Protein Phosphorylation of Human Brain Glutamic Acid Decarboxylase (GAD)65 and GAD67 and Its Physiological Implications†

Jianning Wei,‡ Kathleen M. Davis,§ Heng Wu,‡ and Jang-Yen Wu*,‡

Department of Biomedical Sciences, Florida Atlantic University, Boca Raton, Florida 33431, and Department of Pharmacology and Toxicology, University of Kansas, Lawrence, Kansas 66045

Received February 10, 2004; Revised Manuscript Received March 25, 2004

ABSTRACT: Previously, we reported that protein phosphorylation plays an important role in regulating soluble L-glutamic acid decarboxylase (GAD) [Bao, J. (1995) *J. Biol. Chem. 270*, 6464−6467] and membrane-associated GAD activity [Hsu, C. C. (1999) *J. Biol. Chem. 274*, 24366−24371]. Here, we report the effect of phosphorylation on the two well-defined GAD isoforms, namely, GAD65 and GAD67, using highly purified preparations of recombinant human brain GAD65 and GAD67. GAD65 was activated by phosphorylation, while GAD67 was inhibited by phosphorylation. The effect of phosphorylation on GAD65 and GAD67 could be reversed by treatment with protein phosphatases. We further demonstrate that protein kinase A (PKA) and protein kinase C isoform ϵ are the protein kinases responsible for phosphorylation and regulation of GAD67 and GAD65, respectively. Direct phosphorylation of GAD65 and GAD67 was demonstrated by incorporation of [^{32}P] from [γ-^{32}P]ATP into purified GAD65 and GAD67 and immunoblotting assay using anti-phosphoserine/threonine antibodies. We have identified one specific phosphorylation site, threonine 91 (T91), in hGAD67 that can be phosphorylated by PKA using MALDI−TOF. Site-directed mutation of T91 to alanine abolished PKA-mediated phosphorylation and inhibition of GAD activity. Furthermore, mutation of T91 to aspartic acid or glutamic acid mimics the effect of phosphorylation. A model depicting the effect of phosphorylation on GAD activity upon neuronal stimulation is also proposed.

L-Glutamic acid decarboxylase (GAD[11], EC 4.1.1.15) is a pyridoxal 5′ phosphate (PLP)-dependent enzyme that catalyzes the conversion of L-glutamate to γ-aminobutyric acid (GABA), which is the major inhibitory neurotransmitter in the vertebrate central nervous system. Abnormalities in the function of GAD have been implicated in a number of pathological conditions including Parkinson's disease, stiff-man syndrome, epilepsy, depression, anxiety, and panic disorders (*1*, *2*). Despite its importance, the mechanism underlying the regulation of GAD remains elusive.

It is well-known that reversible protein phosphorylation is a common mechanism responsible for the regulation of protein functions. Regulation of neurotransmitter systems by phosphorylation of their synthesizing enzymes has also been reported. For instance, the rate-limiting enzyme for dopamine synthesis, tyrosine hydroxylase, has been reported to be regulated by direct phosphorylation both in vitro and in vivo (*3*, *4*). Previously, we showed that protein phosphorylation and dephosphorylation plays an important role in the regulation of GAD activity in the brain (*5*, *6*). It was reported that soluble GAD (sGAD) was inactivated upon phosphorylation, presumably by protein kinase A (PKA), and activated upon dephosphorylation by protein phosphatase 2B (PP2B, calcineurin) (*5*). On the contrary, membrane-bound GAD (mGAD) was activated by phosphorylation and inactivated by dephosphorylation (*6*). The kinase responsible for the phosphorylation of mGAD has not yet been identified. Although it is generally believed that GAD65 is enriched in mGAD preparation and that GAD67 is predominantly present in sGAD preparation, GAD65 and GAD67 are certainly present in both soluble and membrane fractions of the brain preparations (*7*). Herein, it became imperative to use a well-defined GAD preparation, namely, GAD65 or GAD67, so that definitive information regarding the effect of protein phosphorylation on GAD65 and GAD67 can be determined.

The current paper concentrates on the effect of phosphorylation on the highly purified recombinant human GAD65 (hGAD65) and human GAD67 (hGAD67). In this paper, we present evidence showing the regulation of hGAD65 and hGAD67 by direct protein phosphorylation using [^{32}P] incorporation and an immunoblotting test. We further identi-

† This work was supported by NIH (NS37851), NSF (IBN-9723079), Charles E. Schmidt Family Foundation, and Center of Excellence for Biomedical and Marine Biotechnology, Florida Atlantic University, Florida (all to J.Y.W.).

* To whom correspondence should be addressed: Department of Biomedical Sciences, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431. Tel: 561-297-0167. Fax: 561-297-0174. E-mail: jwu@fau.edu.

‡ Florida Atlantic University.

§ University of Kansas.

[1] Abbreviations: GAD, L-glutamic acid decarboxylase; hGAD65, human L-glutamic acid decarboxylase with molecular weight of 65 kDa; hGAD67, human L-glutamic acid decarboxylase with molecular weight of 67 kDa; GST, glutathione-*S*-transferase; AET, 2-aminoethylisothiouronium bromide; PLP, pyridoxal 5′ phosphate; cAMP, cyclic adenosine monophosphate; CaMKII, calcium/calmodulin-dependent protein kinase II; MALDI−TOF, matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry; PMA, phorbol 12-myristate 13-acetate; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PP2B, protein phosphatase 2B; PP2Cα, protein phosphatase 2Cα.

10.1021/bi0496992 CCC: $27.50
Published on Web 05/01/2004

FIGURE 1: Amino acid sequencing of the truncated human GAD67. Alignment of N-terminal amino acid sequencing for full-length hGAD67 (A) and two truncated hGAD67 (B). X indicates the amino acids not identified in the analysis.

