The Hydrophilic Isoform of Glutamate Decarboxylase, GAD67, Is Targeted to Membranes and Nerve Terminals Independent of Dimerization with the Hydrophobic Membrane-anchored Isoform, GAD65*

(Received for publication, August 5, 1999, and in revised form, September 30, 1999)

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GAD67, the larger isoform of the γ-aminobutyric acid-synthesizing enzyme glutamic acid decarboxylase, is a hydrophilic soluble molecule, postulated to localize at nerve terminals and membrane compartments by heterodimerization with the smaller membrane-anchored isoform GAD65. We here show that the dimerization region in GAD65 is distinct from the NH₂-terminal membrane-anchoring region and that a membrane-anchoring GAD65 subunit can indeed target a soluble subunit to membrane compartments by dimerization. However, only a fraction of membrane-bound GAD67 is engaged in a heterodimer with GAD65 in rat brain. Furthermore, in GAD65−/− mouse brain, GAD67, which no longer partitions into the Triton X-114 detergent phase, still anchors to membranes at similar levels as in wild-type mice. Similarly, in primary cultures of neurons derived from GAD65−/− mice, GAD67 is targeted to nerve terminals, where it co-localizes with the synaptic vesicle marker SV2. Thus, axonal targeting and membrane anchoring is an intrinsic property of GAD67 and does not require GAD65. The results suggest that three distinct moieties of glutamate decarboxylase localize to membrane compartments, an amphiphilic GAD65 homodimer, an amphiphilic GAD65/G67 heterodimer, tethered to membranes via the GAD65 subunit, and a hydrophilic GAD67 homodimer, which associates with membranes by a distinct mechanism.

Glutamic acid decarboxylase (GAD)1 (EC 4.1.1.15) is the key enzyme in the synthesis of γ-aminobutyric acid (GABA) (1). GAD is expressed at comparable levels in GABA-ergic neurons in the central nervous system and in the insulin-producing β cells in the islets of Langerhans (2, 3). GABA has been established as the major inhibitory neurotransmitter in the central nervous system, whereas its role in islet cell function remains elusive (4).

Mammalian species express two isoforms of GAD designated GAD65 and GAD67 in accordance with their relative molecular masses in kDa (3, 5, 6). GAD65 and GAD67 are highly conserved in evolution. Although the two proteins differ substantially in the first 95 NH₂-terminal amino acids, they share a significant homology in the remaining part of the molecule, which contains the catalytic portion of the enzyme (~78% identity, ~95% similarity). GAD65 and GAD67 show significant differences in their levels of expression in different brain regions and steady state saturation with the co-enzyme pyridoxal 5'-phosphate (PLP) (1). More than half of GAD in rat brain is present as the PLP-free apoenzyme (7, 8), and GAD65 constitutes the majority of this reservoir (9–11). GAD67 constitutes a small fraction of the apo-GAD reservoir in the brain (11) and is predominantly found as a holoenzyme tightly associated with PLP (9–11). GAD65, but not GAD67, is a major target of autoimmune responses directed against pancreatic β cells in type 1 diabetes (12, 13) and toward GABA-ergic neurons in a rare disease of the central nervous system, stiff-man syndrome (14).

Most biochemical studies on the quaternary structure of purified GAD are consistent with the idea that the basic subunit structure is dimeric (1, 15). High resolution mapping of conformational epitopes in GAD65, together with two- and three-dimensional structure prediction and alignment of structural folds with decarboxylases of known structure, resulted in a model of the GAD65 dimer (16). In this model, the two GAD65 subunits are joined in the middle region (amino acids 201–461) and related by a P2₁ symmetry. In a separate analysis, several residues in the middle domain were predicted to reach into the cofactor-binding site of the other subunit and interact directly with the phosphate group of PLP and with residues in the other subunit of the enzyme (17). Thus, the middle domains of GAD65 and GAD67 seem to be responsible for the basic homodimeric structure of the enzyme. GAD65 and GAD67 can also associate with each other to form heterodimers (9, 18, 19), and this association has been attributed to the NH₂-terminal domain of GAD65 (18). It is estimated that GAD65/GAD67 heterodimers constitute ~28% of the total GAD activity in cerebellar membranes (19).

