Association of L-Glutamic Acid Decarboxylase to the 70-kDa Heat Shock Protein as a Potential Anchoring Mechanism to Synaptic Vesicles*

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Recently we have reported that the membrane-associated form of the γ-amino butyric acid-synthesizing enzyme, L-glutamate decarboxylase (MGAD), is regulated by the vesicular proton gradient (Hsu, C. C., Thomas, C., Chen, W., Davis, K. M., Foos, T., Chen, J. L., Wu, E., Floor, E., Schloss, J. V., and Wu, J. Y. (1999) J. Biol. Chem. 274, 24366–24371). In this report, several lines of evidence are presented to indicate that L-glutamate decarboxylase (GAD) can become membrane-associated to synaptic vesicles first through complex formation with the heat shock protein 70 family, specifically heat shock cognate 70 (HSC70), followed by interaction with cysteine string protein (CSP), an integral protein of the synaptic vesicle. The first line of evidence comes from purification of MGAD in which HSC70, as identified by the vesicular proton gradient (Hsu, C. C., Thomas, C., Chen, W., Davis, K. M., Foos, T., Chen, J. L., Wu, E., Floor, E., Schloss, J. V., and Wu, J. Y. (1999) J. Biol. Chem. 274, 24366–24371). In this report, several lines of evidence are presented to indicate that L-glutamate decarboxylase (GAD) can become membrane-associated to synaptic vesicles first through complex formation with the heat shock protein 70 family, specifically heat shock cognate 70 (HSC70), followed by interaction with cysteine string protein (CSP), an integral protein of the synaptic vesicle. The first line of evidence comes from purification of MGAD in which HSC70, as identified from amino acid sequencing, co-purified with GAD. Second, in reconstitution studies, HSC70 was found to form complex with GAD65 as shown by gel mobility shift in non-denaturing gradient gel electrophoresis. Third, in immunoprecipitation studies, again, HSC70 was co-immunoprecipitated with GAD by a GAD65-specific monoclonal antibody. Fourth, HSC70 and CSP were co-purified with GAD by specific anti-GAD immunopurification columns. Furthermore, studies here suggest that both GAD65 and GAD67 are associated with synaptic vesicles along with HSC70 and CSP. Based on these findings, a model is proposed to link anchorage of MGAD to synaptic vesicles in relation to its role in γ-amino butyric acid neurotransmission.

L-Glutamate decarboxylase (GAD; EC 4.1.1.15) is the rate-limiting enzyme involved in the synthesis of γ-amino butyric acid (GABA), a major inhibitory neurotransmitter in the mammalian brain (1). There are two well characterized GAD isoforms in the brain, namely GAD65 and GAD67, referring to GAD with molecular masses of 65 and 67 kDa, respectively (for review, see Ref. 2). GAD67 is mostly soluble and is distributed evenly throughout the cell, whereas GAD65 is concentrated at the nerve terminals (3) and constitutes the majority of the membrane-associated GAD (MGAD) (4, 5). Despite its importance, our knowledge regarding the regulation of GAD activity is quite limited. Recently, we have shown that soluble GAD (SGAD) is activated by dephosphorylation, mediated by a Ca2+- dependent phosphatase, calcineurin, and is inhibited by phosphorylation, mediated by a CAMP-dependent protein kinase A (6, 7). Conversely, MGAD is activated by protein phosphorylation, which depends on the integrity of the electrochemical gradient of synaptic vesicles (5). Hence, GAD activity appears to be regulated differently depending on whether it exists as a soluble or membrane-anchored protein. Judging from the amino acid sequences, it is unlikely that GAD65 and/or GAD67 can be integral membrane components, since neither contains a stretch of hydrophobic amino acids long enough to span the membrane (greater than 20 residues), a typical feature for integral membrane proteins. Furthermore, both isoforms lack the appropriate consensus sequences for the attachment of GAD to membranes through fatty acylation via esterification, or N-myristoylation (2).

