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Amino Acid Residues 24–31 but not Palmitoylation of Cysteines 30 and 45 Are Required for Membrane Anchoring of Glutamic Acid Decarboxylase, GAD₆₅

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Abstract. The smaller isoform of the GABA synthesizing enzyme glutamic acid decarboxylase, GAD₆₅, is synthesized as a soluble protein that undergoes posttranslational modification(s) in the NH₂-terminal region to become anchored to the membrane of small synaptic-like microvesicles in pancreatic β cells, and synaptic vesicles in GABA-ergic neurons. A soluble hydrophilic form, a soluble hydrophobic form, and a hydrophobic firmly membrane-anchored form have been detected in β cells. A reversible and hydroxylamine sensitive palmitoylation has been shown to distinguish the firmly membrane-anchored form from the soluble yet hydrophobic form, suggesting that palmitoylation of cysteines in the NH₂-terminal region is involved in membrane anchoring. In this study we use site-directed mutagenesis to identify the first two cysteines in the NH₂-terminal region, Cys 30 and Cys 45, as the sites of palmitoylation of the GAD₆₅ molecule. Mutation of Cys 30 and Cys 45 to Ala results in a loss of palmitoylation but does not significantly alter membrane association of GAD₆₅ in COS-7 cells. Deletion of the first 23 amino acids at the NH₂ terminus of the GAD₆₅ 30/45A mutant also does not affect the hydrophobicity and membrane anchoring of the GAD₆₅ protein. However, deletion of an additional eight amino acids at the NH₂ terminus results in a protein which is hydrophilic and cytosolic. The results suggest that amino acids 24–31 are required for hydrophobic modification and/or targeting of GAD₆₅ to membrane compartments, whereas palmitoylation of Cys 30 and Cys 45 may rather serve to orient or fold the protein at synaptic vesicle membranes.

Flexible membrane association is an important feature of several proteins involved in membrane trafficking and intracellular signaling (Fischer et al., 1990; Magee et al., 1987; Pate Skene and V'ng, 1989; Levis and Bourne, 1992). It is also a characteristic of some enzymes involved in the biosynthesis of neurotransmitters. Thus glutamic acid decarboxylase, the smaller form of the γ-amino butyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase, GAD₆₅,¹ (Christgau et al., 1991, 1992), tyrosine hydroxylase, the initial and rate limiting enzyme in the synthesis of catecholamines (Kuhn et al., 1990), and dopamine β-hydroxylase, the final enzyme in the synthesis of norepinephrine (Bon et al., 1991), share flexible membrane anchoring to secretory vesicles that store and release their neurotransmitter, suggesting an important regulatory role of membrane localization.

GAD is encoded for by two distinct non-allelic genes, GAD₆₇ and GAD₆₅, which are probably derived from a common ancestor gene and share significant homology except in the first 95 amino acids (Erlander et al., 1991). Both proteins are synthesized in the cytoplasm as hydrophilic and soluble molecules, and GAD₆₇ remains in this form. GAD₆₅ is posttranslationally modified and becomes anchored to the membrane of small synaptic-like microvesicles (Christgau et al., 1991, 1992). The two GAD proteins appear to comprise a dual system for GABA synthesis (Erlander et al., 1991). GAD₆₇ is tightly associated with the coenzyme pyridoxal 5'-phosphate (PLP), and may be responsible for constitutive production of GABA. A significant fraction of GAD₆₇ is detected as the apoenzyme and is PLP inducible. Thus activation of GAD₆₅ can be regulated by PLP levels, and this form may be responsible for generation of GABA in situations of sudden increases in GABA demand (Martin et al., 1991). The membrane localization of GAD₆₅ may facilitate delivery of GABA directly to a synaptic vesicle or synaptic like microvesicle for rapid accumulation and secretion. In contrast GABA synthesized by GAD₆₇, if destined for regulatory secretion, has to be transported from the cytosol and taken up by synaptic vesicles.

°Flexible membrane association
The GAD₆₅ protein is unusually susceptible to becoming a target of autoimmune immunity that affects its major sites of expression, pancreatic β cells and GABA-ergic neurons. Thus GAD₆₅ is a major target antigen in both Type 1 diabetes, which results from an autoimmune destruction of β cells (Baekkeskov et al., 1990; Tisch et al., 1993; Kaufman et al., 1993), and in stiff-man syndrome which affects GABA-ergic neurons (Solimena et al., 1990). In contrast, GAD₇ does not seem to play an independent role as an autoantigen. Rather, GAD₆₅ appears to be recognized only when the immune response to GAD₆₅ involves cross-reacting epitopes in the two proteins (Velloso et al., 1993; Hagopian et al., 1993; Seißler et al., 1993). We have proposed that the membrane anchoring of GAD₆₅ to a secretory vesicle is of significance for the presentation of this form to the immune system and its selective role as an autoantigen (Christgau et al., 1992).