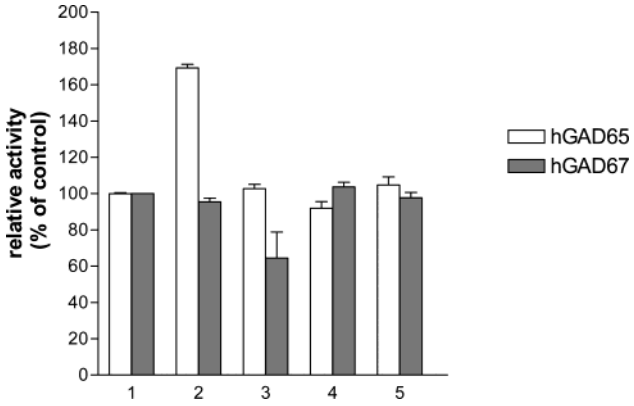

FIGURE 2: Effect of the protein kinase treatment on hGAD65 and hGAD67 activity. Lane 1, hGADs alone; lane 2, hGADs incubated with the PKC isoforms mixture; lane 3, hGADs incubated with PKA; lane 4, hGADs incubated with CaMKII; lane 5, hGADs incubated with 200 μM ATP only. Open bar = hGAD65 activity, and closed bar = hGAD67 activity. The error bars indicate the standard deviation with $n = 3$.

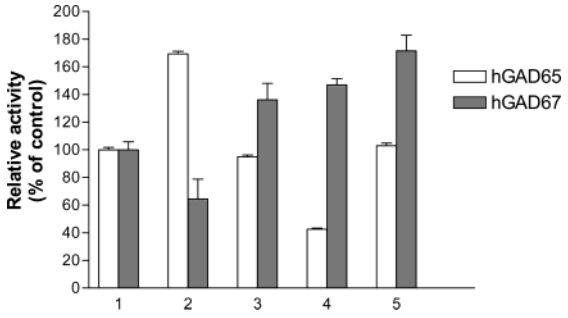

FIGURE 3: Effect of the protein phosphatase treatment on hGAD65 and hGAD67 activity. Lane 1, hGADs alone; lane 2, hGAD65 treated with the PKC isoforms mixture and hGAD67 treated with PKA in the presence of ATP; lane 3, hGADs after the protein kinase treatment, incubated with PP1; lane 4, hGADs after the protein kinase treatment, incubated with PP2A; lane 5, hGADs after the protein kinase treatment, incubated with PP2B (calcineurin). Open bar = hGAD65 activity, and closed bar = hGAD67 activity. The error bars indicate the standard deviation with $n = 3$.

fied the protein kinases involved in regulating GAD65 and GAD67. Moreover, the site of phosphorylation in GAD67, i.e., threonine 91 (T91), was identified by MALDI−TOF analysis. In addition, the results from the site-directed mutation studies on T91 illustrate the importance of this site in regulating GAD67 activity.

MATERIALS AND METHODS

Materials. 2-Aminoethylisothiouronium bromide (AET), PLP, cyclic adenosine monophosphate (cAMP), benzethonium hydroxide, protein kinase C (PKC), phosphatidylserine, diacylglycerol, and Factor Xa were purchased from Sigma. The PKC isoforms (α, β, and γ) mixture, PKCϵ, PKA catalytic subunit, Ca^{2+}/CaM-dependent protein kinase II (CaMKII), protein phosphatases 1, 2A, 2B, and 2Cα, PKC inhibitor peptide, PKA inhibitor peptide, and anti-phospho-Ser/Thr antibody were purchased from Upstate Biotechnology. Phorbol 12-myristate 13-acetate (PMA) was purchased from Tocris. pGEX-6P-1 and pGEX-3X vectors, Factor Xa, PreScission protease, and [L-^{14}C]glutamic acid were purchased from Amersham Biosciences. Quickchange site-directed mutagenesis kit was purchased from Stratagene. [γ-^{32}P]ATP was purchased from Perkin−Elmer. hGAD65 and hGAD67 cDNA in a pGEX-3X vector were generous gifts from Dr. Allen J. Tobin (University of California, Los Angeles, CA).

Purification of Recombinant hGAD65 and hGAD67. Full-length hGAD65 and hGAD67 were expressed separately as a glutathione-*S*-transferase (GST) fusion protein in *Escherichia coli DH5*α using a pGEX-3X vector. The recombinant hGAD65 and hGAD67 were purified as described before (*8*). Briefly, GST fusion proteins were affinity-purified by glutathione affinity agarose. After the GST tag was removed by Factor Xa, the free GAD proteins were further purified by glutathione affinity agarose. Alternatively, hGAD65 and hGAD67 cDNA were subcloned in a frame into a pGEX-6P-1 vector and expressed as a GST fusion protein in *E. coli* BL21. The recombinant proteins were purified as described above except that the GST tag was removed by PreScission protease cleavage.

Table 1: Effect of PKC$\alpha\beta\gamma$ and PKCϵ on hGAD65 Activity

group	control	PKC$\alpha\beta\gamma$	PKCϵ
relative activity (% ± SD)	100.0 ± 2.7	102 ± 6.6	136.8 ± 1.1

Site-Directed Mutation of T91 in hGAD67. The mutation of T91 in hGAD67 to alanine [hGAD67(T91A)], aspartic acid [hGAD67(T91D)], or glutamic acid [hGAD67(T91E)] was carried out using the Quickchange site-directed mutagenesis kit. The primers used for [hGAD67(T91A)] were 5′-GCCCGCTTCCGGCGCGCAGAGACTGACTTCTC-3′ and 5′-GAGAAGTCAGTCTCTGCGCGCCGGAAGCG-GGC-3′. For [hGAD67(T91D)], the primers used were similar to [hGAD67(T91A)] except that the underlined codons were changed to GAC and GTC, respectively. For [hGAD67(T91E)], the primers used were similar to [hGAD67-(T91A)] except that the underlined codons were changed to GAA and TTC, respectively. The mutations were later verified by automated DNA sequencing.