Now evidence is presented here to show that GAD can become membrane-associated through interactions with a member of the heat shock protein 70 family, heat shock cognate 70 (HSC70), which is then anchored to synaptic vesicles through interactions with an intrinsic synaptic vesicle protein, cysteine string protein (CSP). In addition, a model is also proposed to show the anchoring mechanism of GAD to synaptic vesicles and a functional link between GABA synthesis and vesicular GABA transport at the nerve terminals.

EXPERIMENTAL PROCEDURES

Materials—Fresh porcine brains were obtained from a local abattoir. Bovine brain HSC70 and biotinylated HSC70 were obtained from StressGen Biotechnologies Corp. (Collegeville, PA). Anti-GAD65 and anti-GAD67 are polyclonal rabbit antibodies raised against recombinant human GAD65 (HGAD65) and human GAD67 (HGAD67) expressed in separate bacterial systems (8). Subtype-specific polyclonal antibodies of GAD65 and GAD67 were prepared by preadsorbing anti-HGAD65 serum with an excess of recombinant HGAD65 to remove GAD67-specific antibodies. Similarly, the specific anti-HGAD67 serum was obtained by pretreatment of anti-HGAD67 serum with an excess of recombinant HGAD67 as described previously (8). GAD67-specific monoclonal antibody, was purchased from Developmental Studies Hybri-dom Bank (University of Iowa, Iowa City, IA). Rabbit polyclonal anti-HSP70 sera were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-CSP rabbit sera were purchased from Chemicon In-

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ternational, Inc. (Temecula, CA). Benzenthionium hydroxide (hyamine base, 1 m solution in methanol), pyridoxil 5'-phosphate (PLP), 2-aminoethyliothiuronium bromide (AET), dithiothreitol, ATP, AMP-PNP, Nycodenz, and Triton X-100 were purchased from Sigma. Ceramic hydroxyapatite, acrylamide, bisacrylamide, tetramethylethylenediamine, ammonium persulfate, and β-mercaptoethanol and protein assay kit were purchased from Bio-Rad. [L-34C]Glutamic acid was purchased from NEN Life Science Products, and Western-Lite Plus immunodetection kit was purchased from Tropix Inc. (Bedford, MA). HiTrap protein A columns, N-hydroxysuccinimide-activated Sepharose columns, and ECL reagent were purchased from Amersham Pharmacia Biotech. All other chemicals were of the purest grade commercially available.

**Enzyme Assay—**GAD was assayed by a radiometric method measuring the formation of [14C]CO₂ from [L-34C]glutamic acid as described previously (9).

**Preparation of Synaptosomal Membranes—**Unless mentioned otherwise, all purification procedures were carried out at 4 °C, and in standard GAD buffer containing 50 mM potassium phosphate, 1 mM AET, 0.2 mM PLP at pH 7.2. In a typical preparation, a 15% (w/v) porcine brain homogenate was made with a glass-Teflon homogenizer in standard GAD buffer containing 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, and 1 mM theophylline. Preparation of crude synaptosomes was performed as described previously (6, 7). Briefly, fresh porcine brains were homogenized in 0.32 M sucrose (w/v, 15 g/100 ml), and the rate was centrifuged at 100,000 g for 10 min to remove cell debris and the nuclear fraction. The post-nuclear supernatant solution was centrifuged at 23,000 g for 30 min, and the pellet thus obtained was the crude synaptosomal fraction referred to as P₂.

