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Proteolytic processing of the Alzheimer disease-associated presenilin-1 generates an *in vivo* substrate for protein kinase C

(protein phosphorylation/muscarinic receptors/phorbol ester/signal transduction)

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ABSTRACT **The majority of familial Alzheimer disease mutations are linked to the recently cloned presenilin (PS) genes, which encode two highly homologous proteins (PS-1 and PS-2). It was shown that the full-length PS-2 protein is phosphorylated constitutively within its N-terminal domain by casein kinases, whereas the PS-1 protein is not. Full-length PS proteins undergo endoproteolytic cleavage within their hydrophilic loop domain resulting in the formation of ≈20-kDa C-terminal fragments (CTF) and ≈30-kDa N-terminal fragments [Thinakaran, G.,** ***et al.*** **(1996)** ***Neuron*** **17, 181–190]. Here we describe the surprising finding that the CTF of PS-1 is phosphorylated by protein kinase C (PKC). Stimulation of PKC causes a 4- to 5-fold increase of the phosphorylation of the ≈20-kDa CTF of PS-1 resulting in reduced mobility in SDS gels. PKC-stimulated phosphorylation occurs predominantly on serine residues and can be induced either by direct stimulation of PKC with phorbol-12,13-dibutyrate or by activation of the m1 acetylcholine receptor-signaling pathway with the muscarinic agonist carbachol. However, phosphorylation of full-length PS-1 and PS-2 is not altered upon PKC stimulation. In addition, a mutant form of PS-1 lacking exon 10, which does not undergo endoproteolytic cleavage [Thinakaran, G.,** ***et al.*** **(1996)** ***Neuron*** **17, 181–190] is not phosphorylated by PKC, although it still contains all PKC phosphorylation sites conserved between different species. These results show that PKC phosphorylates the PS-1 CTF. Therefore, endoproteolytic cleavage of full-length PS-1 results in the generation of an** ***in vivo*** **substrate for PKC. The selective phosphorylation of the PS-1 CTF indicates that the physiological and/or pathological properties of the CTF are regulated by PKC activity.**

Alzheimer disease (AD) is by far the most common form of dementia worldwide, currently affecting approximately 17 million people. Pathologically, AD is characterized by the invariant accumulation of senile plaques predominantly composed of the amyloid-β peptide (Aβ; ref. 1). Aβ is a proteolytic breakdown product of the β-amyloid precursor protein (βAPP; ref. 2). In most cases the disease occurs sporadically in an age-dependent manner; however, in approximately 10% of the cases, AD can be inherited as an autosomal dominant trait [familial Alzheimer disease (FAD)]. FAD mutations have been linked so far to three different genes (for review see refs. 3 and 4). In addition, a polymorphism in the allele encoding apolipoprotein E was found to be a major risk factor (for review see ref. 5). Mutations within the βAPP gene account for only a very small minority of FAD cases. The majority of FAD cases were linked to mutations within a gene located on chromosome 14 (6–8). This gene was cloned recently and called presenilin (PS)-1 (9). Shortly after, a second gene involved in chromosome 1-linked FAD (10) was identified and named PS-2 (11, 12). Both genes encode highly homologous proteins with multiple trans-membrane domains (refs. 9, 11, and 12; for review see refs. 13 and 14). So far more than 30 FAD-causing mutations were shown to occur within PS-1, whereas only 2 mutations were found within PS-2 (13, 14). Recent work analyzing the C termini of Aβ secreted from primary fibroblasts, or detected within plasma from family members with FAD-linked PS-1 as well as PS-2 mutations, clearly showed that these mutations cause an increased production of Aβ42/43 (15). Similar results were also obtained by overexpressing a variety of mutant PS cDNAs in tissue culture cells and transgenic mice (16–19). Moreover, *in vivo* these mutations clearly cause the enhanced formation of Aβ42/43-containing amyloid plaques (20, 21). Sequence homologies to the *Caenorhabditis elegans* genes *spe-4* and *sel-12* suggest that PS genes might be involved in vesicular trafficking or notch signaling (22, 23). Indeed, recent studies (24, 25) showed that human PS-1 can reconstitute the *sel-12* mutant phenotype, strongly suggesting that PS proteins are involved directly or indirectly in notch signaling.