In Vitro Phosphorylation of hGAD65 and hGAD67. Highly purified hGAD65 and hGAD67 were first dialyzed extensively against a phosphorylation buffer (15 mM Tris-Acetate, 1 mM AET, 0.2 mM PLP at pH 7.2) before the protein kinase treatment. After dialysis, hGAD65 or hGAD67 (~10−20 μg) was added to a 150-μL reaction buffer containing 15 mM Tris-Acetate (pH 7.2), 1 mM AET, 0.2 mM PLP, 1 mM $CaCl_2$ (in the case of the PKCϵ treatment, 1 mM $CaCl_2$ was omitted), 5 mM $MgCl_2$, and 200 μM ATP. For the PKC treatment, 25 ng of PKC$\alpha\beta\gamma$ or PKCϵ, a final concentration of 103 μg/mL phosphatidylserine, 20 μg/mL diacylglycerol,

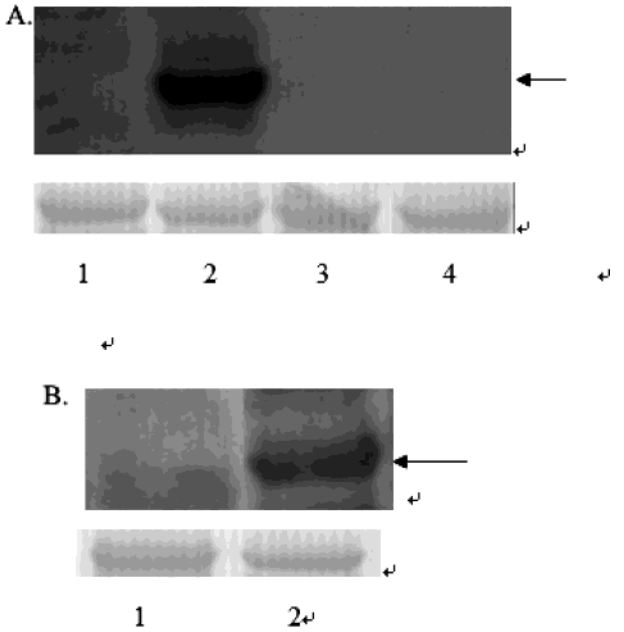

FIGURE 4: Demonstration of the phosphorylation of hGAD65 with different protein kinases by immunoblotting using anti-phosphoserine/threonine antibody. (A) Upper panel, immunostaining using anti-phosphoserine/threonine antibody. Lane 1, control; lane 2, treatment of full-length hGAD65 by PKCϵ; lane 3, treatment of full-length hGAD65 by PKA; lane 4, treatment of full-length hGAD65 by CaMKII. Lower panel, protein staining to ensure equal loading. (B) Upper panel, immunostaining using anti-phosphoserine/threonine antibody. Lane 1, treatment of full-length hGAD65 by PKC$\alpha\beta\gamma$; lane 2, treatment of full-length hGAD65 by PKCϵ. Lower panel, protein staining to ensure equal loading. The arrow indicates the position of hGAD65.

and 80 μM PMA were added to the reaction buffer. For the PKA treatment, 1 μg of PKA catalytic subunit and a final concentration of 0.01 mM *c*AMP were added to the reaction buffer. For the CaMKII treatment, 100 ng of CaMKII and a final concentration of 0.3 mM calmodulin were added. The resulting reaction mixture was further incubated at 30 °C for 30 min. For the control experiments, the condition was the same as the kinase assay condition except that the kinases were excluded.

Dephosphorylation of hGAD65 and hGAD67 by Protein Phosphatases. hGAD65 was first phosphorylated by PKC as described above. The reaction was stopped by adding 0.5 mM PKC inhibitor peptide and further incubated for 5 min. At the end of incubation, the dephosphorylation experiment was carried out as follows: For the PP1 and PP2A treatments, 1 unit of PP1 or PP2A was added directly to the incubation mixture and the dephosphorylation reaction was carried out at 30 °C for 30 min. For the PP2B treatment, a mixture containing 1 unit of PP2B, $NiCl_2$, $CaCl_2$, and calmodulin was added to a final concentration of 1 mM Ni^{2+}, 1 mM Ca^{2+}, and 0.3 mM calmodulin and the dephosphorylation reaction was carried out at 30 °C for 30 min.

hGAD67 was first phosphorylated by PKA as described above. The reaction was stopped by adding 6 mM PKA inhibitor peptide and further incubated for 5 min. At the end of incubation, dephosphorylation experiments were carried out as described for hGAD65.

Enzyme Assay. GAD activity was assayed by a radiometric method measuring the formation of $^{14}CO_2$ from [L-^{14}C]-glutamic acid as described previously (*9*).

Immunoblotting Assay. Highly purified hGAD65 or hGAD67 was phosphorylated according to the method described above and separated on 10% SDS−PAGE followed by transferring to a nitrocellulose membrane. Phosphorylated protein was detected by anti-phosphoserine/threonine antibody (1:500 dilution). Antibody−antigen binding was detected using anti-rabbit horseradish peroxidase (HRP)-linked IgG (1:5000 dilution) as a secondary antibody and was visualized using the enhanced chemiluminescence (ECL).

[^{32}P] Incorporation. Highly purified hGAD65 or hGAD67 was phosphorylated according to the method described above except that 50 μCi [γ-^{32}P]ATP was added to the phosphorylation mixture. After phosphorylation, the reaction mixture was separated by 10% SDS−PAGE. The gel was dried, and the ^{32}P incorporation signal was visualized by a *cyclone* phosphoimager.