**Purification of MGAD—**Purification of MGAD was conducted, as described previously with slight modifications (10–12). The P₂ fraction obtained from the preceding step was first lysed in standard GAD buffer, followed by centrifugation at 100,000 g for 1 h. The liquid thus obtained was referred to as supernatant (S₁). The resulting pellet, S₁M, was solubilized with 0.5% Triton X-100 in standard GAD buffer solution. The solubilized MGAD was further purified through conventional column chromatography consisting of an anion exchange (DEAE-52), an adsorption (ceramic hydroxyapatite), and a gel filtration column (Sephadex G-200). In addition to the conventional column procedures, MGAD was also purified by anti-GAD immunofinity column as described previously (13) with slight modifications. Specific anti-GAD IgG was prepared from anti-GAD sera using protein A column chromatography. Briefly, anti-GAD sera were first loaded on protein A columns, followed by washing with eight column volumes of buffer containing 20 mM sodium phosphate at pH 7. The column was then eluted with six column volumes of buffer containing 0.1 M sodium citrate, pH 3.0, and the eluate was neutralized to pH 7.0 with pre-titrated amount of 1 N NaOH. Anti-GAD IgG thus purified was coupled to N-hydroxysuccinimide-activated Sepharose according to the manufacturer’s instructions (Amersham Pharmacia Biotech). Anti-GAD IgG immunofinity columns were then used for purification of GAD and GAD-associated protein complex as detailed in the following section.

**Recombinant Human Brain GADα and GADβ—**Recombinant human brain GADα and GADβ, referred to as HGADα and HGADβ, respectively, were prepared as described recently (8). Briefly, HGADs were overexpressed in DH5α Escherichia coli, which were transformed with either a HGADα or HGADβ recombinant pGEX-3X plasmid, as glutathione S-transferase (GST) fusion proteins. GST-HGAD fusion proteins were first purified by glutathione affinity column chromatography, followed by cleavage of the fusion proteins with factor Xa. Final purification of free HGADα and HGADβ were achieved using repetitive glutathione affinity column (8).

In Vitro Reconstitution Assay Using Non-denaturing Gradient Polyacrylamide Gel Electrophoresis (NG-PAGE)—Biotinylated bovine HSC70 (2 μg) was incubated at 37 °C for 1 h with either HGADα (2 μg) or HGADβ (2 μg) in a final volume of 10 μl of 50 mM Tris-citrate (pH 7.2). The resulting protein complexes were analyzed by electrophoresis on 5–25% NG-PAGE under nonreducing conditions. Running conditions were modified from the procedure described previously (14). One volume of the protein sample was mixed with two volume of sample buffer containing 50 mM Tris-Cl, pH 6.8, 500 mM glycine, and 2.5 mM β-mercaptoethanol. The samples were loaded into the wells of SDS-polyacrylamide gel electrophoresis slab gels. After electrophoresis, the gel was fixed with 10% acetic acid/50% methanol and stained with Coomassie blue overnight and destained with water. The gels were visualized either with an alkaline phosphatase-conjugated streptavidin using Western-Light Plus (Tropix Inc.) or by isospecific anti-GAD antibody and the ECL kit (Amer sham Pharmacia Biotech).

**Purification of Synaptic Vesicles—**Synaptic vesicles were purified from rat brains as described previously (15). Briefly, synaptic vesicles were isolated from the microsomal fraction by density gradient centrifugation. Nycodenz gradient centrifugation were further purified sequentially on Sepacryl S-100 and CPG-3000 size-exclusion columns in buffer consisting of 0.16 M KCl, 5 mM NaHPO₄, pH 6.6, 1 mM EGTA, 0.02% NaN₃, 1 mM dithiothreitol, and saturated (~1 μM) 3,5-di-butyryl-4-hydroxybenzyl ether. Final purification was achieved by equilibrium centrifugation at 100,000 g on a 4–26% gradient of Ficoll (Mr, 400,000).

Co-immunoprecipitation of HSC70 with GADα—Five hundred microliters of Triton X-100-solubilized and partially purified MGAD preparations from porcine brain P₂M sample were used as the starting material for immunoprecipitation. GAD samples (500 μl) were first precleared by incubation with 100 μl of 50% (w/v) protein A-Sepharose at 4 °C for 2 h. The supernatant solutions obtained after centrifugation were modified from the procedure described previously (15). The MgAD fusion proteins were first purified by glutathione affinity column chromatography, followed by cleavage of the fusion proteins with factor Xa. Final purification of free HGAD65 and HGAD67 were achieved using the Quantity One™ imaging densitometer (model GS-700; Bio-Rad).

