Phosphorylation of Serine Residues 3, 6, 10, and 13 Distinguishes Membrane Anchored from Soluble Glutamic Acid Decarboxylase 65 and Is Restricted to Glutamic Acid Decarboxylase 65α*

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GAD65, the smaller isoform of the γ-aminobutyric acid-synthesizing enzyme glutamic acid decarboxylase is detected as an αβ doublet of distinct mobility on SDS-polyacrylamide gel electrophoresis. Glutamic acid decarboxylase (GAD) 65 is reversibly anchored to the membrane of synaptic vesicles in neurons and synaptic-like microvesicles in pancreatic β-cells. Here we demonstrate that GAD65α but not β is phosphorylated in vivo and in vitro in several cell types. Phosphorylation is not the cause of the αβ heterogeneity but represents a unique post-translational modification of GAD65α. Two-dimensional protein analyses identified five phosphorylated species of three different charges, which are likely to represent mono-, di-, and triphosphorylated GAD65α in different combinations of phosphorylated serines. Phosphorylation of GAD65α was located at serine residues 3, 6, 10, and 13, shown to be mediated by a membrane bound kinase, and distinguish the membrane anchored, and soluble forms of the enzyme. Phosphorylation status does not affect membrane anchoring of GAD65, nor its Km or Vmax for glutamate. The results are consistent with a model in which GAD65α and -β constitute the two subunits of the native GAD65 dimer, only one of which, α, undergoes phosphorylation following membrane anchoring, perhaps to regulate specific aspects of GAD65 function in the synaptic vesicle membrane.

Glutamic acid decarboxylase (GAD)1 (EC 4.1.1.15) catalyzes the α-decarboxylation of glutamate generating γ-aminobutyric acid (GABA). GABA is a major inhibitory neurotransmitter in the central nervous system (1), and may also act as a paracrine signaling molecule in pancreatic islets, where it is synthesized in β-cells (2, 3). GABA can also function as a metabolic intermediate and be fueled into the Krebs cycle via the GABA shunt (4, 5). In mammalian species two highly homologous non-allelic forms of the enzyme have been identified and designated GAD65 and GAD67 in accordance with their relative molecular masses in kDa (6, 7). GAD65, but not GAD67, is unusually susceptible to becoming an autoantigen in two human diseases that affect its major sites of expression, pancreatic β-cells and GABA-ergic neurons. Thus GAD65 is a major autoantigen in insulin-dependent diabetes mellitus in man (8), and the nonobese diabetic mouse (9, 10), and in a rare neurological disease, stiff-man syndrome in man (11).

GAD65 and GAD67 differ with regard to their steady state saturation with the co-enzyme pyridoxal 5′-phosphate (PLP). At least 50% of GAD in brain is present as the PLP free apoenzyme (12, 13). GAD65 constitutes the majority of this enzyme reservoir (14), which can be activated by influx of co-factor, or perhaps by targeting the protein to compartments in the cell where PLP is available. In contrast to GAD65, the majority of GAD67 seems to be perpetually saturated with PLP (14).

The efficacy of neurotransmitter production by GAD65 may also be regulated by its subcellular location. GAD65 is isolated both in a cytosolic and a firmly membrane anchored form (15, 16). The enzyme is synthesized as a cytosolic soluble protein, but undergoes a stepwise post-translational modification in the NH2-terminal domain to become hydrophobic and anchored to synaptic vesicle-like microvesicles (16, 17). The modifications involve palmitoylation of cysteines 30 and 45, but this modification is not essential for membrane anchoring of the protein in COS-7 cells (18). Membrane anchoring is reversible, suggesting that trafficking of GAD65 between membranes and cytosol may regulate its proximity to synaptic vesicles and the efficacy by which its product GABA can be accumulated for secretion (16). Association of GAD65 with vesicles could place the protein in the proximity of a hypothetical GABA transporter, perhaps serving the double function of loading the neurotransmitter into the vesicle that secretes it, as well as removing GABA from the proximity of the enzyme, to prevent product inhibition of enzyme activity (19).

GAD purified from rat, pig, and human brain under native conditions forms dimers (20–22). The native form of both GAD65 and GAD67 is a covalently associated homodimer detected by gel filtration and native gel electrophoresis. Both the GAD67 and GAD65 dimers dissociate into monomers on SDS-PAGE under either reducing or nonreducing conditions. Reducing SDS-PAGE resolves GAD65 but not GAD67 into two

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1 The abbreviations used are: GAD glutamic acid decarboxylase; AET, 2-aminoethylisothiouronium bromide; BHK, baby hamster kidney; GABA, γ-aminobutyric acid; hGAD65, human GAD65; NephGE, non-equilibrium pH-gradient gel electrophoresis; PKA, cAMP-dependent kinase; PLP, pyridoxal 5′-phosphate; rGAD65, rat GAD65; TLE, thin layer electrophoresis; PAGE, polyacrylamide gel electrophoresis; Tricine, N-[2-hydroxy-1,1,1-tris(hydroxymethyl)ethyl]glycine.

2 J. S. Petersen and S. Baekkeskov, unpublished results.
bands of distinct mobility, which have been designated α and β (15, 16, 23). The nature of the differences between α and β is not known. Both α and β are palmitoylated, their ratio is always identical in different subcellular fractions and no differences have been detected in their hydrophobicity (15, 16).

Two-dimensional gel electrophoretic studies of GAD65 in human and rat islets (15, 23, 24) and brain (8) in the presence of enzyme inhibitors suggested to us that the protein might be phosphorylated. The present study demonstrates that GAD65 is phosphorylated in vivo as well as in vitro, that phosphorylation distinguishes GADα and -β, and is a prominent modification of the membrane anchored, but not the cytosolic form of the enzyme.

