribution of the two isoforms in islet cells and the gap in knowledge of GAD function in the islet, it is hoped that future studies using these isoform-specific antibodies will help to dissect the function and regulation of GAD isoforms in islet cells and neurons.

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Literature Cited