Immunopurification of GAD Protein Complex—Anti-GADα and anti-GADβ, immunofinity columns as described in the preceding section were used to purify GAD-associated protein complex, using crude brain extracts of S₁ and solubilized P₂M fraction. In a typical experiment, 80 ml of S₁ or P₂M extract (2 mg/ml) in Tris-citrate buffer (50 mM Tris-citrate, pH 7.0, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium fluoride, and 5 mM benzamidine, 1 mM AET, 0.2 mM PLP) was recirculated into 1 ml of anti-GADα or anti-GADβ immunofinity column at 4 °C for 8 h. The columns were washed with 80 column volumes of the same Tris-citrate buffer, except that the concentration of Tris-citrate was increased to 500 mM. The columns were further eluted with eight column volumes (8 ml) of elution buffer containing 0.1 M citric acid, pH 3.0, at 4 °C. A titrated amount of 1 N NaOH solution was added to the collecting tubes to neutralize and bring the pH up to 7.0 immediately after each collection. Protein concentration in each fraction was monitored at 280 nm. The fractions containing the highest protein concentration were then pooled (~3 ml) and dialyzed twice with 1 liter of 5 mM Tris-citrate buffer, pH 7.0, containing 1 mM AET and 0.2 mM PLP at 4 °C. The dialyzed samples were concentrated with a SpeedVac concentrator and then dialyzed against 50 mM sodium phosphate, pH 7.2, and immunoblots with anti-HSP70 and anti-CSP. Quantitative densitometry analyses of immunoblots were made with the Quantity One™ imaging densitometer (model GS-700; Bio-Rad).

Effect of Protein Complex Formation on GAD Activity in vitro—Reconstitution Studies—Mixtures of 2 μl of highly purified HGADα (1 μg/ml), 2 μl of HSC70 (1 μg/ml), and/or 5 μl of highly purified synaptic vesicles were incubated at 37 °C for 10 min with constant agitation. At the end of the incubation, the entire mixture was transferred to a test tube and GAD activity was determined. SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Unless mentioned otherwise, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10% acrylamide/bisacrylamide gels) was carried out under reducing conditions with the presence of 0.05% β-mercaptoethanol in the sample buffer. The running conditions of SDS-PAGE were followed as described by Laemmli (16). Immunoblotting was conducted as described (6, 7). Briefly, blotting was carried out at 4 °C for 18 h in a LKB 2005 transfer unit containing 25 mM Tris-HCl (pH 6.8), 0.192 mM glycine, 0.5% SDS, and 20% methanol followed by overnight incubation with primary antibody at 4 °C and 2 h of secondary antibody incubation in room temperature. Immunocomplex was visualized using Western-Light Plus or ECL reagents. Quantitative densitometry analyses of the Coomassie Blue-stained protein bands on SDS-PAGE were made with the Quantity One™ imaging densitometer (model GS-700; Bio-Rad).

Amino Acid Sequencing—Highly purified MGAD preparation was digested with trypsin, followed by separation of tryptic peptides on a C₁₈ reverse column using high pressure liquid chromatography as described previously (17). Selected peptides were then sequenced for 10–15 Edman degradation cycles on a Precise Sequencer (Applied Biosystems, Foster City, CA), according to the manufacturer’s specifications. Pro-
tein sequence alignment was performed using the BLAST program, with searches of the SwissProt data bank.