The posttranslational membrane anchoring of GAD₆₅ involves at least two steps. The first step is irreversible and results in a hydrophobic molecule which is still mainly localized in the cytosol. The second step is reversible and results in a firm membrane anchoring of the protein (Christgau et al., 1991). The soluble and membrane associated forms of GAD₆₅ show identical location on two-dimensional gels, suggesting that they are distinguished by small noncharged lipid moieties (Christgau et al., 1991). The modifications of GAD₆₅ do not seem to involve polyisoprenylation, myristoylation, or a phosphatidylinoositol glycan. However, palmitoylation distinguishes the membrane-associated and soluble forms of GAD₆₅, suggesting that this fatty acid is involved in membrane anchoring (Christgau et al., 1992). GAD₆₅ has a proteolytic hot spot 69–80 amino acids into the molecule. Trypsin cleavage at amino acid (aa) 69 or 70 results in a soluble hydrophilic COOH-terminal fragment and a small membrane bound palmitoylated fragment, demonstrating that the NH₂-terminal region is the site of lipid modification and membrane anchoring of GAD₆₅ (Christgau et al., 1992). Consistent with this result, a hybrid molecule containing the NH₂-terminal 1–83 amino acids of the GAD₆₅ molecule and amino acids 89–593 from GAD₆₇ is membrane associated (Solimena et al., 1993).

In this study we have analyzed the structural requirements of palmitoylation of GAD₆₅ and assessed its role in membrane anchoring. Our results suggest that palmitoylation does not play the major role in membrane anchoring of GAD₆₅. Rather a small segment of amino acids adjacent to the palmitoylation sites is required for targeting and membrane anchoring of GAD₆₅.

Materials and Methods

Generation of GAD₆₅ Mutants

A 2.4-kb cDNA clone of rat GAD₆₅ (a gift from Dr. A. Tobin, University of California, Los Angeles, CA) was used in this study (Erlander and Tobin, 1991). The cDNA carried the entire coding region and 80- and 400-bp 5' and 3' untranslated sequences, respectively, and was originally cloned into the EcoRI site of the Bluescript vector (Stratagene, La Jolla, CA). The sequence spanning the first AUG was altered to match the Kozak consensus sequence for optimum translation efficiency (Kozak, 1991) by site directed mutagenesis. The resulting plasmid was named pBS-Y10. Oligonucleotide-mediated in vitro mutagenesis was carried out according to Kunkel (1987). Sequencing of mutant DNA with Sequenase (Boehringer Mannheim Corp., Indianapolis, IN) was carried out using protocols provided by the manufacturer. The wild-type cDNA with a Kozak sequence was subcloned from pBS-Y10 into the KpnI and NotI sites of the expression vector pSV-SPORT ( Gibco BRL, Gaithersburg, MD). The resulting plasmid was named pSV-S10 (see Fig. 2, WT). Amino acid substitution mutants were generated by changing Cys to Ala or Ser residues using pBS-Y10 as template DNA. The resulting mutant DNAs were subcloned into the NotI and HindIII sites of the pCDM8 vector (Seed and Aruffo, 1987) or KpnI and XbaI sites of the pSV-SPORT vector. Expression vectors that carry Cys to Ala substitutions were pCDM-Y35 (see Fig. 2 A, Cys 30 to Ala, 30A), pCDM-Y35 (see Fig. 2 A, Cys 45 to Ala, 45A), and pSV-Y35/35 (see Fig. 2 A, Cys 30 and Cys 45 to Ala, 30/45A). The expression vectors that carry Cys to Ser substitutions were pCDM-Y35 (see Fig. 2 A, Cys 73 to Ser, 73S), pCDM-Y35 (see Fig. 2 A, Cys 73 to Ser, 75S), pCDM-Y35 (see Fig. 2 A, Cys 75 and Cys 77 to Ser, 73/75S), pCDM-Y35 (see Fig. 2 A, Cys 80 and Cys 82 to Ser, 80/82S), pCDM-Y35 (see Fig. 2 A, Cys 73, Cys 75, Cys 80, and Cys 82 to Ser, 75/80/82S), and pCDM-Y35 (see Fig. 2 A, Cys 101 to Ser, 101S). Plasmid pMTY51 carried an insertion of a cDNA fragment lacking the NH₂-terminal 10 amino acids at the EcoRI site of the expression vector pMT2 (a kind gift from R. J. Kaufman, Genetics Institute, Cambridge, MA). The deletion fragment was generated by oligonucleotide-directed mutagenesis by changing the Cys 101 into the first AUG codon flanked by a Kozak consensus sequence (Kozak, 1991) and an EcoRI site 5' to the consensus sequence.

Expression plasmids for wild-type and mutant p21H-ras (181/4S) were kindly donated by Dr. J. Hancock (Onyx, Emeryville, CA) (Hancock et al., 1990).