Cellular expression of the PS genes showed that both PS proteins are predominantly located within the endoplasmic reticulum (ER) (26–29). Interestingly, PS-2 but not PS-1 was shown to be constitutively phosphorylated. PS-2 phosphorylation was mapped to three serine residues within an acidic sequence of the N-terminal domain, which is missing in PS-1 (26). Recently, it was shown that PS proteins undergo endoproteolytic processing (30, 31). The cleavage occurs within the large loop between trans-membrane domain 6 (TM6) and TM7 (of an eight-trans-membrane domain model; ref. 32), which results in the production of ≈30-kDa N-terminal and ≈20-kDa C-terminal fragments (CTFs) (refs. 30 and 31; see also Fig. 1). Radio sequencing of the C-terminal fragment indicated the production of heterogeneous fragments beginning at amino acids 291–299 (D. J. Selkoe and D. B. Teplow, personal communication). Moreover, it appears that *in vivo* PS proteins predominantly occur as proteolytic breakdown products, whereas very little if any full-length PS can be detected within the tissues and cell lines analyzed (30). Here, we report the

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Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer disease; βAPP, β-amyloid precursor protein; CTF, C-terminal fragment; ER, endoplasmic reticulum; FAD, familial Alzheimer disease; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PS, presenilin; TM, trans-membrane domain.

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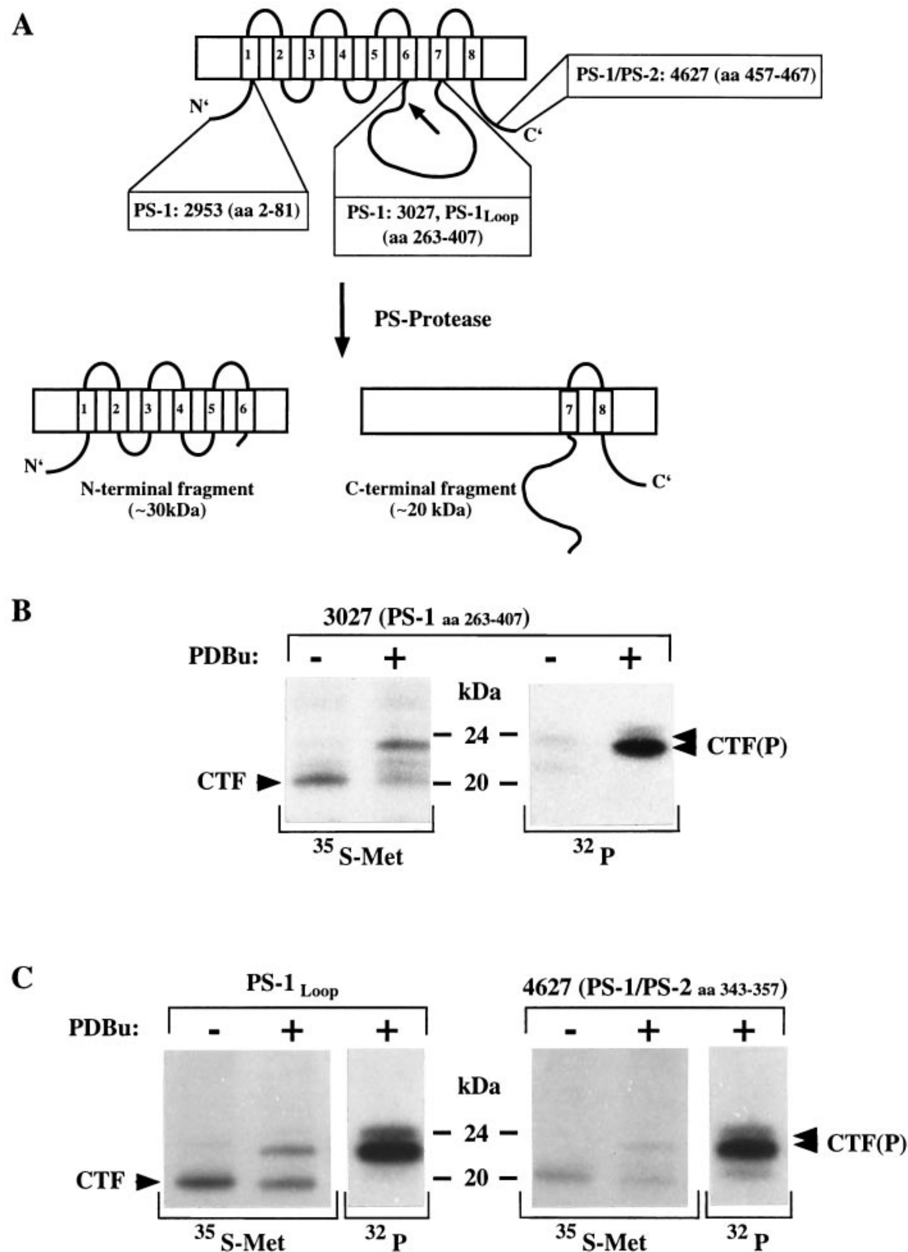