RESULTS

Co-purification of HSC70 with Membrane-associated GAD—The protein pattern of partially purified MGAD fractions after hydroxylapatite column chromatography is shown in Fig. 1A. During purification of MGAD, two proteins at the approximate molecular mass of 67 kDa were found to correlate with GAD activity (Fig. 1B) throughout the purification steps as shown in the highlighted box of Fig. 1A and lane 1 of Fig. 2. Partial amino acid sequencing identified HSC70 as a major constituent of these protein bands with the following sequence: HWPFVVNDAGRPK, which matched in 13 out of 14 amino acid residues to the 89HWPFMVVNDAGRPK102 stretch of HSC70 amino acid sequence that is analogously present across species of murine, human, and bovine. In addition, immunoblotting tests with anti-HSP70 (Fig. 2, lane 2), GAD6 (Fig. 2, lane 3), and anti-GAD67, indicate that the higher band contained both HSP70 and GAD67, whereas the lower band contains GAD65. These results reveal co-purification of HSC70 and isoforms of GAD65 and GAD67, as identified by a polyclonal anti-HSP70 (lane 2), a monoclonal anti-GAD65 antibody (lane 3), and a polyclonal anti-GAD67 (lane 4).

Protein Complex Formation of GAD65 and HSC70 but Not of GAD67 and HSC70—To elucidate the possible protein interaction between HSC70, GAD65, and GAD67, we utilized reconstitution assays using purified HGAD65, HGAD67, and purified HSC70 (~90% pure). The HSC70-HGAD complexes were separated from free HSC70 and HGAD by NG-PAGE and analyzed by immunoblot. Results in Fig. 3 (lanes 2 and 3) show the formation of protein complex between HGAD65 and HSC70, as indicated by the parallel gel mobility shift of HSP70-HGAD.
complex, recognized by GAD6 and the biotin-labeled HSC70. Without HSC70, GAD65 migrated to a lower molecular weight position in the gel (Fig. 3, lane 1). HGAD67 and HSC70 do not form a protein complex and thus do not share this parallel shift in gel mobility (Fig. 3, lanes 5 and 6). Unlike GAD65, the mobility of GAD67 in the gel is unaffected by the addition of HSC70 (Fig. 3, lanes 4 and 5). In addition, lack of immunostaining by anti-GAD67 at the position of HSC70 (Fig. 3, lane 5) indicates that no cross-reactivity was detected between anti-GAD67 and HSC70. These results suggest the possibility of protein complex formation specifically between HSC70 and HGAD65, but not between HSC70 and HGAD67.

**GAD65/HSC70 Protein Complex Formation as Detected by Immunoprecipitation**—If GAD65 does indeed form a protein complex with HSC70, one would expect the antibodies recognizing GAD65 to also immunoprecipitate HSC70. Indeed, the GAD65-specific monoclonal antibody, GAD6 (18), was able to immunoprecipitate GAD65 in a highly purified porcine brain MGAD preparation. Under stringent washing conditions, the presence of HSC70 in the GAD-anti-GAD65 immunoprecipitate was detected, as visualized by immunoblotting to polyclonal anti-HSP70 (Fig. 4, lane C). The MGAD anti-GAD immunoprecipitate was subjected to SDS-PAGE and immunoblot analysis with anti-HSP70 polyclonal antibodies. The MGAD anti-GAD immunoprecipitate was subjected to SDS-PAGE and immunoblot analysis with anti-HSP70 polyclonal antibodies. HSC70 was found to associate with GAD-anti-GAD65 complex in the immunoprecipitate (lane C) as indicated by anti-HSP70 staining, whereas no detectable HSC70 was observed in the supernatant after immunoprecipitation (lane B).

**Presence of GAD65, GAD67, HSC70, and CSP on Synaptic Vesicles**—To determine whether GAD65, GAD67, HSC70, and CSP are present on synaptic vesicles, highly purified synaptic vesicle preparations devoid of soluble proteins (15) were probed with various antibodies on immunoblots. As shown in Fig. 5 (lane 1), a protein band at the position of CSP is strongly stained with anti-CSP polyclonal antibodies, although a few other protein bands are also visible. Anti-HSP70 identified a single protein band at the position corresponding to a molecular mass at about 67 kDa (Fig. 5, lane 2). Anti-GAD65 and anti-GAD67 preadsorbed by respective antigens recognized single bands representing specific isoforms of GAD65 and GAD67 (Fig. 5, lanes 3 and 4), whereas the non-preadsorbed anti-GAD65 and anti-GAD67 polyclonal antibodies revealed both bands as well as an occasional lower band at a molecular mass range of 60–67 kDa (results not shown). The identity of GAD65 was further confirmed by GAD6, which stained a protein band (Fig. 5, lane 5) at the same position as indicated by the preadsorbed GAD65 antibodies (Fig. 5, lane 3). These results clearly indicate the presence or association of GAD65, GAD67, HSC70, and CSP with synaptic vesicles.

