Proteolytic Fragments of the Alzheimer's Disease Associated Presenilins-1 and -2 Are Phosphorylated in Vivo by Distinct Cellular Mechanisms†

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ABSTRACT: The majority of familial Alzheimer's disease mutations are linked to the recently cloned presenilin (PS) genes, which encode two highly homologous proteins (PS-1 and PS-2). Full-length PS proteins undergo endoproteolytic cleavage within their hydrophilic loop domain resulting in the formation of C-terminal (CTF) and N-terminal fragments (NTF). PS-2 was found to be phosphorylated as a full-length protein within its N-terminal domain. In contrast, PS-1 is phosphorylated selectively after proteolytic processing within its ∼20 kDa CTF involving protein kinase C (PKC) and/or protein kinase A (PKA). We now have found that the CTF of the highly homologous PS-2 is also phosphorylated. Surprisingly, the PS-2 CTF is not phosphorylated by PKC or PKA. Instead, the PS-2 CTF is constitutively phosphorylated in vivo by serine/threonine protein kinases, which are independent of phorbol ester and intracellular cAMP. In vitro the large hydrophilic loop of PS-2 between transmembrane domains 6 and 7 can be phosphorylated by casein kinase-1 (CK-1) and CK-2, but not by PKA or PKC. Quantitative analysis of in vitro phosphorylation demonstrates the presence of two phosphorylation sites for CK-1 and a single site for CK-2. A deletion analysis revealed that the CTF of PS-2 is phosphorylated in vivo within an acidic sequence containing three potential phosphorylation sites for CKs (serines 327, 330, and 335). These data suggest that CK type protein kinases phosphorylate the CTF of PS-2 within its hydrophilic loop domain in vivo. Interestingly, the potential phosphorylation sites are located directly adjacent to the recently identified caspase cleavage sites.

Mutations in the presenilin (PS)[1] genes are the most common cause of early onset familial Alzheimer's disease (FAD). Two PS genes, PS-1 and PS-2, have been identified on chromosomes 14 and 1, respectively (*1*−*3*). Both genes encode highly homologous proteins with multiple transmembrane (TM) domains (*4*). So far at least 40 FAD-causing mutations were shown to occur within PS-1 while only two mutations were found within PS-2 (*5*−*8*). All PS mutations analyzed so far elevate the production of elongated β-amyloid peptide (Aβ) ending at amino acid 42/43 (Aβ42/43). Increased production of Aβ42/43 due to the expression of PS mutations was observed in primary fibroblasts, human plasma (*9*), and transfected culture cells as well as brains of transgenic mice (*10*−*14*). In vivo, these mutations cause the preferential precipitation of Aβ42/43 into amyloid plaques (*15*, *16*).

Cellular expression of the PS genes showed that both PS proteins are predominantly located within the endoplasmic reticulum (ER) and early Golgi (*17*−*20*). PS proteins undergo endoproteolytic cleavage (*13*, *21*, *22*,) within the hydrophilic loop between TM 6 and TM 7 [of an eight transmembrane domain model (*4*)], which results in the production of ∼30 kDa N-terminal and ∼20 kDa C-terminal fragments. It appears that PS proteins in vivo predominantly occur as proteolytic breakdown products, whereas very little full-length PS can be detected within the tissues and cell lines analyzed (*21*). Recently, an alternative proteolytic pathway was identified. In this pathway proteases of the caspase family were demonstrated to cleave PS-1 (*23*) and PS-2 (*24*, *25*) further C-terminal within the hydrophilic loop domains.

Full-length PS-2 but not PS-1 was shown to be constitutively phosphorylated in transiently transfected cells (*19*, *20*). PS-2 phosphorylation was mapped to three serine residues within an acidic sequence of the N-terminal domain, which is missing in PS-1 (*19*). Recently, we and others found that the 20 kDa CTF of PS-1, but not full-length PS-1, can be phosphorylated by PKC and/or PKA (*26*, *27*).

Here we present data on the phosphorylation of proteolytic processing products of PS-2. Both proteolytic fragments are phosphorylated in vivo. In contrast to the PS-1 CTF, the PS-2 CTF is not phosphorylated by PKA or PKC. In examinations using several purified protein kinases in vitro, CK-1 and CK-2 were found to phosphorylate a recombinant polypeptide representing large portions of the PS-2 hydrophilic loop. Analysis of the phosphate incorporation in vitro by these kinases revealed at least three different phosphorylation sites present within the PS-2 loop domain. As shown in cultured cells, deletion of potential phosphorylation sites for CKs within the PS-2 loop domain abolishes phosphory-

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[1] Abbreviations: Aβ, amyloid β-peptide; CK-1, casein kinase-1; CK-2, casein kinase-2; CTF, C-terminal fragment; NTF, N-terminal fragment; ER, endoplasmic reticulum; MBP, maltose-binding protein; PDBu, phorbol 12, 13-dibutyrate; PKC, protein kinase C; PKA, protein kinase A; PS, presenilin; TM, transmembrane domain.