Phosphorylation Sites Identification by MALDI−TOF. hGAD67 after the PKA treatment was separated on 10% SDS−PAGE. The band corresponding to hGAD67 was excised and in-gel digested by trypsin. The resulting peptides were analyzed by MALDI−TOF. Peptide mass fingerprinting and peptide sequencing were searched from a database using MS−Fit and MS−Tag, respectively (http://prospector.ucsf.edu). The MALDI−TOF was carried out by the Proteomic

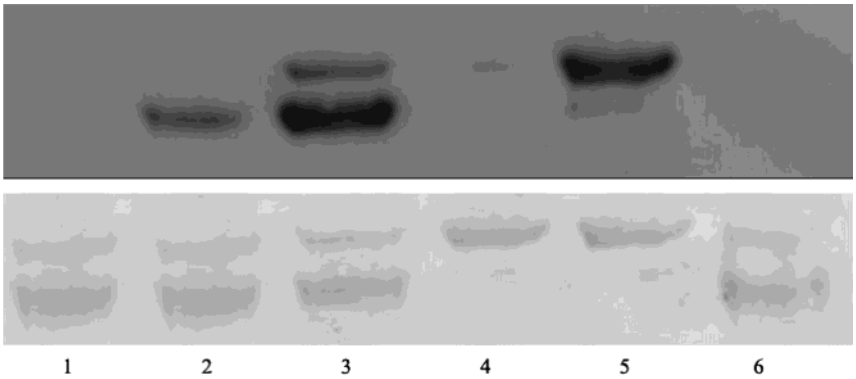

FIGURE 5: Demonstration of the phosphorylation of hGAD67 with different protein kinases by immunoblotting using anti-phosphoserine/threonine antibody. Upper panel, immunostaining using anti-phosphoserine/threonine antibody. Lane 1, control; lane 2, phosphorylation of hGAD67 with a mixture of the full-length and truncated forms by PKC; lane 3, phosphorylation of hGAD67 with a mixture of the full-length and truncated forms by PKA; lane 4, phosphorylation of full-length hGAD67 by PKC; lane 5, phosphorylation of full-length hGAD67 by PKA; lane 6, phosphorylation of hGAD67 with a mixture of the full-length and truncated forms by CaMKII. Lower panel, protein staining to ensure equal loading.

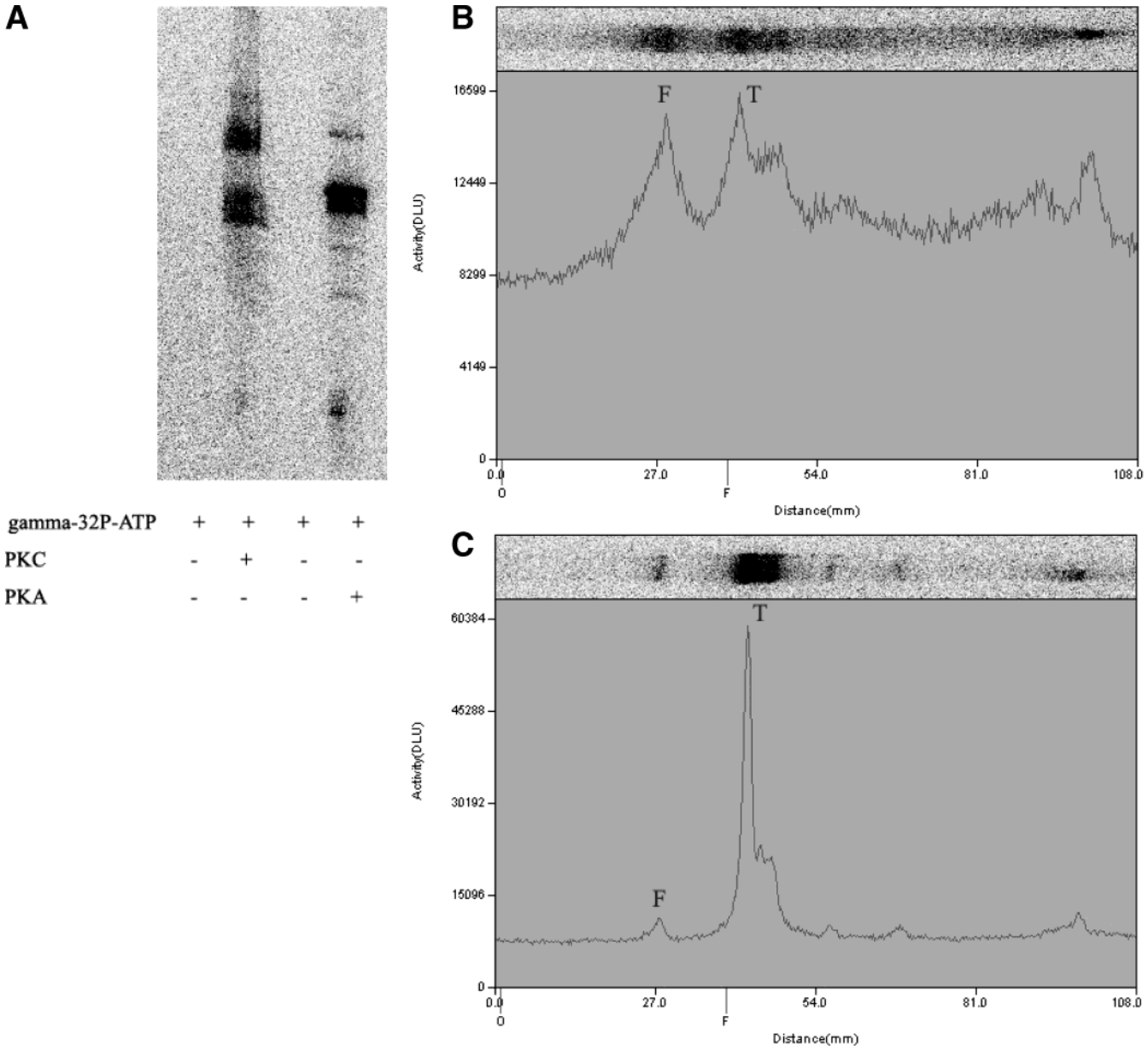

FIGURE 6: Demonstration of direct phosphorylation of hGAD65 and hGAD67 by [^{32}P] incorporation. (A) Phosphorylation of hGAD65 and hGAD67. Lane 1, control containing a mixture of the full-length and truncated forms of hGAD65 and [γ-^{32}P]ATP; lane 2, the same as lane 1 except PKC was included; lane 3, control containing a mixture of the full-length and truncated form of hGAD67 and [γ-^{32}P]ATP; lane 4, the same as lane 3 except PKA was included. The signals were visualized by a *cyclone* phosphoimager. (B) Densitometry scan of the signals from lane 2 of A. (C) Densitometry scan of the signals from lane 4 of A. Peaks indicate the position of the full-length and truncated forms (F = full-length form, and T = truncated form).