MATERIALS AND METHODS

Site-directed Mutagenesis—Oligonucleotide-mediated mutagenesis of serines in the NH₂-terminal region and deletion of the first 8 and 15 amino acids of rat GAD65 (rGAD65), respectively, were described previously (18, 25). Oligonucleotide-mediated mutagenesis of individual serine residues 3, 6, 10, 13, 17, and 20 in human GAD65 (hGAD65) was performed by the method of Kunkel (26). The S3A/S6A/S10A/S13A mutant was generated in two steps, by first constructing a S3A/S6A mutant, generating a single stranded cDNA, and undertaking a second round of mutagenesis to convert serine to alanine. A template for these mutants was the hGAD65 cDNA cloned into a BlueScript KS vector (18).

For expression in COS-7 cells (Life Technologies, Inc., Gaithersburg, MD), mutant hGAD65 cDNAs were subcloned into the SV40-based expression vector pSVSport (Life Technologies, Inc.) at the EcoRI and XbaI sites. For expression in BHK-21 cells (Life Technologies, Inc.), PC12 cells (American Type Tissue Collection, Rockville, MD), and βTC3 cells (27), wild type hGAD65, and the S3A/S6A/S10A/S13A mutant were subcloned into the cytomegalovirus based vector pcDNA3 (Invitrogen, San Diego, CA) at the EcoRI, and XbaI sites.

Cell Lines and Expression of Recombinant Wild-type and Mutant GAD65—Baby hamster kidney cells (BHK-77-3) stably expressing hGAD65 (a generous gift from W. Hagopian, University of Washington) were derived from tk⁻ts13 BHK cells (28), which were originally isolated as temperature-sensitive BHK-21 cells. BHK-77-3 cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, and for BHK-77-3 cells 800 nM methotrexate and for BHK-77-3 cells 800 nM methotrexate. COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), and 800 nM methotrexate. COS-7 cells were grown in 0.5 ml of HMAP buffer containing 1 mM phenylmethylsulfonyl fluoride and 0.1 mM p-chloromercuribenzenesulfonyl acid (HMAP-P buffer). The cells were lysed on ice using a glass homogenizer, warmed to 37 °C, and incubated for an additional 5 min at the same temperature in the presence of 150 µCi of [γ-32P]ATP (30 Ci/mmol, Amersham). Labeled samples were cooled on ice for 5 min and supplemented with 100 µl of HMAP-P buffer containing EDTA, sodium fluoride, NaF, YO, and Triton X-114 to yield final concentrations similar to those in HMAP-PIT buffer, extracted for 30 min and cleared by centrifugation at 15,000 × g for 30 min. The supernatants were immunoprecipitated and analyzed by SDS-PAGE.

Phosphoamino Acid Analysis—In vivo 32P-labeled hGAD65 was isolated by immunoprecipitation with the GAD6 antibody. The immunoprecipitates were subjected to SDS-PAGE, transferred to Immobilon-P membranes, and immunostained with the 170 kDa antisera. The GAD65α and α’ bands were excised and subjected to hydrolysis for 2 h in 6 N HCl at 110 °C using a Waters Picotag hydrolysis station (Waters, Milford, MA). Hydrolyzed samples were evaporated to dryness, redissolved in water, and spotted on a cellulose thin layer chromatography plate (Espey, Philadelphia, PA) at pH 8.9. The phosphoamino acids were separated by ascending chromatography in the second dimension in pyridine/1-butanol/acetic acid/water (50:75:15:60). The phosphopeptides were detected by autoradiography and identified by co-migration with phosphoamino acid standards.

Two-dimensional Phosphopeptide Mapping—Tryptic phosphopeptide analyses were performed essentially as described (32). Briefly, lysates of in vivo 32P-labeled BHK-77-3 cells (10-cm plate) were immunoprecipitated with GAD6 and the immunoprecipitates subjected to SDS-PAGE followed by autoradiography of the unphosphorylated gel. GAD6 was excised from the gel and electroeluted into 0.5 ml of 50 mM NH₄HCO₃, 0.1% SDS on a C.B.S. Scientific (DelMar, CA) electroelution apparatus (33). The eluted protein was lyophilized, acetone precipitated, redissolved in 50 µl of 50 mM ammonium bicarbonate, and digested with 8 µg of sequencing grade modified trypsin (Promega, Madison, WI) overnight at 37 °C. The tryptic digest was lyophilized, re-dissolved in water, and applied to a thin layer cellulose plate (Baker). The phosphopeptides were separated in the first dimension by electrophoresis at pH 4.4 (acetone/pyridine/acetic acid/water, 8:1:2.4) or at pH 8.1 (acetone/pyridine/acetic acid/water, 8:1:2.4) on a Savant TLE apparatus (Savant, Farmingdale, NY) at constant voltage (900 V). The TLE plates were stained with ninhydrin and placed on an x-ray film (Kodak Biomax, Eastman/Kodak, Rochester, NY). Phosphoamino acids were detected by autoradiography and identified by co-migration with ninhydrin stained standards.

Two-dimensional Tryptic Peptide Mapping—Tryptic phosphopeptide analyses were performed essentially as described (32). Briefly, lysates of in vivo 32P-labeled BHK-77-3 cells (10-cm plate) were immunoprecipitated with GAD6 and the immunoprecipitates subjected to SDS-PAGE followed by autoradiography of the unphosphorylated gel. GAD6 was excised from the gel and electroeluted into 0.5 ml of 50 mM NH₄HCO₃, 0.1% SDS on a C.B.S. Scientific (DelMar, CA) electroelution apparatus (33). The eluted protein was lyophilized, acetone precipitated, re-dissolved in 50 µl of 50 mM ammonium bicarbonate, and digested with 8 µg of sequencing grade modified trypsin (Promega, Madison, WI) overnight at 37 °C. The tryptic digest was lyophilized, re-dissolved in water, and applied to a thin layer cellulose plate (Baker). The phosphopeptides were separated in the first dimension by electrophoresis at pH 4.4 (acetone/pyridine/acetic acid/water, 8:1:2.4) or at pH 8.1 (acetone/pyridine/acetic acid/water, 8:1:2.4) on a Savant TLE apparatus (Savant, Farmingdale, NY) at constant voltage (900 V). The TLE plates were stained with ninhydrin and placed on an x-ray film (Kodak Biomax, Eastman/Kodak, Rochester, NY). Phosphoamino acids were detected by autoradiography and identified by co-migration with ninhydrin stained standards.