Generation of Deletion Mutants by PCR

Systematic deletions of the amino terminus of the rat GAD₆₅ 30/45A mutant were generated by polymerase chain reactions (PCRs) using anchored primers. The 5' primers carried an overhang containing the Kozak consensus sequence (Kozak, 1991), and the 3' primers carried an overhang containing a translation termination codon. The 5' and 3' primer also carried a KpnI site upstream from the Kozak consensus sequence and an XbaI site downstream of the termination codon to facilitate unidirectional insertion of the amplified expression cassette into the KpnI and XbaI sites of the COS expression vector pSV-SPORT. Plasmid pCDM-Y35/35 was used as template DNA. The high fidelity Pfu thermostable DNA polymerase (Stratagene) was used to minimize the rate of mismatches in the amplification reaction.

Sequence of the deletion mutants was carried out with an automated sequencing apparatus (Applied Biosystems, Inc., Foster City, CA). The deletion fragments were subcloned into the KpnI and BglII sites of the expression vector pSV-S10 replacing the first 357 amino acids of GAD₆₅ with a deleted NH₂-terminal region. Expression vectors that carry the NH₂-terminal deletions were psV-S156, Δ1–8; psV-S156, Δ1–15; psV-S156, Δ1–23; psV-S156, Δ1–31; and psV-S156, Δ1–38 (Fig. 2 B).

Culture of Cells and Transfection

For transient transfection, COS-7 cells were seeded on 60-mm petri dishes and cultured for 15–24 h to 60–70% confluency. The cells were transfected using DOTAP (Boehringer Mannheim Corp.) by adding 100 μl mixture containing 5 μg DNA and 25 μl DOTAP in transfection buffer (20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl). Transfected cells were refreshed after 12–18 h and incubated for additional 48 h before biosynthetic labeling and immunoprecipitation studies or harvesting for subcellular fractionation and/or Triton X-114 partition studies.

Antisera

The antisera used in this study included two rabbit polyclonal antisera, 1266 and 1267, and two mouse monoclonal antibodies, GAD6E and Ab-1. The 1266 and 1267 antisera (a gift from J. S. Petersen, Hagedorn Research Laboratory, Gentofte, Denmark) and the 1701 serum were raised in rabbits against a synthetic peptide containing the last 19 amino acids of the COOH-terminal region of GAD₆₇. These antisera recognize GAD₆₅ and GAD₆₇ equally well on western blots (Kim et al., 1993). The GAD₆ monospecific antibody (Chang and Gottlieb, 1988) (a gift from Dr. D. Gottlieb, Washington University, St. Louis, MO) is specific for GAD₆₇ (Kim et al., 1993). Monospecific antibody Ab-1 against p21H-ras was from Oncogene Science Inc. (Uniondale, NY).
Biosynthetic Labeling and Immunoprecipitation

For labeling with [[35]S]methionine, cells were washed twice in methionine free DME medium and incubated for 4 h in 2.5 ml of the same medium supplemented with 100 μCi [[35]S]methionine, 1% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For labeling with [[H]palmitic acid, cells (10⁶ in a 60-mm petri dish) were washed twice in DME, followed by incubation for 4 h in 2.5 ml of DME supplemented with 0.5 mCi [[H]palmitic acid, 1% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, nonessential amino acids (50 μM Alanine, 100 μM each of asparagine, aspartic acid, glutamic acid, glycine, proline, and serine), and 5 mg/ml fatty acid free bovine serum albumin. After labeling, cells were washed once in ice cold DME, then twice in cell harvesting buffer (20 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 10 mM benzamidine/HCl). Washed cells were extracted in 0.5 ml hypotonic Hepes buffer (10 mM Hepes/NaOH, pH 7.4, 1 mM MgCl₂, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM PMSF, 0.2 mM PLP, 0.1 mM Na₃VO₄), followed by centrifugation to remove insoluble debris. The soluble extracts (500 μl) were subjected to immunoprecipitation analysis. Immunoprecipitation of labeled GAD₆₅ proteins was performed as described previously by Christgau et al., (1991, 1992) using the monoclonal antibody GAD6 for GAD₆₅ and its point mutants, and antiserum 1266 for deletion mutant mPMTYS1I. Before immunoprecipitation with the 1266 antiserum, the cell extract was supplemented with 0.1% SDS. Immunoprecipitates were analyzed by 10% SDS-PAGE and fluorography (Baeckeskov et al., 1989).