**Association of Bacterial Homologue of HSC70, DnaK, with Recombinant GAD65 and GAD67**—Since the E. coli system in general does not share similar posttranslational modifications with mammalian cells, it would be of interest to see whether recombinant HGAD65 and HGAD67 can also form protein complexes with the bacterial homologue of HSC70, DnaK (19). As shown in Fig. 6, both GST-HGAD65 and GST-HGAD67 fusion proteins purified by GSH affinity columns (8) contain GAD and DnaK as indicated by positive identification in immunoblotting tests using specific anti-HSP70 and anti-GAD sera. Because the difference of the molecular masses between GAD65, GAD67,
Immunoblot analysis, with anti-HSP70 (CSP, respectively.

GST-GAD67 (Fig. 6, lanes 3, 4, 5, and 6). The eluate were subjected to SDS-PAGE and immunoblot analysis, with anti-HSP70 (lanes 5–8) and anti-CSP (lanes 1–4). The upper and lower arrows indicate the position of HSC70 and CSP, respectively.

and HSC70 is small, the larger proteins, namely GST-GAD65 and GST-GAD67, fusion proteins (91–93 kDa), were specifically used in immunoblots to show clear separation of these proteins and the lack of cross-reactivity between anti-HSC70 antibodies and GST-GADs. As shown in Fig. 6, no immunostaining was observed by anti-HSP70 at the gel position corresponding to GST-GAD67 (Fig. 6, lane 2) and GST-GAD65 (Fig. 6, lane 4), thus indicating the lack of cross-reactivity between anti-HSP70 and GST-GAD67 or GST-GAD65.

Demonstration of Protein Complex of GAD, HSC70, and CSP in Immunoaffinity-purified GAD Preparations—Protein complexes of GAD, HSC70, and CSP were isolated from crude brain subcellular extracts of S1 and P2M using anti-GAD65 and anti-GAD67 immunoaffinity columns. The conditions used were of high stringency to prevent nonspecific protein binding during the affinity column purification. The columns were washed extensively with high salt buffer solution after application of the brain extract. The results showed that the GAD preparations purified by anti-GAD immunoaffinity columns also contain HSC70 and CSP in addition to GAD (Fig. 7). This suggest that HSC70 and CSP form a protein complex with GAD and hence were co-purified by the anti-GAD immunoaffinity columns. Retention of HSC70 was observed in all S1 and P2M fractions, suggesting that GAD-HSC70 complex is soluble as well as membrane-anchored. Slight retention of GAD65 in the anti-GAD67 columns and GAD67 in the anti-GAD65 columns was observed in both S1 and P2M fractions as indicated by immunoblotting tests.2 These results showed that both GAD65 and GAD67 are present as soluble as well as the membrane-bound forms. Interestingly, quantitative analysis of these immunoblots indicated that GAD65 protein is roughly distributed equally between the soluble and membrane fractions, whereas GAD67 protein is present largely (∼80%) as soluble form. Although the crucial amino acid sequences involved in membrane anchoring of GAD65 have been determined (20), the nature of membrane association of GAD67 remains largely uncharacterized. One hypothesis is that the membrane association characteristic of GAD67 is due to the heterodimer formation of GAD67 to the membrane-associated GAD65 (21, 22). Judging by the intensity of the CSP band on immunoblots, most CSP retained by the affinity columns is present in the membrane fractions (Fig. 7, lanes 2 and 4) with slight retention of CSP in the S1 fractions by anti-GAD65 affinity column (Fig. 7, lane 1). It is unlikely that anti-GAD65 and anti-GAD67 may cross-react with HSC70 or CSP, since the results shown in Figs. 5 and 6 clearly indicate the lack of immunostaining between anti-GAD65 as well as anti-GAD67 with either HSC70 or CSP. Therefore, the retention of HSC70 (Fig. 7, lanes 5–8) and CSP by anti-GAD immunoaffinity columns is the result of protein-protein interaction between GAD, HSC70, and CSP.