FIG. 1. (*A*) Schematic drawing showing the potential eight-trans-membrane domain structure (32) and the proteolytic processing of the PS proteins (30). The proteolytic cleavage (indicated by an arrow) occurs within the large hydrophilic loop between TM6 and TM7 generating an ≈30-kDa N-terminal fragment and an ≈20-kDa CTF (30). The epitopes of the antibodies used are indicated. (*B*) PDBu stimulates phosphorylation of the CTF of PS-1. Untransfected COS-7 cells were radiolabeled in the presence (+) or absence (−) of PDBu with [^{35}S]methionine (^{35}S-Met) as well as [^{32}P]orthophosphate (^{32}P). Cell lysates were immunoprecipitated with antibody 3027. After [^{35}S]methionine labeling a major polypeptide of ≈20 kDa (CTF) was observed under control conditions. Upon PKC stimulation with PDBu the ≈20-kDa band shifted to ≈23 kDa. Labeling with [^{32}P]orthophosphate revealed two weakly phosphorylated polypeptides of ≈22 kDa and ≈23 kDa under control conditions. PDBu treatment resulted in the detection of a highly phosphorylated polypeptide of ≈23 kDa and a weaker band of ≈24 kDa [CTF(P)]. (*C*) Results very similar to *B* were obtained with two independent antibodies. Untransfected COS-7 cells were radiolabeled in the presence (+) or absence (−) of PDBu with [^{35}S]methionine (^{35}S-Met) as well as [^{32}P]orthophosphate (^{32}P). Cell lysates were immunoprecipitated with antibodies BOS 4627 (26) and PS-1$_{loop}$ (30).

surprising finding that the CTF of PS-1 is selectively phosphorylated *in vivo* by PKC, whereas no PKC-dependent phosphorylation of full-length PS was observed. Our results suggest that endoproteolytic cleavage of PS-1 results in the generation of a PKC substrate. Because phosphorylation of the CTF is modulated by activation/inhibition of muscarinic acetylcholine receptors, the physiological and/or pathological properties of the CTF might be regulated by PKC.

MATERIALS AND METHODS

Antibodies. Polyclonal antibodies 3027 and 2953 were raised to the loop domain (amino acids 263–407) and the N terminus (amino acids 2–81) of PS-1, respectively. The respective coding regions were amplified by PCR using the primers 5′-CCGAATTCTGTCCGAAAGGTCCA-3′ and 5′-CCG-GATCCCTAGGTTGTGTTCCAGTC-3′ for the PS-1 loop domain (3027), and 5′-CCGGATCCACAGAGTTACCTG-CACC-3′ and 5′-TGCGGTCGACATGCTTGGCGC-CATATT-3′ for the PS-1 N terminus (2953). The resulting fragments were cloned into the *Eco*RI/*Bam*HI (3027) restriction site and the *Bam*HI/*Sal*I (2953) restriction site, respectively, of pMAL-c2 (New England Biolabs). The fusion proteins were expressed in *E. coli* DH5α, purified on amylose resin (New England Biolabs) according to the supplier's instruc-

tions, and inoculated into rabbits. Antibody PS-1_{Loop} is described by Thinakaran *et al.* (30). The polyclonal anti-peptide antibodies BOS4627 raised to PS-$1_{457–467}$ and BOS4624 raised to PS-$1_{343–357}$ were described previously (26). The epitopes of the antibodies used in this study are shown in Fig. 1*A*.

Cell Culture. All cell lines were cultured in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum. The kidney 293 cell lines stably expressing PS-1 wild type and PS-1 Δexon 10 were described previously (17). Transient transfections were carried out using DOTAP (Boehringer Mannheim). All cDNA constructs used are encoding the VRSQ motif.

Metabolic Labeling and Immunoprecipitation. Metabolic labeling and immunoprecipitations were carried out as described by Haass *et al.* (33), except that the immunoprecipitates were heated to 68°C before they were separated on 8% or 10% SDS polyacrylamid gels containing 4 M urea.

***In Vivo* Phosphorylation.** A confluent cell monolayer was incubated for 45 min in phosphate-free media (Sigma). The media were aspirated and the respective fresh media were added, together with 13–36 MBq [^{32}P]orthophosphate, and cells were incubated for 2–4 h at 37°C. The conditioned media were then aspirated, and cells were washed twice with ice-cold PBS and immediately lysed on ice with lysis buffer containing 1% Nonidet P-40 for 10 min. Cell lysates were centrifuged 10 min at 14,000 × *g*, and supernatants were immunoprecipitated with specific anti-PS-antibodies for 2–3 h. Radiolabeled proteins were separated by SDS/PAGE as described above and visualized by autoradiography. Quantification was done by phospohorimaging (Molecular Dynamics) using the IMAGEQUANT program as described by Hung *et al.* (34).

Stimulation and Inhibition of Protein Kinase C. To activate protein kinase C during *in vivo* labeling with [^{32}P]orthophosphate, phosphorylation was carried out in the presence of 1 μM phorbol 12,13-dibutyrate (PDBu; Sigma). To inhibit protein kinase C activity, the specific inhibitor GF109203X (Biomol, Plymouth Meeting, PA; final concentration, 0.1 μM) was added to the media during periods of labeling. To activate or inhibit muscarinic acetylcholine receptors, U373 cells stably transfected with the m1-type muscarinic receptor cDNA were treated with 1 mM carbachol (Sigma) or 10 μM atropine (Sigma), respectively.