Subcellular Fractionation

Transfected COS 7 cells from a 6-cm petri dish (10⁶) were washed twice with phosphate buffered saline and homogenized at 4°C in 0.5 ml of hypotonic Hepes buffer containing 1 mM Na₃VO₄. The homogenate was centrifuged at 800 g for 10 min at 4°C to pellet the nuclei. The postnuclear supernatant was centrifuged at 100,000 g for 1 h to separate cytosol (S) and particulate fractions. The resulting pellet was resuspended and incubated for 1 h at room temperature with membrane washing buffer (10 mM Hepes/NaOH, pH 7.4, 0.5 M NaCl, 0.1 mM p-chloromercuriphenyl sulfonic acid, 5 mM EDTA, 0.25% trysylol, 5 mM NaF, 10 mM benzamidine/HCl, 0.1 mM Na₃VO₄, 0.2 mM PLP, 3 mM PMSF), followed by ultracentrifugation at 100,000 g for 1 h to pellet the washed membranes. Washed membranes were extracted for 2 h at 4°C with hypotonic Hepes buffer containing 1% Triton X-114 followed by centrifugation for 45 min at 100,000 g to separate the particulate extract (P) from insoluble debris. The cytosol proteins and proteins extracted from washed membranes were subjected to Triton X-114 partition assays.

Triton X-114 partitioning assays were performed on the subcellular fractions described above or on Triton X-114 detergent extracts of total cells, using a modification of the procedure of Bordier (1981). For comparative analyses of the amphiphilic properties of GAD₆₅ in subcellular fractions, buffer compositions were adjusted to achieve identical conditions in each fraction. Triton X-114 phase transition of each fraction was induced by incubation at 37°C for 2 min. Aqueous (A) and detergent (D) phases were separated by centrifugation at 12,000 g for 2 min. The detergent and aqueous phases were analyzed by 10% SDS-PAGE followed by immunoblotting according to Christgau et al. (1991, 1992) using antiserum 1266 or 1267 (dilution 1:1,000) for GAD₆₅ and its mutant proteins and Ab-1 (dilution 1:500) for p21H₅ and its mutant proteins.

Immunofluorescence Analyses

COS cells seeded in cover slips were cultured to 60-70% confluency before transfection with a GAD₆₅ expression construct or a control plasmid. 20 h later the cells were processed for immunofluorescence analysis. Each manipulation was preceded by washing of cells three times in PBS. Cells were fixed in methanol for 5 min at -20°C, before incubation in blocking buffer (PBS, 2.5% fetal bovine serum, 0.02% azide) for 10 min at room temperature. The cells were incubated with the 170I antiserum (diluted 1:500 in blocking buffer) for 30 min at room temperature followed by incubation with a secondary antibody (rhodamine conjugated goat anti–rabbit antisemum; Jackson Immunoresearch Laboratories, Inc., West Grove, PA) diluted 1:100 in blocking buffer. After the final wash, the cover slips were inverted onto slides with mounting medium (1 mg/ml p-phenylenediamine in 10 mM Tris-HCl pH 8.5, 90% glycerol), sealed with nail polish, and observed through a Zeiss Axiopt microscope (Carl Zeiss, Inc., Thornwood, NY).

Results

Palmitoylation and Membrane Anchoring Are Intrinsic Properties of the NH₂-terminal Region of GAD₆₅

Analyses of trypsin fragments of GAD₆₅ have suggested that palmitoylation and membrane anchoring reside in a small NH₂-terminal fragment of GAD₆₅ (Christgau et al., 1992). To confirm this finding, a NH₂-terminal deletion mutant of GAD₆₅, lacking the first 101 amino acids (GAD₆₅Δ1-101), was generated and analyzed for its palmitoylation and membrane anchoring properties by transient expression in COS-7 cells. Wild-type (wt) GAD₆₅ incorporates [[H]palmitic acid in COS-7 cells (Fig. 1, lane 1) and becomes membrane anchored. In contrast the GAD₆₅Δ1-101 mutant is not palmitoylated (Fig. 1, compare lanes 5 and 10). Furthermore, immunoblotting analysis of soluble and particulate fractions isolated from transfected COS-7 cells detected the GAD₆₅Δ1-101 protein exclusively in soluble fractions (results not shown). Those results provide strong evidence that palmitoylation and membrane anchoring is mediated by the NH₂-terminal 101 amino acids of GAD₆₅.

Site-directed Mutagenesis of Cys 30 and Cys 45 Abolishes Palmitoylation of the GAD₆₅ Molecule

The palmitoylation in the NH₂-terminal domain of GAD₆₅ is hydroxylamine sensitive suggesting a thioester linkage (Christgau et al., 1992). To localize the site(s) of palmitoyla-