Both GAD65 and GAD67 are synthesized in the cytosol as hydrophilic and soluble molecules (20). GAD65 undergoes a stepwise post-translational modification in the NH₂-terminal domain to become hydrophobic and then reversibly membrane-anchored to synaptic-like microvesicles in β cells and synaptic vesicles in neurons (21, 22). A pool of GAD65 is also detected in the Golgi complex region of neurons, pancreatic β cells, and transfected CHO and COS-7 cells (23, 24). The nature and significance of the association of GAD65 with membranes is not
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Experimental Procedures

Antibodies

The GAD65-specific mouse monoclonal antibodies, GAD6 (27), which recognizes a linear epitope localized in the last 41 amino acids of GAD65 (28), and FL-17 (a gift from Dr. Manfred Ziegler), which recognizes a linear epitope in the first 8 amino acids of GAD65, 2 were used in the immunoprecipitation experiments. The 1701 rabbit antiserum was raised against a 19-amino acid peptide in the COOH terminus of rat GAD67 and recognizes GAD65 and GAD67 equally well on Western blots (28). The following antibodies were used in immunofluorescence experiments: (i) the GAD65-specific human monoclonal antibodies MICA 2, 3, and 6, derived from islet cell antibody positive patients, were a gift from Dr. Wiltrud Richter (29); (ii) K2 rabbit antiserum, which recognizes primarily GAD67 on Western blots (9), was purchased from Chemicon (Temecula, CA); (iii) a mouse monoclonal antibody against the SV2 antigen in synaptic vesicle membrane (30) was kindly donated by H. Schwartz, F. Lu¨hder, J. Kim, C. Turck, and S. Baekkeskov, West Grove, PA.

Expression of Wild-type and Mutant Proteins in COS-7 Cells

Wild-type rat GAD65, a deletion mutant of rGAD65 lacking amino acids 1–38 and having the palmitoylation site, Cys45, mutated to Ala (rGAD65/45AΔ1–38), and a deletion mutant of rGAD65 lacking amino acids 1–101 (rGAD65Δ1–101) in the COS expression vector pSVSPORT were described previously (26). A 2.0-kb cDNA for mouse GAD67 (a gift from Dr. Roland Tisch, University of North Carolina, Chapel Hill) containing the entire coding region and 92 and 181 bp of 5′- and 3′-translational sequences, respectively, was excised from a Bluescript II SK+ (Stratagene, La Jolla, CA) as an XhoI–XhoI fragment and subcloned into the SmI site of the pSVSPORT vector. COS-7 cells were cultured and transiently transfected with LipofectAMINE (Life Technologies, Inc.) as described previously (25).

Detergent Extraction of COS-7 Cells and Brain

Transfected COS-7 cells from an 80–90% confluent 10-cm plate were washed twice in ice-cold cell harvesting buffer (20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 10 mM benzamidine/HCl) and extracted by incubation on a shaker for 1 h at 4 °C in 0.5 ml of hypotonic HEPES buffer (HMAP buffer) (10 mM Hepes/NaOH, pH 7.4, 1 mM MgCl2, 1 mM 2-aminoethylisothiouronium bromide, 0.2 mM PLP, 5 mM EDTA, 0.1 mM p-chloromercuriphenyl sulfonic acid, 1 mM leupeptin, 1 mM phenyl-methylsulfonyl fluoride, 10 mM benzamidine, 0.1 mM Na3VO4, 5 mM NaF, and 5 mM N-ethylmaleimide (NEM) containing 1% Triton X-114). The lysate was centrifuged at 150,000 ×g for 30 min to remove insoluble debris.