Mass Spectrometry Service Lab, University of Massachusetts, Medical Center, MA.

RESULTS

Purification of Recombinant hGAD65 and hGAD67. Recently, we found that when GST-tagged hGAD65 was subjected to Factor Xa cleavage, truncated hGAD65 lacking amino acids 1−69 from the N terminus was released simultaneously with the full-length form (*10*). A similar observation was found for hGAD67, and the release of truncated hGAD67 was confirmed by N-terminal amino acid sequencing (Figure 1). For hGAD67, two truncated forms were produced; one is cleaved between arginine 70 and glutamine 71, and the other one is between arginine 90 and T91. To obtain the full-length hGAD65 and hGAD67 only, the cDNA coding for full-length hGAD65 and hGAD67 were subcloned into a pGEX-6P-1 vector, which contains the PreScission protease cleavage sequence (Leu-Phe-Gln-Gly-Pro) and cleaves between Gln and Gly. When it was cleaved with the PreScission protease, GST fusion proteins would only release the full-length forms (data not shown).

Recombinant hGAD65 and hGAD67 were affinity-purified as described previously (*8*). A mixture of both full-length and truncated forms were obtained when purified from the pGEX-3X expression system, and only the full-length forms were obtained from the pGEX-6P-1 expression system. In the following experiments, GAD preparations purified from both expression systems were used as indicated specifically.

Effects of the Protein Kinase Treatment on hGAD65 and hGAD67 Activity. Consistent with our previous findings on

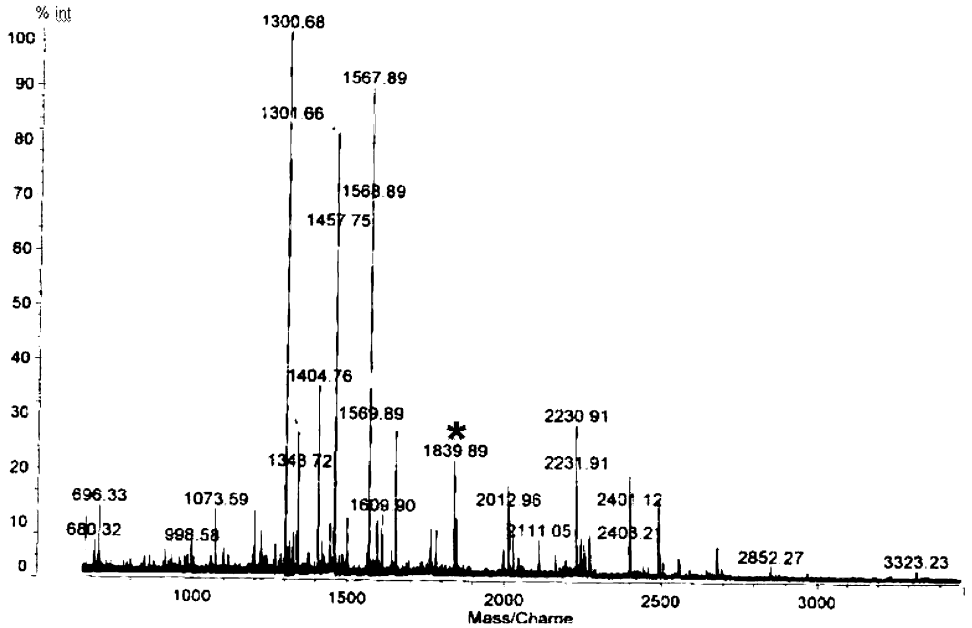

FIGURE 7: Mass spectrum of PKA-treated hGAD67 analyzed by MALDI−TOF. The asterisk indicates the phosphopeptide in hGAD67, $FRRT_{(P)}ETDFSNLFAR$.

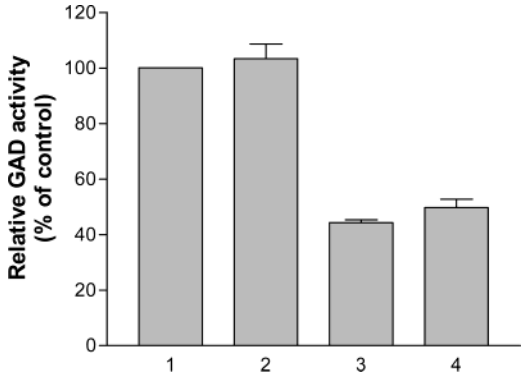

FIGURE 8: Effect of site-directed mutation on GAD67 activity. (1) Wild-type hGAD67, (2) GAD67(T91A), (3) GAD67(T91E), and (4) GAD67(T91D). The error bars indicate the standard deviation with $n = 3$.

mGAD (*6*), hGAD65 activity increased upon phosphorylation (open bars of Figure 2). The treatment of hGAD65 with a mixture of PKC isoforms increased hGAD65 activity by ~65% (open bar of lane 2 of Figure 2), while PKA and CaMKII had little effect on hGAD65 activity (open bars of lanes 3 and 4 of Figure 2). Interestingly, when a mixture of PKCα, -β, and -γ isoforms alone was tested, no significant effect was obtained on hGAD65 activity. A novel PKC isoform ϵ, PKCϵ, which is Ca^{2+}-independent, was found to increase hGAD65 activity by ~35% (Table 1). On the contrary, hGAD67 activity was inhibited by phosphorylation. The PKA treatment inhibited hGAD67 activity by ~35% (closed bar of lane 3 of Figure 2). PKC and CaMKII had no obvious effect on hGAD67 activity (closed bars of lanes 2 and 4 of Figure 2). ATP at the concentration of 200 μM alone had no significant effect on both hGAD65 and hGAD67 activity (lane 5 of Figure 2).