Dephosphorylation by Alkaline Phosphatase. Immunoprecipitates of PS-1 CTFs were incubated for 1 h at 37°C in the absence or presence of alkaline phosphatase (calf intestine; Boehringer, Mannheim) according to the supplier manual.

Phosphoamino Acid Analysis. ^{32}P-labeled CTFs of PS-1 were isolated by immunoprecipitation, separated by SDS/PAGE, electrotransferred onto PVDF membrane, and localized by autoradiography. Bands were cut out and two-dimensional phosphoamino acid analyses were carried out according to Boyle *et al.* (35). Briefly, PS fragments were hydrolyzed by incubation in 6 M HCl for 90 min at 110°C. Supernatants were dried in a SpeedVac concentrator (Savant), and the resulting pellets were dissolved in pH 1.9 buffer (7.8% acetic acid/2.5% formic acid) and spotted onto cellulose–TLC plates together with unlabeled phosphoamino acids (P-Ser, P-Thr, P-Tyr; 1 μg each). High voltage electrophoresis was carried out for 20 min (pH 1.9 buffer) at 1.5 kV and for 16 min (pH 3.5 buffer; 5% acetic acid/0.5% pyridine) at 1.3 kV, respectively. Radioactive phosphoamino acids were identified by autoradiography and comparison with ninhydrin-stained standards.

RESULTS

Recently, it was shown that PS proteins undergo proteolytic processing resulting in the generation of ≈20-kDa C-terminal and ≈30-kDa N-terminal fragments (refs. 30 and 31; see Fig. 1*A*). Because the large hydrophilic loop of PS-1 contains seven potential PKC phosphorylation sites (ref. 9; see also Fig. 5), we examined whether the CTF generated by proteolytic cleavage serves as a PKC substrate. Untransfected COS-7 cells were labeled with [^{35}S]methionine and then incubated in the presence or absence of PDBu, a direct stimulator of PKC (36). Cell lysates were immunoprecipitated with antibody 3027 raised to the hydrophilic loop of PS-1 (Fig. 1*A*). This antibody specifically detects PS-1 with no cross-reactivity to PS-2 (data not shown). Under control conditions, antibody 3027 immunoprecipitates a polypeptide of ≈20 kDa (Fig. 1*B*). In PDBu-treated cells, a major ≈23-kDa polypeptide is detected, which is absent in unstimulated cells, while the ≈20-kDa band is weaker compared with control cells. When cells were labeled with [^{32}P]orthophosphate in the absence of PDBu, two weakly phosphorylated polypeptides of ≈22 kDa and ≈23 kDa were detected (Fig. 1*B*). PKC stimulation with PDBu results in the detection of a major ^{32}P-labeled polypeptide with an apparent molecular mass of ≈23 kDa and a weaker band of ≈24 kDa. The ≈20-kDa polypeptide detected in [^{35}S]methionine-labeled cells is not phosphorylated. The weaker ^{32}P-labeled band of ≈24 kDa presumably represents a distinct phosphorylated form of the PS-1 CTF, because the amount of this form increased when cells were treated with PDBu in the presence of the phosphatase inhibitor okadaic acid (data not shown). To prove that the observed polypeptides indeed represent the CTF of PS-1, we performed an epitope mapping using several independent antibodies (Fig. 1*A*). Antibody PS-1_{loop} (30) also detected the same polypeptides specifically (Fig. 1*C*). This antibody was recently used to identify the CTF of PS-1 for the first time (30). Another polyclonal antibody raised to the peptide PS-$1_{457–467}$ (BOS4627; Fig. 1*A*), which specifically detects PS-1 as well as PS-2 (26) identified both the 20-kDa and the 23-kDa peptides (Fig. 1*C*). None of these polypeptides was immunoprecipitated with the corresponding preimmunsera or with antibody 2953 (Fig. 1*A*) raised against the N-terminal domain of PS-1 proteins (data not shown). These data therefore prove that the observed polypeptide represent the C-terminal cleavage product of the PS full-length protein. Moreover, because the antibodies PS-1_{loop} (30) and 3027 are specific for PS-1 and do not cross-react with PS-2, these data show that the observed polypeptides represent endogenous PS-1 CTFs. PDBu-stimulated phosphorylation of the CTF is not cell-specific because it was observed in all cell lines analyzed so far, including human kidney 293 cells (Fig. 3), human primary fibroblasts (data not shown), and human U373 cells (Fig. 5). It should be noted that PDBu treatment had no effect on phosphorylation of the PS-1 N-terminal fragment (data not shown).