Subcellular Fractionation

Subcellular fractionation of cells was carried out using a modification of methods described previously (26). All procedures were carried out at 4 °C. Transfected COS-7 cells, grown to 80–90% confluence, were harvested from 10-cm plate, were washed twice in ice-cold cell harvesting buffer and homogenized on ice using a glass homogenizer with 0.5 ml of HMAP buffer. The homogenate was centrifuged at 800 ×g for 10 min to remove nuclei and cellular debris. The postnuclear supernatant was centrifuged at 150,000 ×g for 1 h in a TLA 100.3 rotor using a Beckman tabletop ultracentrifuge (Beckman, Palo Alto, CA) to separate cytosol and crude membrane fractions. Sedimented membranes were resuspended in 0.5 ml of membrane washing buffer (HMAP buffer containing 0.5 mM NaCl) and incubated for 1 h, followed by ultracentrifugation at 150,000 ×g for 1 h to pellet the washed membranes. Washed membranes were extracted for 1 h with 0.5 ml of extraction buffer (HMAP buffer containing 150 mM NaCl and 1% Triton X-114) followed by centrifugation for 1 h at 150,000 ×g to separate particulate extract from insoluble debris.

For the subcellular fractionation of rat and mouse brain, Harlan Sprague Dawley male rats (250–300 g) were anesthetized with metofane, and the cerebral cortex and cerebellum were dissected quickly. A 15% (w/v) portion of tissue in HMAP buffer was homogenized with the use of a Tissumizer polytron (Tekmar, Cincinnati, OH). The rest of the procedure was carried out as described above for COS-7 cells.

Triton X-114 Phase Separations

Triton X-114 partitioning assays were performed on the subcellular fractions of mouse brain using a modification of the procedure of BORDER (31). For comparative analyses of the amphiphilic properties of GAD65 and GAD67 in subcellular fractions, buffer compositions were adjusted to achieve identical conditions in each fraction. Aliquots of each fraction were subjected to three rounds of temperature-induced phase transitions using 1% Triton X-114. Following the incubation at 37 °C for 5 min, the detergent and aqueous phases were separated by centrifugation at 12,000 ×g for 2 min at room temperature. The detergent phase from the first and second rounds of phase separations was washed carefully with HMAP buffer containing 150 mM NaCl and resuspended with the same buffer to the original volume. Triton X-114 was added to the aqueous phase obtained after the third round of phase separation to a final concentration of 1%.

Immunoprecipitations

For the immunoprecipitation of GAD65 from cell-free extracts of transfected COS-7 cells, an aliquot of 250 μl from each extract was incubated with 30 μl of the GAD65-specific monoclonal antibody, FL-17, for 2 h at 4 °C with constant shaking. Immunocomplexes were isolated by incubation for 1 h at 4 °C with 50 μl of 50% solution of protein G-Sepharose (PGS; Amersham Pharmacia Biotech, Uppsala, Sweden) in immunoprecipitation buffer (IP buffer, 10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1 g/liter bovine serum albumin, 10 mM benzamidine, and 0.5% Triton X-114). For the immunoprecipitation of GAD65 from rat brain subcellular fractions, 100-μl aliquots from each of the cytosol and membrane extracts were diluted 5-fold in HMAP buffer containing 1% Triton X-114 and 150 mM NaCl and precleared by

2 H. Schwartz, F. Lu¨hder, J. Kim, C. Turck, and S. Baekkeskov, unpublished results.
incubation for 30 min at 4 °C with 100 μl of preswollen protein A-Sepharose (PAS; Amersham Pharmacia Biotech) in IP buffer. Samples were then centrifuged at 750 × g for 1 min, and the resulting supernatants were incubated with 5 μl of GAD65 ascites. After 3 h of incubation at 4 °C with constant shaking, immunocomplexes were isolated by incubation with 100 μl of PGS (100 μl) for 1 h at 4 °C. The PGS-bound PAS-bound immunocomplexes were washed five times with IP buffer, and proteins were eluted from PGS or PAS by boiling for 5 min in SDS-sample buffer followed by centrifugation to remove PGS or PAS.

**Electrophoresis and Immunoblotting**

To estimate the subunit composition of native GAD65 transiently expressed in COS-7 cells, samples from the cell lysate obtained after removing the cell debris were pretreated in non-denaturing and non-reducing sample buffer (0.5% Triton X-100, 8.8, 0.005% bromophen blue, 10% glycerol) and then analyzed by electrophoresis on a native precast 10 or 4–20% linear gradient of polyacrylamide gels (Novex, San Diego, CA). Standard molecular mass standards for native gels were from Amersham Pharmacia Biotech and consisted of thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa). For the analysis under nonreducing conditions to test the presence of disulfide-linked trimers of GAD65, samples were boiled for 3 min in sample buffer containing 2% SDS without reducing agent and then analyzed by SDS-PAGE on 10% polyacrylamide gels. For quantitative analyses of the ratio of GAD65 and GAD67 in immunoprecipitates and in cytosol and membrane fractions of brain, serial dilutions of each fraction were subjected to SDS-PAGE.