Activation of GAD Activity by HSC70 and Highly Purified Synaptic Vesicles—To assess the effect of protein-protein interaction on GAD activity, reconstitution experiments were conducted using purified HGAD65, purified HSC70, and highly purified synaptic vesicle preparations, which have been shown to retain electrochemical proton gradient (23, 24). Since synaptic vesicles and HSC70 alone do not contain detectable GAD activity (Fig. 8, lanes 1 and 2), it is interesting to note that GAD65 activity is markedly increased by incubation with either HSC70 (Fig. 8, lane 6), or synaptic vesicles (Fig. 8, lane 5), as well as HSC70 together with synaptic vesicles (Fig. 8, lane 7).

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Bovine serum albumin, which was included as control protein, showed no effect on GAD activity (Fig. 8, lanes 4), while the crucial amino acid sequences involved in membrane anchoring of GAD65 have been determined (20), the nature of membrane association of GAD67 remains largely uncharacterized. One hypothesis is that the membrane association characteristic of GAD67 is due to the heterodimer formation of GAD67 to the membrane-associated GAD65 (21, 22). Judging by the intensity of the CSP band on immunoblots, most CSP retained by the affinity columns is present in the membrane fractions (Fig. 7, lanes 2 and 4) with slight retention of CSP in the S1 fractions by anti-GAD65 affinity column (Fig. 7, lane 1). It is unlikely that anti-GAD65 and anti-GAD67 may cross-react with HSC70 or CSP, since the results shown in Figs. 5 and 6 clearly indicate the lack of immunostaining between anti-GAD65 as well as anti-GAD67 with either HSC70 or CSP. Therefore, the retention of HSC70 (Fig. 7, lanes 5–8) and CSP by anti-GAD immunoaffinity columns is the result of protein-protein interaction between GAD, HSC70, and CSP.

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Bovine serum albumin, which was included as control protein, showed no effect on GAD activity (Fig. 8, lane 4). These observations are compatible with our hypothesis that GAD65 is anchored to synaptic vesicles through HSC70 and its activity is

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2 C.-C. Hsu, K. M. Davis, H. Jin, T. Foos, E. Floor, W. Chen, J. B. Tyburski, C.-Y. Yang, J. V. Schloss, and J.-Y. Wu, unpublished results.
increased through an electrochemical proton gradient-mediated process (5). Furthermore, the formation of protein complex between GAD$_{65}$ and HSC70 as indicated by co-purification (Figs. 2 and 7), reconstitution assay (Fig. 3), and co-immunoprecipitation (Fig. 4) may induce conformational changes on the GAD$_{65}$ favorable to its stability and catalytic activity.