To further prove that the observed molecular mass shift of PS-1 CTF is due to covalent phosphate incorporation (and not to alternative proteolytic cleavage), immunoprecipitates from PDBu-treated COS-7 cells were incubated in the presence or absence of alkaline phosphatase. Phosphatase treatment resulted in a shift of the 23-kDa form of the PS-1 CTF back to 20 kDa, while incubation in the absence of phosphatase had no effect on its mobility (Fig. 2*A*). These results therefore indicate that the ≈20-kDa CTF undergoes PDBu-stimulated phosphorylation causing a molecular mass shift of about 3–4 kDa. Such a phenomenon is frequently observed for other hyperphosphorylated proteins such as the tau protein (37).

To assess the involvement of PKC in the phosphorylation of the ≈20-kDa CTF, the effect of the PKC-specific inhibitor GF109203X on ^{32}P incorporation was tested. COS-7 cells were labeled with [^{32}P]orthophosphate in the presence or absence of PDBu or GF109203X, and cell lysates were immunoprecipitated with antibody 3027. Inhibition of PKC by GF109203X indeed strongly suppressed phosphorylation of the CTF in the presence of PDBu (Fig. 2*B*), thus clearly showing that PKC catalyses the phosphorylation of the PS-1 CTF. Quantification of the stimulatory effect of PDBu on CTF phosphorylation revealed an approximately 4- to 5-fold increase (Fig. 2*C*). To prove which amino acids of the CTF were phosphorylated by PKC, we performed a phosphoamino acid analysis of the

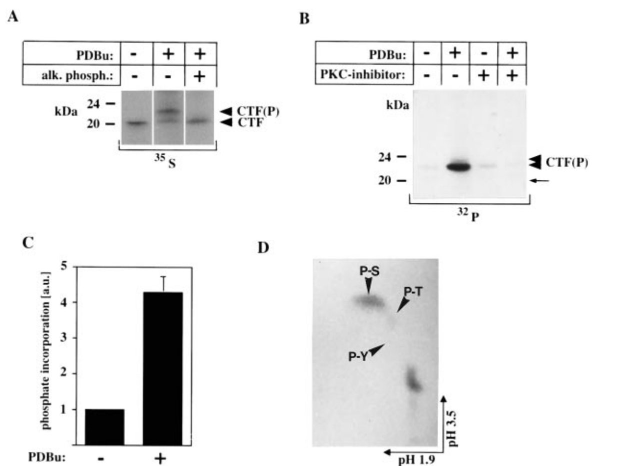

FIG. 2. (*A*) Dephosphorylation reverses the molecular mass shift of the PS-1 CTF. Immunoprecipitates from PDBu-treated COS-7 cells labeled with [^{35}S]methionine were incubated in the presence or absence of alkaline phosphatase. Phosphatase treatment induces a shift of the phosphorylated 23-kDa CTF observed after PDBu treatment back to 20 kDa, which comigrates with the unphosphorylated CTF from unstimulated cells. (*B*) Inhibition of PKC suppresses phosphorylation of the 20-kDa PS-CTF. Untransfected COS-7 cells were radiolabeled in the presence (+) or absence (−) of PDBu with or without (±) the PKC-specific inhibitor GF109203X with [^{32}P]orthophosphate. Cell lysates were immunoprecipitated with antibody 3027. The phosphorylated ≈23-kDa and ≈24-kDa polypeptides are marked by arrowheads [CTF(P)]; the position of the unphosphorylated CTF, which is not visible by ^{32}P labeling is marked by an arrow. This band was detected by parallel labeling with [^{35}S]methionine (data not shown). (*C*) Quantification of PKC-mediated phosphorylation of the PS-1 CTF. Untransfected COS-7 cells were radiolabeled in the presence (+) or absence (−) of PDBu with [^{32}P]orthophosphate (^{32}P). Cell lysates were immunoprecipitated with antibody 3027 and ^{32}P incorporation was quantified by phosphorimaging (34). (Bars = ± SE; $n = 3$.) (*D*) Phosphoamino acid analysis of PKC-phosphorylated CTF. Untransfected COS-7 cells were radiolabeled in the presence of PDBu with [^{32}P]orthophosphate. The CTF was isolated by immunoprecipitation using antibody 3027. The position of phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) is indicated.

phosphorylated CTF after stimulation with PDBu. As shown in Fig. 2*D*, the PS-1 CTF is phosphorylated predominantly on serine residues. This is consistent with the notion that five out of seven potential PKC phosphorylation sites are serines and two are threonines (ref. 11; see Fig. 5).