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lation of the PS-2 CTF in vivo. These data strongly support an involvement of CK-1 and/or CK-2 in the in vivo phosphorylation of the PS-2 CTF.

MATERIALS AND METHODS

Antibodies. The polyclonal antibodies 3027 (directed against the PS-1 loop) and 2972 (directed against the PS-2 N-terminus) have been described (*13*, *27*). Antibody 3711 was raised to the loop domain (amino acids 297−356) of PS-2. The respective coding regions were amplified by PCR using the primers 5′-CCGAATTCGCCATGGTGTGGACG-3′ and 5′-ACGCGTCGACCTCTTCTTCCAGCTC-3′. The resulting fragment was cloned into the EcoR*I*/Sal*I* restriction sites of pMAL-c2 (New England Biolabs). The fusion protein was expressed in *Escherichia coli* DH5α, purified on amylose resin (New England Biolabs) according to the supplier's instruction and inoculated into rabbits. Monoclonal antibody M2 recognizing the FLAG epitope (DYKDDDDK) was obtained from Kodak.

FLAG Epitope Tagged PS-2 CTF Deletion Constructs. The coding region of the PS-2 CTF carrying the FLAG epitope was generated by PCR using the primers 5′-CCGAATTCGCTCCAATGGTGTGGACGGTGG-3′ and 5′-GGCCTCGAGCTACTTGTCGTCATCGTCTTTGTCGTCGATGATGAGCTGATGGGAGGC-3′. The sequence encoding a cluster of CK phosphorylation sites was deleted by PCR. For the PCRs the following primer pairs were used: (1) 5′-GGAGATGGAAGAAGACTACCCCGAAGTCTTTG-3′ and 5′-GGCCTCGAGCTACTTGTCGTCATCGTCTTTGTCGTCGATGATGAGCTGATGGGAGGC-3′ and (2) 5′-CAAAGACTTCGGGGTAGTCTTCTTCCATCTCC-3′ and 5′-CCGAATTCGCTCCAATGGTGTGGACGGTGG-3′. After gel purification, the primary PCR products were mixed and a second PCR was carried out using the primers 5′-CCGAATTCGCTCCAATGGTGTGGACGGTGG-3′ and 5′-GGCCTCGAGCTACTTG-TCGTCATCGTCTTTGTCGTCGATGATGAGCTGATGGGAGGC-3′. Both constructs, the PS-2 CTF wt and the CTFΔ327−335, were cloned into the EcoR*I*/Xho*I* restriction sites of pcDNA3 vector (Invitrogen).

Transient Transfection, Metabolic Labeling, and Immunoprecipitation. Cultured cells were transiently transfected using DOTAP (Boehringer Mannheim) according to the supplier's instructions 2 days before the experiment. Metabolic labeling and immunoprecipitations were carried out as described previously (*27*, *28*) except that the immunoprecipitates were heated to 68 °C before they were separated on 12% SDS polyacrylamide gels containing 4 M urea.

In Vivo phosphorylation. A confluent cell monolayer was incubated for 45 min in phosphate-free (Gibco Lifescience) media. The media were aspirated, and phosphate-free media were added, together with 13−36 MBq of [^{32}P]orthophosphate. 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% NP-40 for 10 min. Cell lysates were centrifuged for 10 min at 14000*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 performed by phosphorimaging (Molecular Dynamics) using the ImageQuant program as described by Hung et al. (*29*).

Stimulation and Inhibition of PKC and PKA. To activate PKC and PKA during in vivo labeling with [^{32}P]orthophosphate, phosphorylation was carried out in the presence of 1 μM phorbol 12,13-dibutyrate (PDBu, Biomol) or 5 μM forskolin (Biomol). To inhibit PKC and PKA activities, the selective inhibitors GF109203X (Biomol; final concentration 0.1 μM) and KT 5720 (Calbiochem; final concentration 0.5 μM) were added to the media during periods of labeling.