Effect of Protein Phosphatase Treatment on hGAD65 and hGAD67 Activity. To test whether the effect of phosphorylation on hGAD65 and hGAD67 activity is reversible, dephosphorylation studies were performed as described in the Materials and Methods. hGAD65 activity was found to be inhibited by dephosphorylation, while hGAD67 was activated (Figure 3). For hGAD65, PKC-mediated activation of GAD activity was completely abolished by all three protein phosphatases tested (Figure 3). It is of interest that protein phosphatase 2A (PP2A) not only abolished PKC-mediated activation, but also decreased the basal activity of hGAD65 by ~55% (open bar of lane 4 of Figure 3). Similarly, the PKA-mediated inhibition of hGAD67 activity was also abolished by all of the phosphatases tested, including PP1, PP2A, and PP2B. In addition, PP1/PP2A and PP2B also significantly increased the basal activity of hGAD67 by 40 and 70%, respectively (closed bars of lanes 3−5 of Figure 3).

Direct Phosphorylation of hGADs. To test whether the effect of kinase treatment was due to the direct phosphorylation of GAD proteins, we carried out the immunoblotting assay using a general anti-phosphoserine/threonine antibody. Among the PKC isoforms tested, only PKCϵ (lane 2 of both parts A and B of Figure 4), but not PKC isoforms α, β, and γ (lane 1 of Figure 4B), could phosphorylate and activate hGAD65. hGAD65 could not be phosphorylated by PKA and CaMKII in vitro (lanes 3 and 4 of Figure 4A, respectively).

When hGAD67 was treated with PKA, both the full-length and truncated hGAD67 were phosphorylated by PKA (lanes 3 and 5 of Figure 5). Interestingly, although PKC has no effect on hGAD67 activity, the truncated hGAD67, but not the full-length hGAD67, could be phosphorylated by PKCα, -β, and -γ treatments (lanes 2 and 4 of Figure 5). CaMKII could not phosphorylate the full-length or the truncated form of hGAD67 (lane 6 of Figure 5).

The results from the immunoblotting test were further confirmed by ^{32}P autoradiography. A direct demonstration of [^{32}P] incorporation into both the full-length and truncated hGAD65 was obtained when hGAD65 was incubated with PKC in the presence of [γ-^{32}P]ATP as shown in parts A (lane 2) and B of Figure 6. Similarly, when treated with PKA, both full-length and truncated hGAD67 showed strong

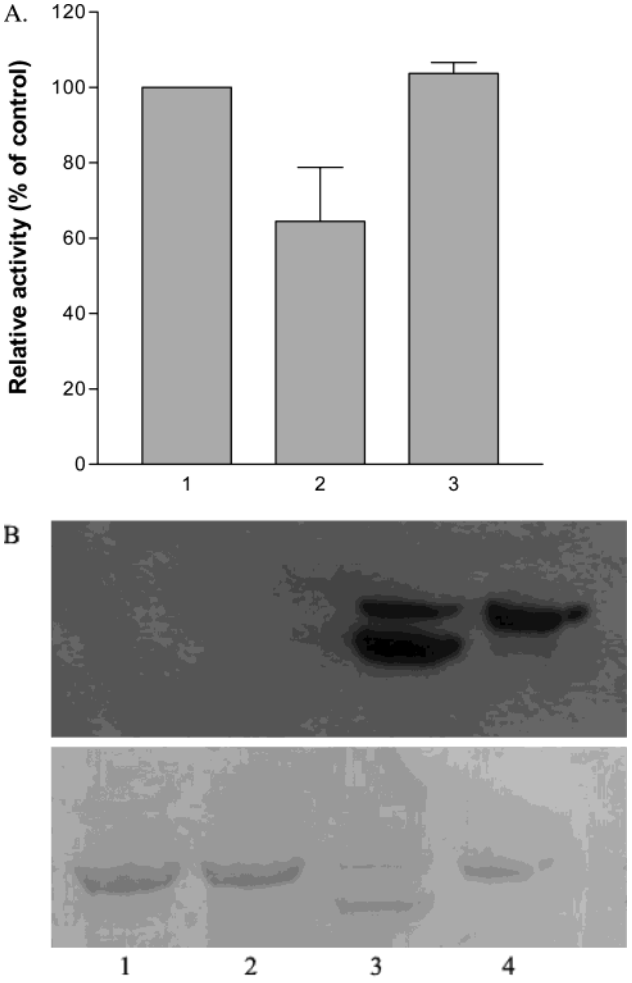

FIGURE 9: Role of T91 in the PKA-mediated regulation of hGAD67 activity through phosphorylation. (A) Lane 1, control; lane 2, wild-type hGAD67 treated with PKA; lane 3, hGAD67(T91A) treated with PKA. (B) Upper panel, immunoblotting using anti-phosphoserine/threonine antibody. Lane 1, hGAD67(T91A) control; lane 2, hGAD67(T91A) treated with PKA; lanes 3 and 4, wild-type hGAD67 treated with PKA served as the positive control (lane 3, a mixture containing both the full-length and truncated forms of hGAD67; lane 4, the full-length form of hGAD67 only). Lower panel, protein staining to ensure equal loading.

incorporation of [^{32}P] (parts A (lane 4) and C of Figure 6). No incorporation of [^{32}P] was observed in the control group (lanes 1 and 3 of Figure 6A).

Identification of the Phosphorylation Site by MALDI−TOF. After the positive demonstration of direct phosphorylation of hGAD67 by [^{32}P] incorporation and immunoblotting using a phospho-specific antibody, we further identified the residue(s) involved in the phosphorylation of hGAD67 by MALDI−TOF as shown in Figure 7. The sequence coverage for trypsin digestion is 24%. By comparing the theoretical spectrum with the experimental spectrum, we identified one phosphopeptide in hGAD67, which contains amino acids 88−101, FRRTETDFSNLFAR, as indicated by an asterisk in Figure 7, because it shows an increase in its mass of 80 Da (*m*/*z* 1839.89) because of the addition of HPO_3^- compared to the theoretical peptide (*m*/*z* 1759.89). Once the phosphopeptide was identified, it is further analyzed via MS/MS to identify the residue, which is phosphorylated. Fragment ions from the phosphopeptide obtained by MS/MS are (*m*/*z*) 516.37, 542.73, 854.70, 888.70, 906.54, 986.31, 1052.73, 1070.62, 1087.95, 1200.06, 1283.01, 1569.08, 1585.77, and 1603.99. These fragment ions were used to search the matched peptide from the database by MS−Tag. When the fragmental ions were compared, two potential phosphorylation sites were identified, i.e., T91 and threonine 93 (T93), in FRRT(91)ET(93)DFSNLFAR. We concluded that T91 is the site that is phosphorylated by PKA based on the following two criteria: first, T91 is within the PKA phosphorylation motif R−X−S/T, and second, phosphorylation of T91 could block the potential cleavage by trypsin between arginine 90 and T91, thus maintaining the whole peptide to remain intact.