One-dimensional Tryptic Peptide Mapping—GAD65 was immunoprecipitated from Triton X-114 lysates of in vivo labeled BHK-77-3 cells and purified by electroelution as described above. The lyophilized and
acetone precipitated sample was dissolved in 100 μl of 50 mM ammonium bicarbonate. A 25-μl aliquot was removed for SDS-PAGE analysis. For an extensive degradation by trypsin, the remaining sample was digested with 4 μg of modified trypsin (Promega), first at 4 °C for 5 min, and then at 37 °C overnight. Aliquots (25 μl) were removed at the two time points and samples were carried out by modification of the methods described by Blindermann et al. (20). To determine the Km and Vmax for glutamate, COS-7 cells were harvested 48 h post-transfection, and lysed in 50 mM HEPES/NaOH, pH 7.0, 0.1 mM PLP, 1 mM 2-aminoethanol-sodium uronide bromide, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine/HCl, and 0.5% Triton X-114. The lysate was incubated at 15,000 × g for 30 min to remove debris, and the supernatant was aliquoted and diluted 1:10 in the same buffer without Triton X-114, but containing 0.2–9 μM [32P]glutamate. Reactions were incubated for 1 h at 37 °C, and quenched by addition of 6 N HCl. Lysates from non-transfected COS-7 cells were used as a negative control at each substrate concentration. GAD65 protein concentrations in the different lysates were measured as percentage of wild-type protein by immunoblotting and densitometry analyses (GE-700 Imaging Densitometer, Bio-Rad) of multiple serial dilutions of each protein. Kinetic constants were calculated by a nonlinear fit to the Michaelis-Menten equation using GraFit (37). Km and Vmax values for PLP were determined in a similar manner except that cells were lysed in the absence of PLP, the assay buffer contained 0.1% bovine serum albumin, and the reactions were carried out at 20 μM [32P]glutamate and at PLP concentrations between 0.1 and 20 μM.

RESULTS

GAD65 Is Phosphorylated in Vitro in Whole Cell Lysates—We initially analyzed whether hGAD65 expressed in BHK-77-3 and COS-7 cells could be phosphorylated in cellular lysates. The cells were homogenized, incubated with [γ-32P]ATP, and the labeled protein isolated by immunoprecipitation with a GAD65 specific antibody (GAD6). The isolated protein was analyzed by SDS-PAGE and autoradiography. These experiments demonstrated that 32P-labeled GAD65 is specifically immunoprecipitated from phosphorylated cell lysates of both BHK-77-3 cells and COS-7 cells (Fig. 1A). Similair phosphorylation experiments carried out using lysates of SF9 cells expressing GAD65 showed that GAD65 is also phosphorylated in vitro, using this material (Fig. 1B, lanes 5 and 6).

Under native conditions, GAD65 is a noncovalently associating homodimer of two 65-kDa subunits. Heating of the protein in SDS in the presence or absence of reducing agents results in dissociation of the dimer into monomer subunits.2 Under reducing conditions, high resolution SDS-PAGE and immunoblotting analyses of GAD65 reveals the doublet of GAD65 bands designated α and β (Fig. 1B) as described earlier (15, 16, 18), and sometimes a more slowly migrating band designated α' (Fig. 1B, lanes 1 and 2). Analyses of 32P autoradiograms of the immunoblots revealed one major phosphorylated band which co-migrated with the GAD65α band, and in some experiments, a weaker band which co-migrated with the GAD65α' band. No 32P-labeled band co-migrated with GAD65β. The co-migration of phosphorylated GAD65 with α, but not with β, suggested either that phosphorylation is a cause of the α/β heterogeneity or that phosphorylation is an exclusive property of α.

The first possibility seemed unlikely, because the presence or absence of phosphate inhibitors in phosphorylation experiments did not seem to affect the α/β ratio (data not shown). Furthermore, a treatment of GAD65 immunoprecipitated from in vitro labeled BHK-77-3 cells with calf intestinal phosphatase, resulted in a significant decrease in the 32P signal, yet the α/β ratio was not affected (data not shown). Hence, it is unlikely that phosphorylation is the principal cause of the observed α/β heterogeneity. This result has been confirmed by two-dimensional protein analyses (see below).

GAD65 Is Phosphorylated in Live Cells—We next addressed the question whether GAD65 is phosphorylated in vivo. BHK-
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77-3 cells in culture were labeled with [35S]labeled cells and subjected to immunoprecipitation with GAD65. Immunoblotting of the immunoprecipitates revealed the GAD65 α/β bands (Fig. 1B, lane 8). Autoradiography of the immunoblots demonstrated that the major in vivo phosphorylated band co-migrated with α (Fig. 1B, lane 7). Thus phosphorylation seems to be restricted to GAD65α both in vivo and in vitro. Phosphorylation of GAD65 was several-fold more efficient in vivo than in vitro. In vivo labeled cells were therefore used for all of the following experiments except where indicated.