**DISCUSSION**

Since the structures of various isoforms of GAD have been determined (for review, see Ref. 2), it becomes clear that, in general, GAD is not an integral membrane protein due to lack of a stretch of hydrophobic amino acids long enough to span the membrane. Nevertheless, some populations of both GAD$_{65}$ and GAD$_{67}$ are still firmly anchored to membranes despite various ionic extraction methods (20–22). GAD can interact with membranes by ionic or hydrophobic mechanisms. Fonnum (25) reported that GAD could become associated with membrane in the presence of Cu$^{2+}$. Martin and Martin (26) have shown that apoGAD has a strong affinity for polyamines, e.g. hexasulfate, and thus may be anchored to synaptic vesicles through ion interactions, since the cytoplasmic face of synaptic vesicles is enriched in acidic phospholipids (27). However, Chang and Gottlieb (18) have demonstrated that 60% of GAD is membrane bound and can be released only with detergent (e.g. 0.2% Triton X-100) but not with high salt, e.g. 1 M NaCl or 1 M KCl, suggesting that the interaction between GAD and membranes is predominantly hydrophobic. Subsequent studies attempting to characterize this hydrophobic interaction included studies in palmitoylation as a membrane anchorage mechanism of GAD$_{65}$ (28). However, a more recent study showed that palmitoylation is not required for anchoring GAD$_{65}$ to membranes since mutation of amino acids involved in palmitoylation has no effect on membrane anchorage (20). Recent efforts in elucidating the membrane association mechanism of GAD by protein phosphorylation (29) and GAD dimerization (21, 22) have deciphered the respective crucial amino acid sequences involved. Still, the mechanism of GAD anchorage to membranes and synaptic vesicles and its effect on GAD activity remains unclear.

Besides a direct interaction with membranes as discussed above, proteins can also become membrane-anchored through chaperone proteins. One example is the 70-kDa family of heat shock proteins (HSP70), which have been shown to serve as molecular chaperons in promoting protein folding and facilitating protein transport to intracellular organelles, including mitochondria, nuclei, endoplasmic reticulum, and lysosomes (30–32). The ubiquitous binding partners of HSP70 include a wide range of unfolded peptides as well as native proteins. Two native proteins found in the nerve terminal that bind with the constitutively expressed member of the heat shock protein 70 family, HSC70, are the clathrin triskelions of coated vesicles (33, 34) and the intrinsic synaptic vesicle protein, CSP (35–37).

In this study, several lines of evidence are presented to support the hypothesis that GAD can become membrane-associated or anchored to synaptic vesicles through protein complex formation first with HSC70, followed by protein-protein interaction involving GAD-HSC70 complex and CSP on synaptic vesicles. The first line of evidence comes from purification of MGAD in which HSC70, as identified from amino acid sequencing, co-purified with GAD. Second, in reconstitution studies, HSC70 was found to form a complex with GAD$_{65}$ as shown in gel shift mobility in NG-PAGE. Third, in immunoprecipitation studies, again, HSC70 was found to co-immunoprecipitate with GAD by a GAD$_{65}$-specific monoclonal antibody. Fourth, HSC70 and CSP were co-purified with GAD by specific anti-GAD immunoprecipitation columns. In light of our previous findings that MGAD is activated by protein phosphorylation, which depends on the integrity of the proton gradient on synaptic vesicles and is inhibited by dephosphorylation (5), whereas SGAD is activated by dephosphorylation and inhibited by phosphorylation (6, 7), the physiological significance of GAD association to synaptic vesicles and its activation by interaction with HSC70 and synaptic vesicles can be summarized in Fig. 9. Once GABAergic neurons are stimulated, GABA is released by exocytosis (3). Synaptic vesicles are then retrieved through coated vesicle formation. Clathrin-coated pits are then dissociated from vesicles through interaction with HSC70 (2). The uncouated synaptic vesicles thus replenish their proton gradient through action of the vacuolar proton pump (V-ATPase) (3). Once the vesicular proton gradient is restored, MGAD, which is anchored to synaptic vesicles through HSC70 and CSP, is activated by membrane-bound protein kinase (4). Newly synthesized GABA is then transported into synaptic vesicles by the vesicular GABA transporter to replenish vesicular GABA (3). In addition, activation of the GABA neurons triggers the influx of Ca$^{2+}$ into the terminals (6), which activates calcineurin (PrP 2B) (7), and results in increased GABA synthesis by SGAD. GABA synthesized by SGAD may also be transported into synaptic vesicles (8) or be catabolized by GABA-transaminase to provide ATP (9), which may be utilized by V-ATPase to maintain the proton gradient on synaptic vesicles, a condition favoring GABA synthesis by MGAD and GABA transport by vesicular GABA transporter.

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