Figure 1. Site-directed mutagenesis of Cys 30 and Cys 45 abolishes palmitoylation in GAD₆₅. SDS-PAGE of wt GAD₆₅ and mutant proteins labeled with [H]palmitic acid or [[35]S]methionine. COS-7 cells transiently expressing wt GAD₆₅, or the 30A, 45A, Δ1-101, and 30/45A mutant proteins were labeled with either [H]palmitic acid (lanes 1-5) or [[35]S]methionine (lanes 6-10) and immunoprecipitated with either the GAD₆ antibody (lanes 1-4 and 6-9) or the 1266 antisera (lanes 5 and 10). 30A: replacement of Cys 30 with Ala; 45A: replacement of Cys 45 with Ala; Δ1-101: deletion of the NH₂-terminal 101 amino acids; 30/45A: replacement of both Cys 30 and Cys 45 with Ala. To obtain similar intensity of radioactive proteins on fluorograms, the ratio between immunoprecipitated [[35]S]methionine and [H]palmitic acid-labeled proteins subjected to SDS-PAGE was 1:40.
Figure 2. Sequence of Cys substitution mutants and NH2-terminal deletion deletion mutants of GAD65. (A) Mutations of cysteine residues in the NH2-terminal region of GAD65. The wt GAD65 sequence is given at the top with the positions of the cysteines to be mutated given in bold face. The substitutions for these cysteines are indicated for each mutant. The dashed lines denote amino acids which are identical in wt and mutant proteins. (B) NH2-terminal deletions of the GAD65/40/45A mutant. The wild-type sequence is indicated at the top. The dashed lines indicate amino acids which are identical in wt and mutant protein. None of the deletion mutants carry the palmitoylation sites (Cys 30 and Cys 45). An additional substitution of Phe to Val was introduced into the AI-8 deletion mutant to generate a Kozak DNA consensus sequence for translation efficiency (Kozak 1991).

and investigated the role of this fatty acid in membrane anchoring, cysteine residues in the NH2-terminal region were systematically substituted by alanine or serine residues using site-directed in vitro mutagenesis (Fig. 2 A). The substitution mutants were transiently expressed in COS-7 cells and analyzed for incorporation of [3H]palmitic acid by biosynthetic labeling experiments. All of the single cysteine substitution mutants incorporated [3H]palmitic acid (not shown). Analyses of a series of mutants containing substitutions of combinations of two and three cysteines in the NH2-terminal region (Fig. 2 A) identified one mutant that had lost the ability to incorporate [3H]palmitic acid (Fig. 1, lane 4). All the other combinations of double and triple cysteine mutations shown in Fig. 2 A incorporated palmitic acid (not shown). In the non-palmitoylated mutant (30/45A) Cys 30 and Cys 45 were replaced by alanine. The result suggests that Cys 30 and Cys 45 are the sites of posttranslational palmitoylation in GAD65.

Non-palmitoylated Form of GAD65 Remains Hydrophobic and Membrane Bound in COS-7 Cells

To investigate the effect of loss of palmitoylation on the hydrophobicity of GAD65, Triton X-114 extracts of COS-7 cells expressing either wt GAD65 or the 30/45A mutant were subjected to temperature induced Triton X-114 phase transition. The detergent and aqueous phases were separated and analyzed by immunoblotting (Fig. 3). These analyses showed that the 30/45A mutant partitioned into the Triton X-114 detergent phase at a similar ratio as the wt protein (Fig. 3, compare lanes 1 and 2 with lanes 13 and 14) demonstrating that the non-palmitoylated 30/45A mutant is still amphiphilic. This result is consistent with the finding that palmitoylation is not the first step in the posttranslational hydrophobic modification of GAD65, but rather a subsequent step that distinguishes the hydrophobic soluble form, which has undergone the first modification, from a hydrophobic membrane anchored form (Christgau et al., 1991, 1992).

To assess the role of palmitoylation in membrane anchoring of the GAD65 molecule, we analyzed the subcellular distribution of wt and the non-palmitoylated GAD65/30/45A mutant expressed in COS-7 cells. The distribution of GAD65 in those experiments was compared with wt and a non-palmitoylated mutant of p21H-~ which were co-transfected into COS-7 cells and analyzed in parallel.

Palmitoylation of Cys 181 and Cys 184 in p21H-~ is required for membrane anchoring and mutation of these residues to serines (p21H-~181/184S) abolishes membrane anchoring (Hancock et al., 1990). COS-7 cells co-transfected with either wt GAD65 and wt p21H-~ (Fig. 4, lanes I-4) or GAD65/30/45A and p21H-~181/184S (Fig. 4, lanes 13-16) were homogenized and separated into a cytosol and a particulate membrane fraction. The membrane fraction was washed in 0.5 M NaCl. Proteins in the cytosolic and washed membrane fractions were subjected to Triton X-114 phase separation. The distribution of wt and non-palmitoylated mutants of GAD65 and p21H-~ into the detergent and aqueous phases of each fraction was analyzed by immunoblotting. The results of these analyses are shown in Fig. 4, lanes I-4 and 13-16.