Evidence for Multiple Phosphorylation of GAD65—Extensive two-dimensional gel electrophoretic analyses using NEPHGE or isoelectric focusing in the first dimension and SDS-PAGE in the second dimension have been carried out to characterize GAD65 isolated from human and rat islets of Langerhans (8, 23, 30). GAD65 immunoprecipitated from total islet cell lysates or isolated membrane fractions resolved into a series of differently charged spots in the first dimension, with the least acidic species having a pI of approximately 6.7 which corresponds to the pI calculated for the unmodified protein based on its amino acid sequence (6). We investigated the two-dimensional pattern of phosphorylated GAD65 isolated from in vivo labeled BHK-77-3 cells, and compared it with the [35S]methionine-labeled and/or immunostained protein on Western blots (Fig. 2). GAD65 was identified in autoradiograms of total cell lysates by Western blot analysis of two-dimensional gels using the 1701 antibody, followed by autoradiography, or by comparison of the mobility of an immunoprecipitated sample of the protein, which was analyzed in a parallel run. Western blot analyses of 35S- and 33P-labeled GAD65 revealed a similar pattern of 4 differently charged spots in both GAD65α and β, ranging in pI from approximately 6.7 to 6.3, and one or two spots in α’ of a charge similar to the most acidic spots of α and β. The two-dimensional pattern observed for GAD65α/β in BHK-77-3 cells is identical to the pattern described previously for the protein in pancreatic β-cells (23). Autoradiography of the Western blot of the 33P-labeled GAD65 and alignment of the autoradiographed and immunostained images demonstrated that all but the most basic spot in α as well as all the spots in α’ were phosphorylated. The most basic spot in GAD65α and all the spots in GAD5β were not 33P-labeled. These analyses confirm that phosphorylation is not the primary cause of the α/β heterogeneity, because they reveal an unmodified α/β doublet at pl 6.7. Phosphorylation, however, seems to give rise to α’, because all the α’ spots are phosphorylated and there is no unmodified α’ spot at pl 6.7.

The acidic charge heterogeneity in GAD65α and in two-dimensional gels represents the phosphorylated species. Phosphorylation is, however, clearly not the cause of the identical charge heterogeneity detected in GAD65β. Two possibilities can be suggested to explain this result. The first is that GAD65β undergoes an acidic modification, which does not affect the mobility on SDS-PAGE, and is distinct from phosphorylation, yet results in identical charge heterogeneity as observed for the phosphorylated GAD65α. Although this possibility cannot be formally excluded it seems unlikely, based on extensive two-dimensional analysis of GAD65 isolated from pancreatic β-cells in different conditions, which preserved the charge heterogeneity or phosphorylation of GAD65α to a varying degree (15, 23, 30). In every analysis the charge heterogeneity of GAD65β has been the exact replica of the GAD65α pattern, indicating that if GAD65β indeed undergoes a series of distinct acidic modifications they would (i) result in identical charge heterogeneity as that imposed by phosphorylation of α in all cases; and (ii) be of identical stability as the phosphorylation of α in a variety of conditions. The probability of a distinct acidic modification that so closely resembles phosphorylation is low. The most likely explanation is that β does not undergo an acidic phosphorylation process.
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 modification, but that α and β constitute the two subunits of the GAD65 dimer. The GAD65 dimer is stable in non-ionic detergent and urea and is therefore unlikely to dissociate during electrophoresis in the NEPHGE dimension. It will, however, come apart under the strongly dissociating conditions of the SDS-PAGE dimension. The charge heterogeneity detected in β in the NEPHGE dimension on two-dimensional gels would therefore not reflect its own heterogeneity, but that of the native GAD65 dimer.

The two-dimensional gel electrophoretic data strongly suggest that phosphorylation is an exclusive property of α. Thus the acidic charge heterogeneity in α would result from phosphorylation of one, two, and three amino acid residues, respectively, without significantly affecting the SDS-PAGE mobility, whereas the phosphorylated spots in α may represent double and/or triple phosphorylation of a different combination of amino acids in GAD65α resulting in a slight mobility shift on SDS-PAGE. It is also possible that the modification of GAD65 is restricted to mono- and di- phosphorylation of the protein, but that the triple charge heterogeneity is caused by differences in protonation of the phosphate groups at the near neutral pH of GAD65 (38).

GAD65 Is Phosphorylated on Multiple Serine Residues in the NH2-terminal Domain.—The amino acid residue(s), which undergo phosphorylation in the GAD65 protein, were characterized by phosphoamino acid analyses. GAD65 from in vivo phosphorylated BHK-77-3 cells was subjected to hydrolysis in 6 N HCl, and amino acids were separated by thin layer electrophoresis. Phosphorylated amino acids were detected by autoradiography. Those analyses detected only one phosphorylated amino acid which was identified as phosphoserine (Fig. 3, panel A). Thus GAD65 seems to undergo phosphorylation exclusively on serine residue(s).

We next assessed the distribution of phosphorylation sites in GAD65 by two-dimensional phosphopeptide mapping. 32P-Labeled GAD65 was purified from BHK-77-3 cells and subjected to tryptic digestion followed by two-dimensional separation of peptides by TLE at pH 4.4 or 8.9 in the first dimension, and TLC in the second dimension. Labeled peptides were detected by autoradiography. TLE at pH 4.4 revealed a single phosphorylated spot on the two-dimensional plate, suggesting that the phosphorylation sites may reside in a single peptide (Fig. 3, panel B). Identical results were obtained for hGAD65 expressed in COS-7 cells (results not shown). TLE at pH 8.9, which enhances the separation of acidic peptides, resolved the phosphorylated spot on the two-dimensional plate, suggesting that the phosphorylation sites may reside in a single peptide (Fig. 3, panel B).

Panel c, autoradiogram of 32P-labeled GAD65 purified from BHK-77-3 cells, and incubated with a high concentration of trypsin at 4°C for 5 min (lane 6) or overnight (O/N) at 37°C (lane 7). Twice as much sample was loaded in lane 7 to ensure disappearance of the 8-kDa band and detection of any minor fragments present.