**Immunofluorescence Analyses**

After 2 weeks of growth, cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature. All of the subsequent steps were carried out at room temperature. Permeabilization of the cells and blocking of nonspecific binding were performed by incubation for 20 min with 1% bovine serum albumin, 0.1% Triton X-100 in phosphate-buffered saline (blocking buffer). For co-localization of both GAD65 and GAD67 in rat hippocampal neurons, the human monomolar antibodies to GAD65 (MICA 2, 3, and 6, diluted 1:250) along with rabbit antibodies to GAD67 (K2, diluted 1:250) were applied in blocking buffer. After 1 h of incubation, immunoreactivity was visualized by secondary antibody staining using donkey anti-human IgG conjugated to Cy3 and goat anti-rabbit IgG conjugated to fluorescein isothiocyanate. Identification of GAD clusters and their co-localization with synaptic markers was accomplished with dual color microscopy using a Nikon ×100 objective (NA 1.4), mercury arc lamp illumination and standard fluorescein and Cy3 filter sets (Omega). Fluorescent images were acquired using a cooled digital CCD camera (Princeton Instruments, Inc.). Minimal bleed-through between channels was confirmed by imaging single-labeled specimens. 12-bit images were linearly written to 8-bit data set after normalizing images to maximize usable contrast range using IPLAP Spectrum software (Syngral Analytics). For display in figures, monochrome and merged colors were processed using Adobe Photoshop software (Adobe Systems, San Jose, CA). In unprocessed raw 8-bit data, synaptic structures (identified as SV2-positive puncta) were considered co-localized with GAD if the intensity of immunoreactivity was >2-fold higher than the background level. Most GAD-positive synaptic structures had fluorescence intensities >4-fold higher than background.

**RESULTS**

**Specific and Nonspecific Oligomerization of GAD65 and GAD67**—Each of the GAD isoforms contains a large number of cysteine residues, suggesting a propensity for forming nonspecific disulfide bridges in nonreducing conditions. It was therefore important to establish conditions for maintaining the native structure of the proteins during analysis.

In analyses of the oligomerization state of GAD65 expressed in COS-7 cells using SDS-gel electrophoresis in the presence or absence of β-mercaptoethanol (β-ME), the protein was detected as a monomer, dimer, and trimer under nonreducing but denaturing conditions, whereas under reducing and denaturing conditions, the protein was detected as a monomer and dimer.
conditions all of the protein was detected as a monomer (Fig. 1A), suggesting the presence of disulfide-linked bridges. The formation of disulfide-linked dimers and sometimes trimers was also observed for GAD65 and GAD67 expressed in mouse and rat brain (Fig. 1C and data not shown). The formation of disulfide-linked oligomers was, however, significantly diminished or prevented when lysis of COS-7 cells was performed under nonreducing (Fig. 1D) and reducing conditions (results not shown). The native subunit structure of rGAD65 expressed in COS-7 cells did not change when lysis of cells was performed in the presence or absence of 200 \( \mu \text{M} \) of the coenzyme PLP or in the presence or absence of 10 \( \text{mM} \) L-glutamate (data not shown). This is in contrast to results obtained for Escherichia coli GAD65, in which the dimer assembly to form a hexamer is enhanced by the addition of PLP (35).