Previously we have shown that full-length PS-2 is phosphorylated *in vivo* within its N-terminal domain, whereas full-length PS-1 is phosphorylated very weakly, if at all (26). PS-2 phosphorylation was mapped to serine residues within an acidic sequence of the N-terminal domain containing potential phosphorylation sites for both casein kinase 1 and 2 (26). However, here we report that PKC phosphorylates the CTF of PS-1 *in vivo*. Because we (26) and others (30) do not detect endogenous full-length PS proteins, kidney 293 cells stably transfected with the PS-1 cDNA (19) were incubated with [^{32}P]orthophosphate in the presence or absence of PDBu. In parallel, cells were radiolabeled with [^{35}S]methionine. Cell lysates were prepared and full-length PS proteins and the CTFs were immunoprecipitated with antibody BOS4627. As shown by [^{32}S]methionine labeling, PS-1 was expressed as full-length protein of 42–45 kDa (Fig. 3*A*). In agreement with our previous report (26), we found very little if any phosphorylation of full-length PS-1 under nonstimulated conditions, which was not increased after PKC stimulation with PDBu (Fig. 3*A*). In contrast, we detected a strong increase of the phosphorylation of the PS-1 CTF in these cells (Fig. 3*A*). Similar results were obtained using COS-7 cells transiently transfected with the human PS-1 cDNA (data not shown).

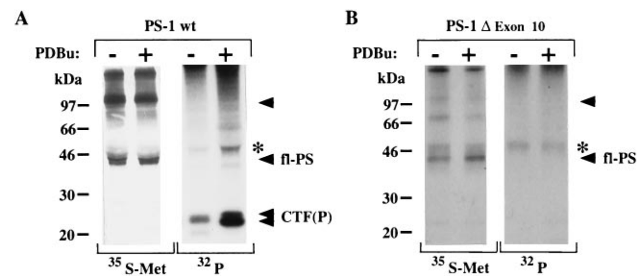

FIG. 3. PKC selectively phosphorylates the PS-1 CTF but not the full-length protein. Human kidney 293 cells stably expressing PS-1 wild-type (*A*) or PS-1 Δexon 10 (17, 38) (*B*) were radiolabeled with [^{35}S]methionine (^{35}S-Met) as well as [^{32}P]orthophosphate (^{32}P) in the presence (+) or absence (−) of PDBu, and PS proteins were isolated by immunoprecipitation using antibody BOS4627. Full-length PS proteins are indicated by an arrow marked fl-PS. Arrowheads indicate dimeric forms of PS proteins (26). Note that no increased phosphorylation of both overexpressed full-length proteins, wild-type and Δexon 10, is observed after PDBu treatment, whereas phosphorylation of the PS-1 CTF is strongly increased in cells expressing wild-type PS-1. Note that no phosphorylated endogenous PS-1 CTF is detected in cells expressing the PS-1 Δexon 10 protein (*B*). The weak band (*) observed after ^{32}P-labeling in cells transfected with the PS-1 is a nonspecific protein that does not comigrate with PS-1 (26).

However, these experiments could not exclude the possibility that PKC phosphorylates full-length PS proteins, thereby inducing a very rapid proteolytic processing. In this case it might be impossible to detect PKC-phosphorylated full-length PS precursors. Therefore, we analyzed the phosphorylation of a naturally occurring PS-1 mutation lacking exon 10 (PS-1 Δexon 10; ref. 38). This mutant splice variant of PS-1 lacks amino acids 290–319 containing the proteolytic cleavage site and was shown not to undergo endoproteolytic cleavage (30). However, PS-1 Δexon 10 was not phosphorylated under both conditions, in the absence and presence of PDBu (Fig. 3*B*), although five out of the seven potential PKC recognition sites were still present (Fig. 5). As a control, labeling with [^{35}S]methionine revealed that expression of PS-1 Δexon 10, like that of wild-type PS-1, was not altered upon PDBu treatment (Fig. 3 *A* and *B*). Therefore, these data indicate that the observed phosphorylated CTF results predominantly from a direct modification by PKC and not from cleavage of the phosphorylated full-length precursor. Notably, in kidney 293 cells stably transfected with the PS-1 Δexon 10 cDNA, we did not detect endogenous PS-1 CTFs, indicating that overexpression of PS-1 Δexon 10 inhibits proteolytic processing of endogenous PS-1.