p21H-~ is synthesized as a hydrophilic soluble molecule (Fig. 4, lanes I and 13) and undergoes a two step posttranslational modification at its carboxyl terminus to become membrane anchored. The first step involves farnesylation of Cys...
Figure 4. Subcellular distribution of GAD$_{65}$, GAD$_{65}$30/45A, and deletion mutants of GAD$_{65}$30/45A in COS-7 cells. A cytosolic fraction (S) and an extract of a washed membrane fraction (P) were prepared from COS-7 cells expressing the indicated proteins and subjected to Triton X-114 phase separation. Wild-type p21$^{H-}$ was co-expressed with the GAD$_{65}$30/45A$^{1-38}$ (lanes 1-4) and the GAD$_{65}$30/45A$^{1-31}$ mutant (lanes 5-8). The non-palmitoylated ras mutant 181/184S was co-expressed with the GAD$_{65}$30/45A$^{13-38}$ (lanes 9-12) and GAD$_{65}$30/45A (lanes 13-16). Aqueous (A) and detergent (D) phases were analyzed by SDS-PAGE and immunoblotting with the antisera 1267 for GAD$_{65}$ and its mutants and antisera Ab-1 for ras proteins. The wild-type ras is detected as an immature cytosolic form present only in the aqueous phases (lanes 1 and 5) and as the fully modified farnesylated and palmitoylated membrane anchored form detected exclusively in the detergent phase (lanes 4 and 8). In the 181/184S mutant of ras, the palmitoylation sites (Cys 181 and Cys 184) are mutated to serine. Lack of palmitoylation abolishes membrane anchoring and hence this mutant accumulates as the immature hydrophilic and cytosolic form (lanes 9 and 13) and as a farnesylated, hydrophobic but soluble form (lanes 10 and 14). In contrast, lack of palmitoylation of the GAD$_{65}$ protein does not result in a significant change in membrane anchoring or distribution between aqueous and detergent phases (compare lanes 1-4 with lanes 13-16). However deletion of amino acids 1-31 and 1-38 in the non-palmitoylated GAD$_{65}$30/45A mutant result in hydrophilic membrane anchored forms demonstrated for this protein (Christgau et al., 1991, 1992). The hydrophilic GAD$_{65}$ forms, in contrast to the p21$^{H-}$ forms, never partition completely into the detergent phase but are consistently detected in both aqueous and detergent phases (Christgau et al., 1991, 1992). It is likely that the size of GAD$_{65}$ precludes its quantitative partitioning into the detergent phase (Bjerrum et al., 1983). The majority of the wt GAD$_{65}$ protein is however found together with the wt p21$^{H-}$ protein in the detergent phases of the membrane fraction (Fig. 4, lane 4). The GAD$_{65}$30/45A mutant behaved similarly to the wt protein in those analyses (Fig. 4, compare lanes 1-4 with 13-16). Thus a significant fraction of the non-palmitoylated mutant protein was found in membrane fractions where it distributed into the Triton X-114 detergent phase (Fig. 4, lane 16). In comparison, the p21$^{H-}$181/184S mutant was found exclusively in the cytosol fraction. Thus in contrast to p21$^{H-}$, palmitoylation of the GAD$_{65}$ protein is not a requisite for membrane anchoring. Instead other modification(s) in the NH$_2$-terminal region are implied. Those results motivated an investigation of which part of the NH$_2$-terminal region is required for hydrophilic modification of membrane anchoring of GAD$_{65}$.

Amino Acid Residues 24–31 Are Essential for Membrane Anchoring of GAD$_{65}$

To further localize the sequences in the NH$_2$-terminal region which are required for targeting and anchoring of GAD$_{65}$ to membrane compartments, we analyzed the effect of stepwise deletions of NH$_2$-terminal sequences in the GAD$_{65}$30/45A mutant on the hydrophobicity and membrane anchoring of the resulting proteins (Fig. 2 B). The mutants were transiently expressed in COS-7 cells and analyzed in Triton X-114 partition assays (Fig. 3), in parallel with wt GAD$_{65}$ and the 30/45A mutant from which they were derived. Deletions of up to 23 NH$_2$-terminal amino acids of the 30/45A mutant did not result in significant changes in hydrophobicity of the resulting proteins (Fig. 3, compare lanes 3–8 with lanes 1–2). However, deletion of an additional eight amino acids (Δ1–31) resulted in a protein which did not partition into the Triton X-114 detergent suggesting that this protein does not undergo hydrophobic modifications and hence remains hydrophilic. This mutant protein was expressed at lower levels than the shorter deletion mutants (Figs. 3 and 4) suggesting that it is less stable than the other mutants. However deletion of an additional seven amino acids (Δ1–38) resulted in a protein which was expressed at similar levels as the shorter deletion mutants. The Δ1–38 mutant also did not partition into the Triton X-114 detergent phase demonstrating that it does not undergo hydrophobic modification and therefore remains hydrophilic (Fig. 3). Analyses of subcellular distribution of the deletion mutants in COS-7 cells confirmed that both the Δ1–31 and Δ1–38 mutants were hydrophilic and predominantly localized in the cytosol fraction (Fig. 4, lanes 5 and 9). In conditions (wash of membrane fraction in 0.5 M NaCl) where all of the non-palmitoylated but farnesylated p21$^{H-}$ 181/184S protein was localized in the cytosolic fraction (Fig. 4, lane 10), a small fraction of the non-palmitoylated and hydrophilic Δ1–31 and Δ1–38 GAD$_{65}$ was detected in the aqueous phase of the membrane fraction (Fig. 4, lanes 7 and 11). Thus it appears
that the ΔI-31 and ΔI-38 deletion mutants maintain a low level of membrane avidity in the absence of posttranslational hydrophobic modification(s) perhaps by association with a membrane protein. These data show that deletion of amino acids 24–31 in the non-palmitoylated GAD₆₅ mutant results in a loss of hydrophobic modification and a predominant localization in the cytosol. Thus amino acids 24–31 are required for hydrophobic modification and appropriate membrane anchoring of GAD₆₅.