Site-Directed Mutagenesis. To confirm our conclusion from MALDI−TOF and further address the role of T91 in regulation of hGAD67 activity, we mutated this residue either to alanine [GAD67(T91A)], which cannot be phosphorylated, glutamic acid [GAD67(T91E)], or aspartic acid [GAD67-(T91D)], which would mimic the phosphorylation of GAD67. Mutation of T91 to alanine alone has no significant effect on GAD67 activity as compared to the wild-type GAD67 (Figure 8). However, mutation of T91 to glutamic acid or aspartic acid decreased GAD67 activity by ~60% (Figure 8). Mutation of T91 to alanine also abolished the PKA-mediated inhibition of hGAD67 activity (Figure 9A) and prevented phosphorylation of hGAD67 by PKA because no signal was detected by immunoblotting (lanes 1 and 2 of Figure 9B).

DISCUSSION

The results presented here, together with our previously reported findings (*5*, *6*), strongly support the conclusion that phosphorylation plays an important role in the regulation of GAD activity. GAD65 and GAD67 appear to be regulated differently by phosphorylation. GAD65 is activated upon phosphorylation, while GAD67 is inhibited by phosphorylation. How the phosphorylation causes opposite responses in GAD65 and GAD67 needs to be further characterized. It is conceivable that phosphorylation of GAD65 and GAD67 may change the K_m of the enzymes for their cofactor PLP or their substrate L-glutamic acid differently. Accordingly, for GAD65, phosphorylation might decrease the K_m for PLP and/or L-glutamic acid and the opposite effect would occur to GAD67 upon phosphorylation. The previous studies on mGAD showed that phosphorylation lowers the K_m of mGAD for L-glutamate from 2.1 to 0.9 mM and increases the V_{max} by 50% (*6*). Because the experiments reported here were conducted at a saturating level of PLP (0.2 mM), the effect of the phosphorylation is most likely due to the changes in the K_m for L-glutamic acid.

In regards to the identity of protein kinases and phosphatases involved in the regulation of GAD65 and GAD67, we have screened the three most abundant protein kinases, PKC, PKA, and CaMKII, and three types of protein phosphatases, PP1, PP2A, and PP2B, for their possible roles in the regulation of GAD65 and GAD67 in vivo. PP2Cα was not included because it is less-characterized in neurons than the other three types (*11*). hGAD67 is phosphorylated by PKA and dephosphorylated by calcineurin. Their possible roles in vivo were confirmed by the previous study of sGAD (*5*). Using MALDI−TOF, the phosphorylation site by PKA was identified to be T91. The importance of T91 in regulating hGAD67 activity is supported by the site-directed mutagen-