Fig. 3. GAD65 is phosphorylated on serine residue(s) which may reside in the same tryptic fragment. A, autoradiogram of a TLE analyses of amino acids generated by hydrolysis of hGAD65 purified from in vivo phosphorylated BHK-77-3 cells. P-S, P-T, and P-Y indicate the location of phosphoserine, phosphothreonine, and phosphotyrosine standards; P, inorganic phosphate. B, autoradiograms of two-dimensional TLE/TLC analysis of tryptic fragments of hGAD65 purified from in vivo phosphorylated BHK-77-3 cells using TLE at pH 4.4 (top panel) and 8.9 (bottom panel), respectively. TLE at pH 8.9 enhances the separation of differently charged peptides and resolves the single spot detected at pH 4.4 into three species indicated by arrows.

Fig. 4. Localization of the phosphorylation site(s) to the amino terminus of GAD65 by one-dimensional tryptic phosphopeptide mapping. A, tryptic degradation map of the NH2 terminus of GAD65. B, separation of tryptic phosphopeptides of GAD65 by SDS-PAGE (panels a and b) and Tris/Tricine gel electrophoresis (panel c). Panel a, immunostaining with the COOH-terminal antibody 1701 of a Western blot of SDS-PAGE of 32P-labeled GAD65 purified from in vivo labeled BHK-77-3 cells and incubated with low concentrations of trypsin at 37°C for the indicated times. Panel b, autoradiogram of the immunoblot shown in panel a. The 58-kDa COOH-terminal fragment of GAD65 detected by immunoblotting of lane 2, is not phosphorylated (lane 4). Panel c, autoradiogram of 32P-labeled GAD65 purified from BHK-77-3 cells, and incubated with a high concentration of trypsin at 4°C for 5 min (lane 6) or overnight (O/N) at 37°C (lane 7). Twice as much sample was loaded in lane 7 to ensure disappearance of the 8-kDa band and detection of any minor fragments present.
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GAD65 is phosphorylated on serine residues in the first 15 amino acids of GAD65. Upper panel, autoradiogram of wild-type and mutant rat (lanes 1–7) and human (lanes 8–16). GAD65 immunoprecipitated from transiently transfected and in vivo phosphorylated COS-7 cells and Western blotted to polyvinylidene difluoride membranes. Lower panel, immunostaining of the same Western blots.

Substitution of serines 3 and 6 (lane 2) results in a decrease in phosphorylation and additional removal of serines 10 and 13 results in a complete loss of phosphorylation of rat GAD65 (lane 3). Similarly, mutation of serines 3 and 6, or serines 10, 13, and 17 to alanine but not mutation of serines 58, 61, and 64 caused a decrease in phosphorylation compared to the wild-type rat protein (lanes 4–7). Consistent with the results with rat mutants, mutation of individual serines 6, 10, and 13 in hGAD65 to alanine results in a decrease in phosphorylation signal and, furthermore, a slight mobility shift, whereas mutation of serine 3 results in a decrease in phosphorylation signal only.

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Analysis of single serine substitution mutants in the NH2-terminal peptide of hGAD65 (Fig. 5B, lanes 8–14) revealed a decrease in phosphorylation signal in the hGAD65 S3A, hGAD65 S6A, hGAD65 S10A, and hGAD65 S13A proteins but not in the hGAD65 S17A and hGAD65 S20A mutants, consistent with polyphosphorylation of serines in the NH2-terminal peptide and suggesting that all 4 serines, 3, 6, 10, 13, can be phosphorylated relatively independently of each other. Interestingly, the S6A, S10A, and S13A mutants also showed a slight increase in mobility on SDS-PAGE. Similar shifts in mobility upon removal of phosphorylation sites have been reported for other proteins (40). Substitution of all 4 serines, 3, 6, 10, and 13 in hGAD65 abolished ≥95% of the phosphorylation signal (Fig. 5, lanes 15 and 16).

The mutational analysis, as well as the two-dimensional phosphopeptide analysis and protein studies, suggest that all four serine residues 3, 6, 10, and 13 can be phosphorylated in combinations to generate mono-, di-, and triphosphorylated GAD65a. All these serine residues reside in the same tryptic fragment (aa 1–27). Hence the three spots detected by two-dimensional tryptic phosphopeptide analyses at pH 8.9, are likely to represent the mono-, di-, and triphosphorylated GAD65. The two-dimensional protein analyses reveal three acidic spots which co-migrate with nonphosphorylated α on SDS-PAGE, and one or two spots in α' which align(s) with the most acidic spot(s) in α. Since addition of each phosphate group is likely to result in one charge shift, the three increasingly acidic 32P-labeled spots that co-migrate with nonphosphorylated α are likely to represent GAD65α phosphorylated on one, two, and three serines, respectively, in a combination that does not result in a significant mobility shift in the SDS-PAGE dimension. Similarly, the spot(s) detected in α', may represent double, and/or triple phosphorylation of serines in GAD65α in a combination of serines residues that results in a slight mobility shift in the SDS-PAGE dimension.