In vivo, PKC can be activated by signaling pathways involving muscarinic acetylcholine cell surface receptors (39, 40). We therefore tested whether phosphorylation of the CTF can be induced via receptor activation by the muscarinic agonist carbachol. U373 cells stably expressing the m1 receptor were labeled with [^{35}S]methionine or with [^{32}P]orthophosphate in the absence or presence of 1 mM carbachol. Incubation with carbachol resulted in a strong increase in the phosphorylation of the CTF, which was comparable to that observed by direct activation of PKC with PDBu (Fig. 4 *Lower*). Again, phosphorylation results in a shift in the apparent molecular mass of the 20-kDa CTF to 23 kDa (Fig. 4 *Upper*). When carbachol and the muscarinic antagonist atropine were added to cells simultaneously, phosphorylation of the CTF was inhibited, as shown by reduced phosphate incorporation and lack of the molecular mass shift. The presence of the PKC-specific inhibitor GF109203X during incubation with carbachol also reduced the carbachol-stimulated phosphorylation of the CTF. These data therefore indicate that phosphorylation of the CTF of PS-1 can be induced by stimulating a signaling cascade involving PKC via an m1-type acetylcholine receptor with muscarinic ago-

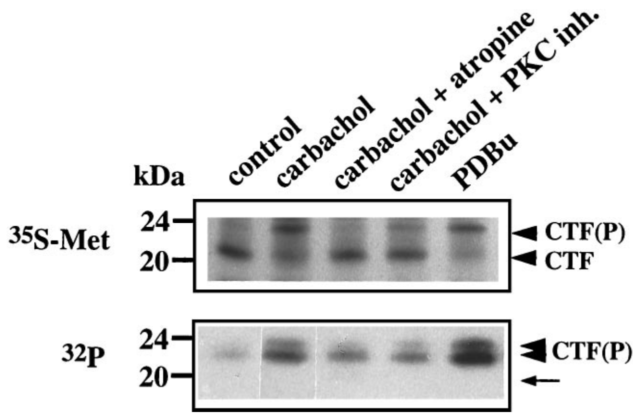

FIG. 4. Stimulation of m1 muscarinic acetylcholine receptors results in the phosphorylation of the CTF of PS-1 by PKC. U373 cells stably transfected with the m1 receptor cDNA were labeled with [^{35}S]methionine (^{35}S-Met; *Upper*) for 14 h and then incubated for 2 h in the absence or presence of carbachol alone or in combination with either atropine or a PKC inhibitor. To activate PKC directly, cells were treated with PDBu for 2 h. The CTFs of PS-1 were immunoprecipitated with antibody 3027. The positions of unphosphorylated CTF (CTF) and phosphorylated CTF [CTF(P)] are marked by arrowheads. To analyze phosphate incorporation, cells were incubated in the presence of [^{32}P]orthophosphate during drug treatments. The CTFs of PS-1 were precipitated from cell lysates with antibody 3027. The ^{32}P-labeled ≈23-kDa and ≈24-kDa polypeptides are marked by arrowheads [CTF(P)], and the position of unphosphorylated CTF (not detectable by ^{32}P labeling) is marked by an arrow. Note that stimulation of m1 receptors with carbachol results in a marked increase in phosphate incorporation as well as a molecular mass shift, similar to that observed after PDBu-mediated PKC stimulation.

nists, suggesting that the physiological or pathological function(s) of the CTF can be biologically regulated.

DISCUSSION

In this study we showed that the C-terminal fragment of PS-1 is specifically phosphorylated by PKC. The identity of the C-terminal cleavage product was proven by epitope mapping using a variety of independent antibodies, including one (PS-1_{Loop}) that was recently used to identify the CTF of PS-1 (30).

The involvement of PKC in the phosphorylation of the CTF was proven by direct activation with PDBu and selective inhibition by a specific PKC inhibitor. Moreover, the CTF was also phosphorylated upon PKC stimulation via m1 receptor-mediated signaling pathways. These results therefore strongly suggest that the CTF of PS-1 serves as an *in vivo* substrate of PKC.

Interestingly, we did not observe PKC-dependent phosphorylation of full-length PS-1 protein. Recently, we reported that full-length PS-2 protein is phosphorylated *in vivo* within its N-terminal domain, most likely by casein kinases, whereas PS-1 undergoes very little if any phosphorylation (26). Here we demonstrated that endoproteolytic cleavage products and full-length PS proteins are modified differently by PKC. Both full-length PS-1 as well as PS-2 were shown not to be phosphorylated by PKC, because neither stimulation nor inhibition of PKC altered their phosphorylation state. To rule out the possibility that full-length PS proteins are phosphorylated and immediately cleaved, in which case phosphorylated full-length proteins might not be detectable, we tested the phosphorylation of the FAD-associated splicing variant of PS-1 missing exon 10 (38), which was shown not to undergo endoproteolytic cleavage (30). It is important to note that this deleted form of PS-1 still contains five out of seven potential PKC phosphorylation sites within the hydrophilic loop domain (Fig. 5), therefore making it possible to test PKC-dependent phosphorylation of full-length PS-1. Moreover, potential PKC phosphorylation sites within exon 10 are not very well conserved between different species, whereas the PKC sites between amino acids 320 and 397 are highly conserved (Fig. 5), suggesting that these sites are biologically relevant. In addition, we (25) and others (24) have shown recently that human PS-1 Δexon 10 can functionally replace mutant sel-12 in *C. elegans.* This shows that PS-1 Δexon 10 is biologically active and can behave like wild-type PS-1, making it therefore an appropriate tool to study its phosphorylation. As observed for wild-type full-length PS proteins, the exon 10-deleted form of PS-1 also is not phosphorylated by PKC. In addition, we did not observe a decrease in the amount of full-length wild-type PS-1 or an increase in the amount of the PS-1 CTF upon PDBu stimulation (Fig. 3), therefore indicating that PKC activation has no effect on proteolytic processing of PS-1. These results strongly suggest that PKC-dependent phosphorylation occurs selectively on the PS-1 CTF after its generation by proteolytic cleavage of the full-length precursor. One explanation for this phenomenon might be that PKC phosphorylation sites are not accessible in the three-dimensional conformation of the full-length protein when the large hydrophilic loop is flanked by TM6 and TM7. Endoproteolytic cleavage of PS-1, which is known to occur shortly after the putative TM6 (ref. 30; D. J. Selkoe and D. B. Teplow, personal communication), might therefore result in a conformational change of the loop domain and facilitated access of the kinase to its recognition sites.