**Immunofluorescence Analyses of Wild Type and Mutants of GAD₆₅ in COS-7 Cells**

GAD₆₅ is anchored to the membrane of synaptic-like microvesicles in pancreatic β cells (Christgau et al., 1992), but remains associated with the Golgi apparatus in transfected CHO cells (Solimena et al., 1993). We analyzed the subcellular distribution of wt GAD₆₅, the 30/45AA mutant, and the ΔI-38 mutant in COS-7 cells by indirect immunofluorescence. In COS-7 cells wt GAD₆₅ is localized in a large membranous perinuclear compartment, and in punctate like structures that may represent membrane vesicles (Fig. 5 a). No significant difference was observed between the wt protein and the 30/45A mutant (not shown). The ΔI-38 mutant was evenly distributed in a diffuse pattern throughout the cytosol (Fig. 5 b), which is similar to the cellular distribution of GAD₆₅ (Solimena et al., 1993; and our unpublished results). The immunofluorescence analyses of the cellular distribution of wt GAD₆₅ and the two mutants are consistent with the results from subcellular analyses and confirm that the ΔI-38 deletion mutant is predominantly cytosolic in contrast to the wt protein which is associated with membrane compartments.

**Discussion**

The NH₂-terminal region distinguishes the two isoforms of mammalian glutamate decarboxylase, GAD₆₅ and GAD₆₇, and is altogether absent in *Drosophila* GAD (Erlander et al., 1991). It is possible that this region has evolved to target the two mammalian enzymes to different sub-cellular compartments. Thus, while the GAD₆₅ protein is localized in the cytosolic compartment, the NH₂-terminal region of GAD₆₇ mediates targeting and anchoring to membranous compartments in both endocrine (Christgau et al., 1992) and non-endocrine cells (Christgau et al., 1992; Solimena et al., 1993).

Pulse-chase analyses of GAD₆₅ in pancreatic β cells suggest that the newly synthesized hydrophilic enzyme is first modified to become a hydrophobic molecule which is cytosolic or of a low membrane avidity. This form then undergoes a second set of modifications that include a hydroxylamine sensitive palmitoylation in the NH₂-terminal region. These modifications result in a firmly membrane-anchored molecule which remains membrane anchored in conditions that release peripheral membrane proteins (Christgau et al., 1991). Because palmitoylation is a distinct property of the membrane anchored form (Christgau et al., 1992) we previously proposed that palmitoylation might be important for membrane anchoring of GAD₆₅. Palmitoylation is clearly required for membrane association of some of the ras proteins, where mutation of palmitoylated cysteines results in loss of membrane anchoring (Hancock et al., 1990). In this study we have identified Cys 30 and Cys 45 as the sites of palmitoylation in the GAD₆₅ molecule by site-directed mutagenesis. Mutation of both of these residues abolishes palmitoylation of GAD₆₅ in COS-7 cells. The non-palmitoylated mutant is still hydrophobic, which is consistent with a biosynthetic pathway in which the protein first undergoes an irreversible hydrophobic posttranslational modification(s) that results in a hydrophobic yet soluble molecule. Surprisingly, the non-palmitoylated GAD₆₅30/45A mutant is still membrane anchored in COS-7 cells, demonstrating that palmitoylation is not essential for membrane anchoring. Our results suggest that the reversible membrane anchoring of the GAD₆₅ molecule (Christgau et al., 1992) is not mediated exclusively by a palmitoylation–depalmitoylation mechanism as it is in some of the ras proteins (Magee et al., 1987;
H Hancock et al., 1989, 1990). Rather GAD_56 undergoes additional posttranslational modification(s) that contribute to its membrane association. The nature of this modification is currently unknown, but our previous work indicates that several lipid modifications known to contribute to membrane anchoring, including polyisoprenylation, phosphorylation, and myristoylation, are not involved in anchoring GAD_56 (Christgau et al., 1992).