**A Membrane-anchoring GAD65 Subunit Mediates a Robust Membrane Association of a Soluble Subunit by Dimerization**—If soluble GAD67 associates with membranes by heterodimerization with a membrane-anchored GAD65 subunit as suggested by Dirkx et al. (18), this would imply that membrane anchoring of only one GAD65 subunit suffices to achieve membrane association of a homo- or heterodimer. To test this possibility, we first addressed the question whether soluble mutants of GAD65, lacking the membrane-anchoring NH\(_2\)-terminal region, can form dimers. As shown in Fig. 1D (lanes 4 and 5), the deletion mutants rGAD65/45A1–38 and rGAD65Δ1–101, which are soluble when expressed in COS-7 cells (22, 26), are detected exclusively as noncovalently linked disulfide bridges, all subsequent studies of GAD oligomerization were performed in the presence of 5 mM NEM. In these conditions the native form of wild-type rGAD65 is a noncovalently linked dimer in both the cytosol and membrane fractions of COS-7 cells, as revealed by nondenaturing gel electrophoresis under nonreducing (Fig. 1D) and reducing conditions (results not shown). The native subunit structure of rGAD65 expressed in COS-7 cells did not change when lysis of cells was performed in the presence or absence of 200 \( \mu \text{M} \) of the coenzyme PLP or in the presence or absence of 10 \( \text{mM} \) L-glutamate (data not shown). This is in contrast to results obtained for Escherichia coli GAD65, in which the dimer assembly to form a hexamer is enhanced by the addition of PLP (35).

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These results show that a firm membrane anchoring of a dimer only requires tethering of one GAD65 subunit to membranes. We have previously shown that the two subunits in a GAD65 dimer undergo distinct post-translational modifications and that only one is phosphorylated in membranes (25). Thus, the two subunits in the GAD65 dimer are not identical and probably serve distinct roles in regulation of membrane trafficking of the protein.

A Pool of Rat Brain GAD67 Forms a Noncovalently Linked Dimer with GAD65 in the Cytosol and Membrane Fractions, and Dimerization Does Not Involve the NH2-terminal Region of GAD65—The formation of heterodimers between GAD65 and GAD67 (18, 19) was suggested to involve the NH2-terminal region of GAD65 (18). Thus, dimerization with and membrane tethering of soluble GAD67 was suggested to be mediated by the same region of GAD65 (18).

Dirks et al. (18) showed that heterodimerization with GAD67 does not involve formation of nonspecific disulfide bridges through cysteines in the NH2-terminal region of GAD65. However, the possibility that a population of GAD65/GAD67 heterodimers detected in vitro is formed by nonspecific formation of disulfide bridges involving other regions of GAD65 was not addressed. We therefore studied the heterodimerization of GAD65 and GAD67 in the presence of 5 mM NEM during lysis and subcellular fractionation of cells and subsequent immunoprecipitation to prevent the formation of nonspecific disulfide bridges between any parts of the two proteins.

GAD67 was detected in both cytosol and membrane fractions of rat brain (Fig. 3A, lanes 1 and 5), confirming that in neurons both GAD65 and GAD67 are associated with membranes (18, 19). Furthermore, GAD67 was recovered in immunoprecipitates with a GAD65-specific antibody, GAD6, in both cytosol and membrane fractions, demonstrating that the two proteins specifically associate into dimers (Fig. 3A, lanes 2–4 and 6–8). Similar results were obtained using the anti-GAD65-specific monoclonal antibody FL-17, which is directed against the first 8 amino acids of GAD65 (data not shown). The identity of the two isoforms in the Western blot (Fig. 3A) was confirmed by stripping bound antibodies and reprobing the blot first with the anti-GAD67-specific antibody, K2, and then with the anti-GAD65-specific antibody, FL-27 (data not shown).

To study the role of the NH2-terminal region of GAD65 in the formation of heterodimers, we tested whether the NH2-terminal deletion mutants of this isoform can associate with GAD67. The GAD65/45AΔ1–38 and rGAD65/45Δ1–38 mutants were each coexpressed with mGAD67 in COS-7 cells and immunoprecipitated with the GAD65-specific monoclonal antibody GAD6. As shown in Fig. 3B, mGAD67 was co-precipitated with both rGAD65/45AΔ1–38 (lane 6) and rGAD65Δ1–101 (lane 10), demonstrating that the NH2-terminal 101 amino acids of GAD65 are not involved in the heterodimerization with GAD67. Thus, hetero-homodimerization and membrane anchoring are mediated by distinct GAD65 regions.