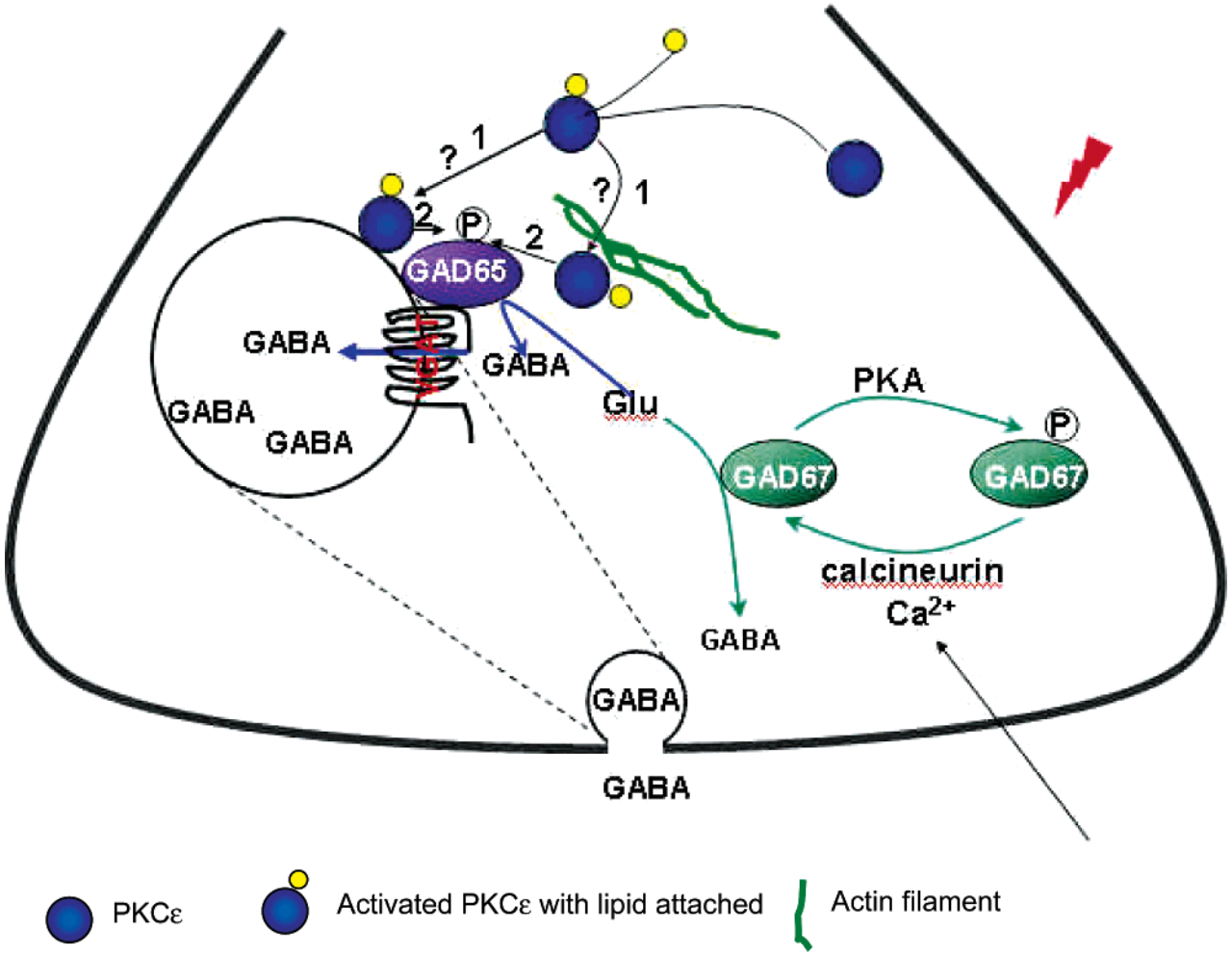

FIGURE 10: Proposed model depicting the role of protein phosphorylation in the regulation of GAD65 and GAD67 and its physiological implications (modified from ref *24*).

esis experiment. When T91 is mutated to alanine, hGAD67 could no longer be phosphorylated by PKA and the effect of PKA-mediated inhibition on hGAD67 is abolished. However, when T91 is mutated to aspartic acid or glutamic acid, which mimics the phosphorylation status of hGAD67, the activity was greatly decreased compared to the wild-type hGAD67. It is interesting that, although PKC had no effect on hGAD67 activity, it could phosphorylate the truncated hGAD67 but not the full-length form. The fact that the full-length hGAD67 cannot be phosphorylated by PKC has two implications: first, the phosphorylation site for PKC is not within the N terminus of hGAD67 (1−70 amino acids); second, the potential phosphorylation sites for PKC are masked in the full-length hGAD67, and the removal of the first 70 amino acids from the N terminus causes a conformational change that exposes the sites that can then be phosphorylated by PKC.

When GAD65 is compared to GAD67, the responsible protein kinases and phosphatases for the regulation of GAD65 are less-characterized. The earlier study on mGAD, presumably GAD65, did not specify the responsible protein kinase but indicated that the phosphorylation of mGAD is through a membrane-bound protein kinase (*6*). CaMKII might be a good candidate because it is also associated with synaptic vesicles and can phosphorylate several synaptic-vesicle-associated proteins (*12*). However, our in vitro studies indicate that CaMKII has no effect on GAD activity and cannot phosphorylate GAD based on the immunoblotting analysis. It is interesting that Ca^{2+}/CaM could induce the dimerization of plant GAD, thus activating plant GAD (*13*). Although this mechanism may be important for plant GAD, it has not been reported in mammalian GAD.

We were unable to identify the phosphopeptide for hGAD65 presumably because of the low recovery of the phosphopeptide. It has been reported that phosphorylation of serine residues 3, 6, 10, and 13 in GAD65 is involved in the anchoring of GAD65 to the membrane and has no effect on GAD65 activity (*14*). In our study, the truncated form with the deletion of amino acids 1−69 from the N terminus was also phosphorylated as shown by ^{32}P incorporation, indicating that the sites that are phosphorylated by PKC are different from the four sites reported by Namchuk et al. (*14*). In their study, they did not identify the protein kinases that are responsible for the phosphorylation of Ser3, -6, -10, and -13. By searching the consensus sequence motif, we found that they are the potential phosphorylation site for mitogen-activated protein kinase (MAPK) and glycogen synthase kinase 3 (GSK-3).

Our current paper indicates that PKC phosphorylates and activates GAD65 in vitro. PKC consists of a family of enzymes: conventional PKC (PKCα, -β, and -γ), which require diacylglycerol, phosphatidylserine, and Ca^{2+}, novel PKC (PKCδ, -ϵ, -η, and -θ), which are Ca^{2+}-independent but do require diacylglycerol, and atypical PKC (PKCζ, -ι,

and -λ), whose regulation is not fully understood. Numerous studies have shown the functional link between PKC isoforms and changes in the neurotransmitter release (*15*, *16*). The 83.4-kDa PKCϵ isoform is particularly interesting, because it is expressed at very low levels in several normal tissues but is expressed at much higher levels in the brain, particularly the presynaptic nerve terminals (*17*). The evidence for the specific involvement of PKCϵ in brain functions is emerging (*18*−*21*). Our study on phosphorylation of mGAD also indicated that the responsible protein kinase is Ca^{2+}-independent, because common Ca^{2+} chelator such as EDTA has no effect on the activation of mGAD by ATP (*6*). This observation together with the property of PKCϵ being Ca^{2+}-independent led us to speculate the involvement of PKCϵ in the regulation of GAD65 in vivo. This notion is supported by our results reported here that PKCϵ, but not the PKCα, -β, and -γ mixture, could phosphorylate and activate GAD65. Furthermore, PKCϵ can be translocated to the membrane fraction in rat cortical synaptosomes upon activation (*22*). Herein, a model to depict the role of PKCϵ in the regulation of GABA biosynthesis is proposed as follows (Figure 10): Upon neuronal stimulation, PKCϵ translocates from cytosol to the synaptic-vesicle (SV) membrane or near the SV (step 1), for example, through anchoring to filamentous actin (*23*), and is activated by phospholipids. This translocation process needs to be investigated and thus is indicated by a question mark. This translocation brings PKCϵ close to GAD65, resulting in phosphorylation and activation of SV-associated GAD65 (step 2). GABA newly synthesized by SV-associated GAD65 is then transported into SVs by a vesicular GABA transporter (VGAT) through a coupling mechanism (*24*). The refilled GABA in the SVs is ready to be released upon arrival of a new action potential.

In summary, this paper demonstrates that GAD65 and GAD67 are regulated in an opposite manner by phosphorylation, and this is compatible to the idea that these two forms of GAD, which have different subcellular distribution, may also be regulated differently in responding to physiological conditions.

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BI0496992