Phosphorylation Does Not Affect $K_m$ or $V_{max}$ for Substrate and Co-enzyme—Bao et al. (41, 42) reported that the enzyme activity of an unspecified soluble isoform of GAD from porcine brain is regulated by phosphorylation. We assessed whether phosphorylation of GAD65 affects $K_m$ or $V_{max}$ for glutamate and PLP. The analyses for glutamate were carried out using detergent extracts of wild-type and phosphorylation mutants of hGAD65 and rGAD65 expressed in COS-7 cells (Table 1). Substitution of the phosphorylated serines in the NH2-terminal domain for alanine either individually or in combinations, that
TABLE I
Kinetic constants for glutamate for wild-type and mutant GAD65 proteins

| Construct | $K_m$ (mM) | Relative $V_{max}$ |
|-----------|------------|--------------------|
| hGAD65    | 2.15 ± 0.35 | 1.0                |
| hGAD65 S3A/S6A/S10A/S13A | 2.13 ± 0.16 | 1.0 ± 0.2           |
| rGAD65    | 1.02 ± 0.15 | 1.0                |
| rGAD65 C30A/C45A | 0.81 ± 0.18 | 1.0 ± 0.1           |
| rGAD65 C10A/C13A | 0.71 ± 0.13 | 0.8 ± 0.1           |
| rGAD65 C30A/C45A | 0.12 ± 0.18 | 1.0 ± 0.5           |
| rGAD65 S10A/S13A/S17A | 0.12 ± 0.14 | 1.0 ± 0.3           |
| rGAD65 C30A/C45A | 0.08 ± 0.04 | 1.3 ± 0.4           |

$K_m$ and $V_{max}$ were determined for mutant and wild-type GAD65 proteins transiently expressed in COS-7 cells. All analyses were done at least in triplicate. The $V_{max}$ measured for the wild-type human and rat protein was arbitrarily given a specific activity of 1.0 and values for the mutant proteins were calculated as relative values compared to the activity of the wild-type protein.

included all serines 3, 6, 10, and 13 in hGAD65, did not affect the $K_m$ or $V_{max}$ of GAD65 for glutamate (Table I). Deletion of the first 15 amino acids of the rGAD65 resulted in a decrease of $K_m$ for glutamate from 1.2 to 0.9 mM. This may, however, reflect a conformational effect of deletion, rather than removal of the phosphorylated residues, since a decrease in $K_m$ was not observed for the hGAD65 tetraserine mutant.

The analysis for PLP was carried out using wild-type hGAD65 and the S3A/S6A/S10A/S13A mutant. Both proteins showed identical deviations from Michaelis-Menten kinetics at low PLP concentrations. At PLP concentrations between 0.5 and 20 μM PLP, however, both wild-type and the tetraserine GAD65 mutant obeyed Michaelis-Menten kinetics, were identical with regard to $V_{max}$, and displayed an identical $K_m$ value of −0.9 μM.

In summary, phosphorylation does not seem to regulate kinetic properties of GAD65 with regard to glutamate and PLP. It is of note, however, that a detection of subtle effects of phosphorylation on the kinetics of GAD65 may require the separation of phosphorylated, and nonphosphorylated fractions of the wild-type protein and purification of each to homogeneity.

Phosphorylation of GAD65 Is Mediated by a Membrane-associated Kinase and Phosphorylated Protein Remains Largely Membrane-associated—Membrane anchoring of GAD65 is reversible (15, 16), but the facts that regulate the trafficking of the protein between cytosol and membranes are unknown. Washing of isolated membranes in buffers containing enzyme inhibitors results in a stably membrane-associated protein that can only be released from membranes by detergent (15, 16). We analyzed the subcellular localization of phosphorylated GAD65. $^{32}P$ in vivo labeled BHK-77-3 cells were homogenized, and a postnuclear supernatant separated into cytosolic and crude membrane fractions. The membranes were subjected to a high salt wash. The cytosol, washed membrane fraction, and high salt membrane wash were subjected to Triton X-114 phase separation to analyze the effect of phosphorylation on the hydrophobicity of the protein. GAD65 in the different fractions was analyzed by immunoprecipitation followed by immunoblotting and autoradiography of the same immunoblots (Fig. 6). As shown previously for the protein in pancreatic β-cells and transiently transfected COS-7 cells (15, 16, 18), GAD65 is found in the cytosol, membrane wash, and washed particulate fraction of BHK-77-3 cells. Similar amounts of total GAD65 were detected in washed membrane and cytosolic fractions (Fig. 6). In contrast, phosphorylated GAD65 was detected either exclusively (results not shown) or predominantly (Fig. 6).

FIG. 6. Phosphorylated GAD65 is predominantly localized in membranes in BHK-77-3 cells and phosphorylation is mediated by a membrane associated kinase. Upper panels are autoradiograms of the immunoblots shown in the lower panels. Panels a and d, GAD65 immunoprecipitated from Triton X-114 detergent phase (d) and aqueous phase (a) separated cytosolic (CYTO), washed membrane (MEM), and high salt membrane wash (WASH) fraction prepared from in vitro phosphorylated BHK-77-3 cells homogenized in lysis buffer containing 5 mM EDTA and 5 mM sodium fluoride. Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting and autoradiography. Panels b and e, GAD65 immunoprecipitated from the same fractions isolated from in vitro phosphorylated BHK-77-3 cells. Panels c and f, GAD65 immunoprecipitated from cytosolic and membrane fractions which were prepared from BHK-77-3 cells and then subjected to in vitro phosphorylation.
Phosphorylation of Membrane-bound GAD65

We have demonstrated that GAD65 expressed in a variety of cell lines is phosphorylated both in vitro and in vivo. Phosphorylation is mediated by a membrane bound kinase, and the phosphorylated protein seems to remain largely membrane associated. This study provides the first evidence that the membrane bound form of GAD65 is phosphorylated. Phosphorylation as well as palmitoylation thus distinguish the membrane bound and soluble forms of the enzyme.