GAD67 Is Associated with Membrane Fractions Independent of GAD65—The results shown above are consistent with an ability of GAD65 to mediate membrane anchoring of GAD67 in a heterodimer. Densitometric analyses of total GAD67 and GAD67 in a complex with GAD65 (GAD6 immunoprecipitate) obtained from three independent rat brain extracts (a representative immunoblot is shown in Fig. 3A), however, indicate that while the ratio between GAD65 and GAD67 is about 1:1 in membrane fractions prepared from rat cerebellum/cerebral cortex (Fig. 3A, lanes 1 and 5), the ratio in immunoprecipitates of GAD65 from those fractions (Fig. 3A, lanes 4 and 8) is approximately 3:1. Similar ratios between GAD65 and GAD67 were obtained for membrane fractions prepared from three extracts.
of mouse cerebellum/cerebral cortex (a representative blot is shown in Fig. 4, lanes 1 and 3). These results suggest that approximately 33% of GAD67 in membrane fractions of rat and mouse brain is present as a heterodimer with GAD65, consistent with results obtained with rat cerebellar membranes in the absence of alkylating reagents (19). Thus, the majority of GAD67 appears to reside in membranes as a homodimer independent of dimerization with GAD65, suggesting that GAD67 can become membrane-anchored by a mechanism distinct from dimerization with GAD65.

To clarify whether GAD67 is strictly dependent on GAD65 for its localization to membranes, the subcellular distribution of GAD67 was analyzed in the brains of GAD65 knock-out mice (GAD65Δ/Δ) and their wild-type littermates. Before extraction with 1% TX-114, membranes were washed three times by incubation on ice for 1 h with HMAP buffer containing 0.5 M NaCl. Proteins were resolved by SDS-PAGE. Immunoblotting was performed with the 1701 antibody. GAD67 is co-precipitated with both rGAD65/45AΔ1–38 (lane 6) and rGAD65Δ1–101 (lane 10).

GAD67 is hydrophilic in rat islets and transfected fibroblasts (20, 22, 23). To test whether the membrane association of GAD67 is mediated by a specific hydrophobic modification of the protein in neurons, we assessed the ability of GAD67 in cytosolic and membrane fractions of mouse brain to partition into the detergent phase following a temperature-induced Triton X-114 phase separation. Whereas a portion of GAD67 in both cytosolic and membrane fractions prepared from wild-type (GAD65Δ/Δ) mouse brains partitioned into the detergent phase (Fig. 5, lanes 2 and 6), all GAD67 remained in the aqueous phase in the absence of GAD65 (Fig. 5, lanes 3, 7, and 11), suggesting (i) that the partition of GAD67 into the detergent phase is mediated by its association with GAD65 in a heterodimer and (ii) that GAD67 is not by itself hydrophobic. Densitometric analysis of the immunoblot (Fig. 5, lanes 2 and 6) demonstrates that the ratio between GAD65 and GAD67 is approximately 3:1 and 2.6:1 in the detergent phase of the cytosolic and membrane fractions, respectively. Since these ratios are similar to that obtained for immunoprecipitates of (GAD65Δ/Δ) mouse brains partitioned into the detergent phase (Fig. 5, lanes 2 and 6), all GAD67 remained in the aqueous phase in the absence of GAD65 (Fig. 5, lanes 3, 7, and 11), suggesting (i) that the partition of GAD67 into the detergent phase is mediated by its association with GAD65 in a heterodimer and (ii) that GAD67 is not by itself hydrophobic. Densitometric analysis of the immunoblot (Fig. 5, lanes 2 and 6) demonstrates that the ratio between GAD65 and GAD67 is approximately 3:1 and 2.6:1 in the detergent phase of the cytosolic and membrane fractions, respectively. Since these ratios are similar to that obtained for immunoprecipitates of
GAD65 from cytosolic and membrane fractions of rat brain (Fig. 3A, lanes 4 and 8), we conclude that association with GAD65 accounts for approximately all GAD67 found in the detergent phase of the cytosol and membrane fractions prepared from wild-type mouse brain. These results suggest that the association of GAD67 to membranes in the GAD65 knock-out mouse brain is not mediated by its own hydrophobicity but rather by interaction with membrane components that are distinct from GAD65 and do not confer hydrophobicity upon GAD67.