Using the palmitoylation minus mutant of GAD_56, we have dissected the NH2-terminal region by deleting segments of the NH2 terminus and analyzing the hydrophobicity and membrane anchoring properties of the resulting mutants. The non-palmitoylated mutant lacking amino acids 1–23 was not significantly different from the wild-type protein with regard to hydrophobicity, subcellular localization and membrane anchoring. Thus the first 23 amino acids in the NH2 terminus of the GAD_56 molecule are not required for hydrophobic modification, subcellular targeting, and membrane anchoring. However, deletion mutants which lacked an additional 8 or 15 amino acids, respectively (aa 24–31, aa 24–38) failed to undergo hydrophobic modification(s) and were predominantly localized in the cytosol. Thus it appears that amino acids 24–31 are required for both the first hydrophobic modification and the subsequent membrane anchoring of GAD_56, suggesting that those events may be closely linked. It is conceivable that the first hydrophobic modification is a prerequisite for the subsequent membrane anchoring modification, as is the case for farnesoylation and palmitoylation in p21H and p21N (Hancock et al., 1989). Alternatively, the first hydrophobic modification and the membrane anchoring moiety in GAD_56 may be independent but spatially linked and thus both affected by deletion of amino acids 24–31. Mass spectrometric analyses will address the question whether the amino acid 24–31 segment itself is posttranslationally modified in the wild-type protein.

It can not be excluded that deletion of amino acids 24–31 in the GAD_56 protein results in a conformational change elsewhere in the molecule which is incompatible with targeting and anchoring of GAD_56 to membrane compartments. It is more likely however that the major effect of deletion is local, which would suggest that the palmitoylated Cys 30 and Cys 45 residues are adjacent to the additional hydrophobic and membrane anchoring moieties in GAD_56.

What is the role of palmitoylation in GAD_56? Our results suggest that palmitoylation is not required for membrane anchoring of GAD_56. One possibility is that palmitoylation presents a redundant or alternative membrane anchoring moiety in the GAD_56 protein perhaps analogous to the situation in the α chain of several of the trimeric GTP-binding proteins, which are both palmitoylated and myristoylated (Linder et al., 1993; Parenti et al., 1993). Alternatively, it is possible that palmitoylation of GAD_56 adjacent to the membrane anchoring region has a different role, which is apparent in certain other membrane proteins. For example, the Vesicular Stomatitis Virus G protein (Rose et al., 1984; CD4 (Crise and Rose, 1992), the transferrin receptor (Jing and Trowbridge, 1987), and the heavy chain of the human class I histocompatibility antigen HLA-B7 (Kaufman et al., 1984; Floegh et al., 1981) each are palmitoylated at cysteines adjacent to their membrane spanning region. In those proteins palmitoylation is not necessary for stable membrane association. Rather palmitoylation may serve a different role.

In the transferrin receptor, lack of palmitoylation increases the rate of endocytosis (Alvarez et al., 1990). In the β2-adrenergic receptor, palmitoylation seems to be important for coupling to the guanyl nucleotide regulatory protein G (Moffett et al., 1993). By analogy palmitoylation of Cys 30 and Cys 45 adjacent to the aa 23–31 segment required for membrane anchoring of GAD_56 may be important for modifying the orientation or folding of the protein at the membrane of synaptic vesicles or synaptic like microvesicles. Such folding may facilitate protein–protein or protein–lipid interactions and be important for the function of the enzyme.

We have shown that a 57-kD hydrophilic soluble COOH-terminal fragment of GAD_56 can be spontaneously released from islet cell membranes as a function of time suggesting an enzymatic reactivity that can cleave the protein downstream from the membrane anchoring region (Christgau et al., 1992). The 57-kD fragment is detected in islet cell (Christgau et al., 1991) and brain extracts (our unpublished results) suggesting that this reactivity is present in both tissues. Thus, in addition to the reversible membrane anchoring of GAD_56, which may confer a flexible and perhaps regulated on-off mechanism of membrane anchoring, there may be a second and irreversible mechanism that can release the protein from membrane compartments as a fragment that has lost the membrane anchoring domain.

It is not known whether the reversible targeting of GAD_56 to the membrane of synaptic vesicles or synaptic-like microvesicles plays a role in its presentation to the immune system and thus its unique autoantigenicity compared with GAD_7. It can be hypothesized that reversible anchoring to the membrane of synaptic vesicles which are part of the endocytic/exocytic membrane network, and perhaps also the proteolytic release mechanism described above, targets the protein for increased degradation and presentation by MHC class I antigens for recognition by cytotoxic T-cells. Alternatively, the membrane-anchored GAD_56 may be targeted to the surface during exocytosis for recognition by autantibodies. Whereas the first mechanism appears relevant for T-cell–mediated destruction of pancreatic β cells, the second possibility may be the only mechanism by which GABA-ergic neurons can present the protein to the immune system in the absence of MHC class I antigen expression.

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