The identity of the phosphorylated protein as GAD65 was established by several criteria. First, the $^{32}$P-labeled protein was immunoprecipitated with a GAD65 specific antibody (GAD6), but not with control antibodies. Second, the phosphorylated protein was recognized by a second GAD antibody (1701) on Western blots of immunoprecipitates. Third, the phosphorylated protein was absent in parent cell lines negative for GAD65, and finally, two-dimensional gel electrophoretic analysis of the phosphorylated protein revealed the well characterized charge and size coordinates of GAD65 from pancreatic β-cells and neurons (8, 15, 23, 24). Phosphorylated GAD65 co-migrated with the α component of the GAD65 αβ doublet described earlier (15, 16, 23) and with a weaker more slowly migrating band on SDS-PAGE, α’, but was not detected in GAD65β. Phosphorylation is the first modification shown to be unique to GAD65α.

GAD65 is polyphosphorylated on the NH$_2$-terminal serine residues 3, 6, 10, and 13. Interestingly, exon 1 (aa 1–25), which encodes the phosphorylated region of GAD65, as well as exons 2 (aa 26–40) and 3 (aa 46–95), share no homology with the analogous regions of the larger isoform of glutamate decarboxylase, GAD67, although the two proteins are 78% identical in the remaining sequences. Thus it seems likely that the phosphorylation of NH$_2$-terminal serines in GAD65 shown here is unique for this isoform.

Two-dimensional NEPHGE/SDS-PAGE analysis of intact GAD65 confirmed the presence of multiple phosphorylated species, and allowed their further characterization. The two-dimensional gel electrophoretic analyses of GAD65 also revealed a heterogeneity in the SDS-PAGE mobility of the two most acidic phosphorylated species, corresponding to the α’ band detected by one-dimensional SDS-PAGE. Since alanine substitutions of individual serine residues in the NH$_2$-terminal region of GAD65 affected the SDS-PAGE mobility of the protein to a different degree, we propose that the $^{32}$P-labeled spots detected in α and α’ represent phosphorylation of different combinations of serine residues 3, 6, 10, and 13.

The nature of differences between the α and β forms of GAD65 is unknown. Both α and β are palmitoylated and analyses of hydrophobicity and subcellular localization of GAD65 have not revealed differences in those parameters between α and β (16). SDS-PAGE analysis of the protein under nonreducing conditions reveals only a single band with the mobility of GAD65 α. Addition of increasing concentrations of β-mercaptoethanol is accompanied by the appearance of the β-band, suggesting that the oxidation and/or folding state of the two forms may differ. The two-dimensional gel electrophoretic analyses show that phosphorylation is not the cause of the α/β heterogeneity, since both exist in a nonphosphorylated form. Rather, phosphorylation seems to be an exclusive property of GAD65α. GAD65β is not phosphorylated and yet consistently displays an identical acidic charge heterogeneity on two-dimensional gels as α. Based on these results, we propose that α and β constitute the two subunits of the native non-disulfide linked GAD65 dimer, and that the protein remains as a dimer in the conditions of the NEPHGE analyses of the first dimension, but then falls apart in the strongly dissociating conditions of the second dimensional SDS-PAGE. Thus studies of native GAD65 would analyze the α/β dimer as one entity and not discern potential differences in the chemical and physical parameters of the two subunits. This model is consistent with the inability of earlier studies to detect differences in Triton X-114 partition patterns of α and β, in spite of their distinct phosphorylation, and the consequent anticipated increase in the hydrophilicity of α (15, 16).

The charge shift separation of phosphorylated and non-phosphorylated spots by two-dimensional NEPHGE/SDS-PAGE allows us to roughly estimate the stoichiometry of GAD65 phosphorylation. Based on several two-dimensional experiments, we estimate that 20–40% of total GAD65 in BHK-77-3 cells is phosphorylated, corresponding to 30–60% of the membrane anchored protein.

A common biological function of protein phosphorylation is regulation of enzymatic activity. Bao et al. (41, 42) reported that phosphorylation of a soluble, nonidentified GAD isofrom from brain by cAMP-dependent kinase (PKA) caused a decrease in enzyme activity, which could be reversed by treatment of the protein with calcineurin. We determined the $V_{max}$ and $K_m$ for wild-type GAD65 as well as a series of phosphorylation mutants in cell free lysates generated from transfected COS-7 cells. In our studies, a decrease or loss in phosphorylation observed for the serine substitution mutants did not result in a detectable effect on the $V_{max}$ and $K_m$ for glutamate or PLP. Furthermore, several lines of evidence suggest that the kinase involved in phosphorylation of GAD65 is not PKA. First, the phosphorylation site we have identified does not contain a PKA consensus sequence; second, the addition of cAMP or staurosporin (a cAMP-dependent kinase inhibitor) to our in vitro labeling reactions did not affect the degree of phosphorylation of GAD65; third, we were unable to detect phosphorylation of purified GAD65 in vitro by PKA. In contrast GAD67 can be phosphorylated by PKA in similar conditions. We therefore suggest that the phosphorylation reported by Bao et al. (41, 42) may be a characteristic of the cystolic isoform, GAD67.

Phosphorylation can play a role in the regulation of membrane association of proteins. Addition of phosphate groups can decrease the membrane avidity of proteins by charge repulsion from membrane phospholipids. Hence phosphorylation of a membrane protein can facilitate its translocation to the cytosol (Ref. 43 and references therein). Conversely, phosphorylation of a cytosolic protein can block its membrane association and retain it in the cytosol (44). GAD65 is an amphiphilic molecule

**DISCUSSION**

Phosphorylation of NH$_2$-terminal serines in GAD65 shown here is analogous regions of the larger isoform of glutamate decarboxylase residues 3, 6, 10, and 13. Interestingly, exon 1 (aa 1–25), which was described earlier (15, 16, 23) and with a weaker more slowly separated cytosolic (CYTO), washed membrane (MEM), and a high salt membrane wash (WASH) fractions prepared from cells (lanes 5–16). Immunoprecipitates were subjected to SDS-PAGE followed by immunoblotting. Removal of the serine phosphorylation sites abolished phosphorylation of GAD65 in βTC3 cells (lanes 1–4) but did not affect the membrane association of the protein in these cells (lanes 5–16).