We next addressed the question whether GAD67 can mediate membrane association of GAD65 mutants (rGAD65/45A,D1–38 and rGAD65/D1–101) that cannot by themselves localize to membranes. Membrane and cytosol fractions of COS-7 cells coexpressing these mutants together with mGAD67 were subjected to immunoprecipitation with the GAD65-specific antibody GAD6. In these experiments, the heterodimers between GAD67 and the soluble mutant subunits were only detected in the cytosol fraction (results not shown). Therefore, although GAD67 can associate with membranes, it cannot mediate membrane association of a soluble GAD65 subunit.

GAD67 Localization to Nerve Terminals in Primary Cultures of Neurons Is Independent of GAD65—It has been suggested that the association of GAD67 with synaptic vesicles from rat brain and its localization to nerve terminals of GABA-ergic neurons, where synaptic vesicles are clustered, is mediated by its interaction with GAD65 (18, 36). However, GAD67 was also found to localize to the terminals of dentate granule cells of the hippocampus, which contain little or no GAD65 (37). To examine the role of GAD65 in targeting GAD67 to nerve terminals, we studied the localization of GAD67 in cultured rat hippocampal neurons prepared from wild-type embryos, where GAD67 is expressed together with GAD65, and in cultured cortical neurons prepared from GAD65 deficient mouse embryos, where GAD67 is expressed alone.

Double immunostaining of 2-week-old primary cultured rat hippocampal neurons with anti-GAD67 and anti-GAD65 antibodies revealed that in addition to its diffuse distribution in the cell body and proximal dendrites, a pool of GAD67 co-localized with GAD65 in the perinuclear region and in punctate structures (Fig. 6). The punctate structure localization of GAD65 and GAD67 was demonstrated to correspond to axon terminals by triple labeling with the antibodies directed against the synaptic vesicle protein SV2 (Fig. 7, white color). Immunostaining of 2-week-old primary cultured cortical neurons prepared from homozygous mutant mouse embryos (GAD65/−/−) and their heterozygous littermates (GAD65+/−), demonstrated that GAD67 is localized to nerve terminals both in the presence (Fig. 6A) and absence of GAD65 (Fig. 6B). We also performed immunostaining of primary cultures of cortical neurons obtained from homozygous mutant GAD67−/− mouse embryos and their wild-type GAD67+/+ littermates. In those cultures, GAD65 was localized to nerve terminals in the presence and absence of GAD67 (data not shown). Taken together, these results indicate that although the nerve terminals of neurons obtained from wild-type mouse embryos were always positive for both GAD67 and GAD65, the two isoforms localize independently to nerve terminals.
DISCUSSION

Targeting to Nerve Terminals and Membrane Anchoring Is an Intrinsic Property of GAD67—The evolution of two different isoforms of glutamic acid decarboxylase in mammals provides a fundamental aspect of regulation of GABA-ergic neurotransmission. This is best evidenced by the distinct phenotypes of GAD65+/− and GAD67+/− mice. GAD67 provides >90% of brain GABA, and GAD67 deficiency results in neonatal death (38, 39). In contrast, GAD65+/− mice are viable and have near normal GABA levels in brain but develop spontaneous seizures and are deficient in the fine tuning of inhibitory neurotransmission involved in responses to a number of stimuli (11, 40, 41). Both forms are synthesized as soluble hydrophilic molecules (20). Based on experiments in pancreatic β cells, hydrophobic modifications and anchoring to the membrane of synaptic vesicles in neurons and synaptic-like microvesicules in pancreatic β cells was described to be an exclusive characteristic of GAD65 (20–22). Thus, targeting of GAD67 to membrane compartments was attributed to heterodimerization with GAD65 (18, 19). We and others have proposed that the anchoring of GAD65 to the membrane of synaptic vesicles distinguishes it from GAD67 and provides a mechanism by which GAD65-generated GABA is more rapidly available for secretion (26). The results presented here, however, show that targeting to nerve terminals and anchoring to membranes are also intrinsic properties of GAD67 and occur independent of an association with GAD65. Thus, the two isoforms share properties that are not attributed to GAD65. It will be important to establish whether the dynamics, location, and control of membrane anchoring differ for the two isoforms and how these properties translate to the differences in their function.