In our study we clearly proved that the CTF of PS-1 is phosphorylated by PKC. Due to the lack of C-terminal PS-2-specific antibodies we were unable to prove phosphorylation of the CTF of PS-2. However, because the CTF of PS-1 contains seven potential PKC recognition sites (9), whereas the putative CTF of PS-2 contains none (Fig. 5), one might speculate that the CTF of PS-2 is not phosphorylated by PKC. Therefore, the biological function of the CTFs of PS-1 and PS-2 might be regulated differently.

Although it is unclear which isoform of PKC phosphorylates the PS-1 CTF, it was reported that the PKC α-isoform translocates from the cytosol to ER membranes upon stimu-

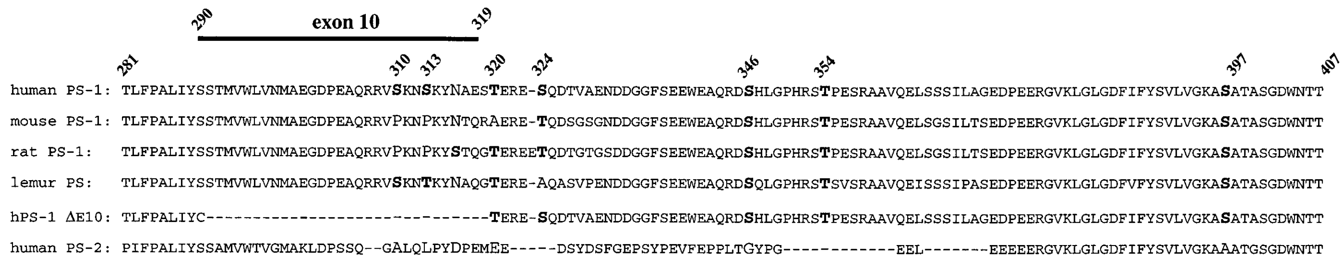

FIG. 5. Conservation of potential PKC phosphorylation sites within the loop domain of PS-1 from different species and of human PS-2. The potential phosphorylation sites, with minimal consensus sequence for PKC [R/K-X-S*/T* or S*/T*-X-R/K (46); phosphate acceptor sites marked by asterisks] are in bold. Amino acid numbering is for the human sequence of PS-1 (9). The sequence encoded by exon 10 (290–319) is marked by a solid bar. Two potential phosphorylation sites (Ser[310] and Ser[313]) are missing in the exon 10-deleted splicing variant. Both sites are not well conserved, in contrast to those downstream of exon 10. Note that the loop domain of human PS-2 contains no potential PKC phosphorylation sites, whereas that of human PS-1 contains seven.

lation with phorbolesters (41, 42). This is the subcellular compartment in which PS proteins are predominantly localized (26, 27–29). In addition, the presented data also strongly suggest that the hydrophilic loop is exposed to the cytoplasmic face of the ER. Taken together with our previous data (26), we would postulate that the N terminus as well as the hydrophilic loop of PS proteins are cytoplasmic (see Fig. 1*A*). This is in agreement with data published recently by Doan *et al.* (32).

Although the biological function of the CTF is unknown, it should be noted that activation of PKC stimulates α-secretory processing of βAPP (43, 44), thus increasing the amount of secreted APP_S-α and decreasing the formation of Aβ peptide (34, 45). In this study, we demonstrate that phosphorylation the CTF of PS-1 parallels the secretion of APP_S. Because the protein substrate(s) of PKC mediating the α-secretory pathway are unknown so far, it will be of great interest if the selective phosphorylation of the PS-1 CTF is involved in the regulation of βAPP processing.

Note Added in Proof. While this manuscript was under review, we learned that similar data were obtained by Gandy and coworkers (47).

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