The two-dimensional gel electrophoretic analyses show that phosphorylation is not the cause of the α/β heterogeneity, since both exist in a nonphosphorylated form. Rather, phosphorylation seems to be an exclusive property of GAD65α. GAD65β is not phosphorylated and yet consistently displays an identical acidic charge heterogeneity on two-dimensional gels as α. Based on these results, we propose that α and β constitute the two subunits of the native non-disulfide linked GAD65 dimer, and that the protein remains as a dimer in the conditions of the NEPHGE analyses of the first dimension, but then falls apart in the strongly dissociating conditions of the second dimensional SDS-PAGE. Thus studies of native GAD65 would analyze the α/β dimer as one entity and not discern potential differences in the chemical and physical parameters of the two subunits. This model is consistent with the inability of earlier studies to detect differences in Triton X-114 partition patterns of α and β, in spite of their distinct phosphorylation, and the consequent anticipated increase in the hydrophilicity of α (15, 16).

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**3.** M. Namchuk and S. Baekkeskov, unpublished results.
which is isolated in a hydrophilic soluble form, a hydrophobic soluble form, and a hydrophobic firmly membrane anchored form (15, 16). The membrane anchoring of GAD65 is reversible (16). The phosphorylated NH2-terminal peptide is proximal to the region of GAD65 that undergoes a set of hydrophobic modifications and is implicated in the membrane anchoring of the protein (16, 18, 45).

The mechanism of membrane anchoring of GAD65 is not known. Palmitoylation of cysteines 30 and 45 is an exclusive property of membrane anchored GAD65 and was originally proposed to be involved in membrane anchoring (16). However, site-directed mutagenesis of the palmitoylated cysteines does not affect the distribution of the protein between cytosol and membrane compartments in COS-7 cells (18). Palmitoylation is therefore not critical for membrane anchoring, but may assist in targeting GAD65 to the right membrane compartment, and/or play a role in protein-protein interaction in synaptic vesicle membranes. Deletion of amino acids 1–23 in GAD65 and concomitant mutation of the palmitoylated cysteines also does not affect the distribution of the protein between soluble and membrane compartments in COS-7 cells. However, removal of the next 8 amino acids (aa 24–31) abolishes membrane anchoring (18). Studies of GAD65/GAD67 chimeras suggest that a substitution of the first 29 amino acids of GAD67 with the first 27 amino acids of GAD65 is sufficient to target the normally cytosolic GAD67 isoform to the perinuclear membrane compartment in CHO cells (45). Thus amino acids 24–27 seem to be critical for membrane anchoring of GAD65. Because of the proximity of the phosphorylated serine residues to the region implicated in membrane anchoring of GAD65, we speculated that phosphorylation might be a mechanism by which GAD65 is released from membranes. To address this possibility, subcellular fractionation experiments were performed on in vivo or in vitro phosphorylated GAD65. These experiments revealed that phosphorylated GAD65 is predominantly localized in membrane compartments, despite the fact that 40–50% of total GAD65 is recovered in the cytosol. A prominent fraction of membrane associated GAD65 (30–60%) was phosphorylated in our experiments. Yet high salt wash of membranes in the presence of phosphatase inhibitors did not release the phosphorylated protein, strongly suggesting that phosphorylation is either not involved in dissociation of GAD65 from membranes, or if required, it is not sufficient for release.

Phosphorylation could play a role in membrane association by mediating association of soluble GAD65 with a membrane protein, thus targeting it to a specific membrane compartment where it can undergo lipid modification(s) by a membrane associated enzyme and become firmly membrane anchored. Two lines of evidence argue against this possibility. First, the hGAD65 S3A/S6A/S10A/S13A in which phosphorylation is almost completely abolished, is membrane anchored similar to wild-type GAD65 in transiently transfected COS-7 cells, βTC3 cells, BHK-21 cells, and PC12 cells, and similar results have been obtained for the rGAD65 Δ1–15 phosphorylation mutant, and the deletion and substitution mutants, which show a significant decrease in phosphorylation in COS-7 cells (Refs. 18 and 25, and this study) demonstrating that phosphorylation of GAD65 is not required for membrane anchoring in those cells. Second, in vitro phosphorylation experiments using separated membrane and cytosolic fractions of BHK-77-3 in the presence of phosphatase inhibitors, showed that GAD65 was exclusively phosphorylated in the membrane fraction. Thus phosphorylation is not involved in anchoring cytosolic GAD65 to membranes, but is an independent modification caused by a membrane associated kinase, and restricted to the fraction of the enzyme, which is already membrane anchored.

In summary we have demonstrated that the membrane bound form of GAD65 is phosphorylated on multiple serine residues in the NH2-terminal domain and that this modification is restricted to GAD65α. The biological function of this modification remains to be established. Phosphorylation of GAD65 does not appear to alter its kinetic behavior with respect to the substrate glutamate, or the co-enzyme PLP, nor does it appear to be involved in the membrane anchoring of the protein in transiently transfected cells.

By necessity, we have studied the effect of phosphorylation of GAD65 on membrane anchoring and enzyme activity in recombinant cell lines. The role of phosphorylation of the enzyme in its natural environment, neurons and primary pancreatic β-cells, may be distinct and perhaps become accessible in the future by genetic manipulations in the mouse.

We speculate that phosphorylation of membrane anchored GAD65 is involved in regulating or facilitating a specific function of the enzyme in synaptic vesicle membranes. One possibility is that phosphorylation mediates interaction of GAD65 with regulatory proteins in the synaptic vesicle membrane or facilitates transport of the product GABA to the lumen of vesicles by docking the enzyme to a hypothetical GABA transporter.

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