GAD65 and GAD67 Form Specific Noncovalently Linked Heterodimers—The native forms of both GAD65 and GAD67 are noncovalently linked homodimers (Ref. 19 and results presented here). However, because of the large number of cysteines, each isoform has a tendency to form nonspecific disulfide-linked dimers and multimers in vitro (Ref. 34 and results presented here). In view of this phenomenon, it was important to establish whether the formation of heterodimers of the two forms (18, 19) was specific or due to an in vitro formation of disulfide bridges. Although Dirkx et al. (18) presented evidence that -SH groups in the NH2-terminal region of GAD65 were not involved in the dimerization with GAD67, a role of other sulphydryl groups was not assessed. In the present study, the tendency of recombinant GAD65 and endogenous GAD65 and GAD67 to form disulfide-linked multimers was almost completely prevented by blocking the reactive cysteines in both isoforms with the alkylation reagents NEM or iodoacetamide. Under alkylation conditions, a significant fraction of GAD65 and GAD67 was still detected as heterodimers, demonstrating that the association between the two isoforms is specific and does not require nonspecific formation of disulfides. We therefore conclude that heterodimers of GAD65/GAD67 are formed in vivo.

Homo- and Heterodimerization of GAD65 Does Not Involve the NH2-terminal Membrane-anchoring Region—Molecular modeling studies have suggested that the middle domain of each subunit is involved in GAD65 dimerization (16, 17). Since there are no proteins of known structure that share sequence homology with GAD65 in the first 100 amino acids at the NH2 terminus of GAD65 (16), a structure prediction is not available for this region. The results presented here show that the GAD65Δ1–101 mutant is exclusively detected as a dimer in nondenaturing gels and therefore provide the first evidence that the NH2-terminal region of GAD65 is not important for homodimerization. Thus, the predicted interactions of several residues in the middle domain of one subunit with PLP in the active site of the second subunit may represent the main bonds between the subunits (17).

Furthermore, the GAD65Δ1–101 mutant forms a heterodimer with GAD67, demonstrating that the NH2-terminal region of GAD65 is not required for heterodimerization. Dirkx et al. (18) linked the first 83 amino acids of GAD65 to β-galactosidase and suggested based on immunofluorescence studies that this protein could mediate localization of GAD67 to the Golgi complex region when the two proteins were co-expressed in CHO cells. Based on this indirect evidence, it was concluded that the NH2-terminal region of GAD65 mediates heterodimerization with GAD67. However, as demonstrated in this study, GAD67 can associate with membranes independent of GAD65. It is, therefore, possible that the localization of GAD67 to the Golgi region observed in CHO cells (18) occurred independent of the GAD65(1–83)/β-gal protein.

The first 101 amino acids of GAD65 are demonstrably not necessary for heterodimerization. Therefore, in light of the high homology between GAD65 and GAD67 in the remainder of the molecule, we propose that the structural interactions involved in heterodimerization may be similar to those for homodimerization (19).

What Is the Role of GAD65/GAD67 Heterodimerization?—The results presented here establish that the two enzymes GAD65 and GAD67 specifically form noncovalently linked heterodimers in addition to homodimers and that each homodimer associates with membranes. Firm membrane association of a dimer can be achieved by only one membrane anchoring-competent GAD65 subunit. This infers that a heterodimer between a hydrophilic GAD67 subunit and a membrane-anchoring GAD65 subunit can localize to membranes. In contrast, a heterodimer between a soluble GAD65 subunit and a membrane anchoring-competent GAD65 subunit does not anchor to membranes. The realization that GAD67 can mediate its own association to membranes but cannot do so for soluble GAD65...
implicates a homodimer-dependent association that is distinct from that of GAD65. We suggest that the GAD67/GAD67 homodimer has a distinct microlocalization within membrane compartments of nerve terminals to the GAD65/GAD65 and GAD65/GAD67 dimers and that this form can therefore impart different GABA secretion responses.

Acknowledgments—We thank Dr. Roger Nicoll and Christian Billette for help in establishing cultures of primary neurons, Dr. Mark von Zastrow for help with the immunofluorescence analyses, Dr. Wiltrud Richter for donating the MICA 2, 4, and 6 supernatants, Dr. Mannfred Ziegler for donating the FL 17 antibody, Dr. Ronald Tisch for mGAD65 cDNA, and Dr. Allan Tobin for a human GAD65 cDNA.

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