In Vitro Phosphorylation Assays. Recombinant rat CK-1 δ (New England Biolabs), recombinant subunits of human CK-2 (gift from Dr. W. Pyerin), and the catalytic subunit of PKA purified from bovine heart (gift from Dr. V. Kinzel) were used for in vitro phosphorylation assays in a buffer containing 20 mM Tris, pH 7.5, 5 mM magnesium acetate, 5 mM dithiothreitol. PKC purified from rat brain (Biomol) was assayed in a similar buffer supplemented with 1 μM PDBu, 0.5 mM calcium chloride, and 100 μg/mL phosphatidylserine under mixed micellar conditions (*30*).

The PS-2 loop domain expressed in *E. coli* was cleaved from the MBP fusion protein by incubation with factor X according to the supplier's instruction (New England Biolabs). The purified loop domain of PS-2 was added to the reaction mixtures, and the phosphorylation reaction was started by addition of [γ-^{32}P]ATP. After various periods of incubation at 32 °C the reaction was stopped by the addition of SDS-sample buffer. Phosphate incorporation was analyzed by autoradiography of dried gels and phosphorimaging. The amount of recombinant PS-2 loop substrate protein was determined by densitometric analysis of Coomassie-stained gels using bovine serum albumin as a standard.

Phosphoamino Acid Analysis. ^{32}P-labeled CTFs and NTFs of PS-2 were isolated by immunoprecipitation, separated by SDS−PAGE, electrotransferred onto PVDF membrane, and localized by autoradiography. Bands were cut out and subjected to one-dimensional phosphoamino acid analysis (*31*). Briefly, PS fragments were hydrolyzed by incubation in 6 N HCl for 90 min at 110 °C. Supernatants were dried in a SpeedVac concentrator and the resulting pellets dissolved in pH 2.5 buffer 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 45 min at 20 mA. Radioactive phosphoamino acids were identified by autoradiography and comparison with ninhydrin-stained standards.

RESULTS

In Vivo Phosphorylation of PS-1 and PS-2 Proteolytic Fragments Involves Different Protein Kinases. Recently, we reported that full-length PS-2 is phosphorylated in vivo within its N-terminal domain (*19*). In contrast, PS-1 was demonstrated to be phosphorylated within its 20 kDa CTF in a PDBu and forskolin dependent manner selectively after proteolytic processing resulting in a characteristic decrease in its gel mobility (*26*, *27*). Like PS-1, the homologous PS-2 also undergoes endoproteolytic cleavage within its hydrophilic loop domain (*13*, *32*). We therefore investigated a potential phosphorylation of the PS-2 CTF by PKC/PKA as well as phosphorylation of the corresponding NTF.

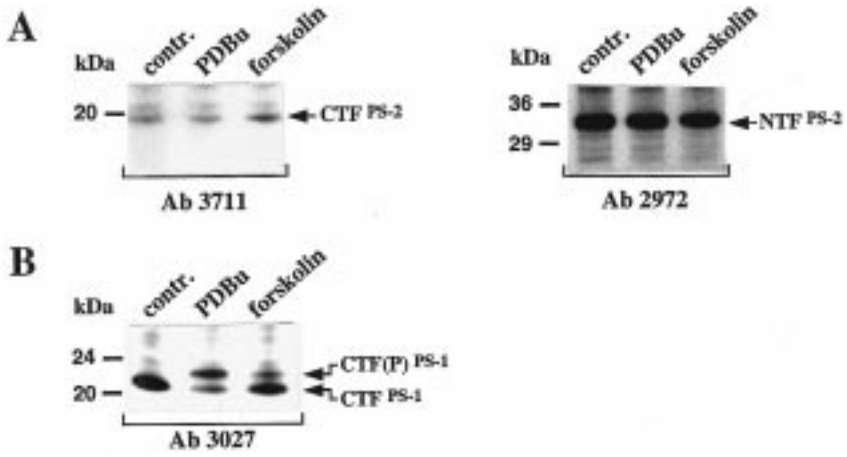

FIGURE 1: Detection of proteolytic processing products of PS-2 in stably transfected kidney 293 cells. (A) Cells were metabolically labeled with ^{35}S-methionine for 8 h, and protein kinase activators PDBu (1 μM) or forskolin (5 μM) were added during the last hour of labeling. Cell lysates were precipitated with antibodies 3711 and 2972, respectively, and separated by SDS−PAGE. (B) In contrast to the PS-2 CTF and NTF, the apparent molecular mass of the PS-1 CTF shifts from 20 kDa to 23 kDa upon PDBu or forskolin treatment, consistent with previous results (*26*, *27*). The CTF of PS-1 was precipitated with antibody 3027. The respective proteolytic processing products are indicated by arrows. CTF(P) indicates the phosphorylated form of the PS-1 CTF, which undergoes a characteristic shift in its gel mobility (*26*, *27*).

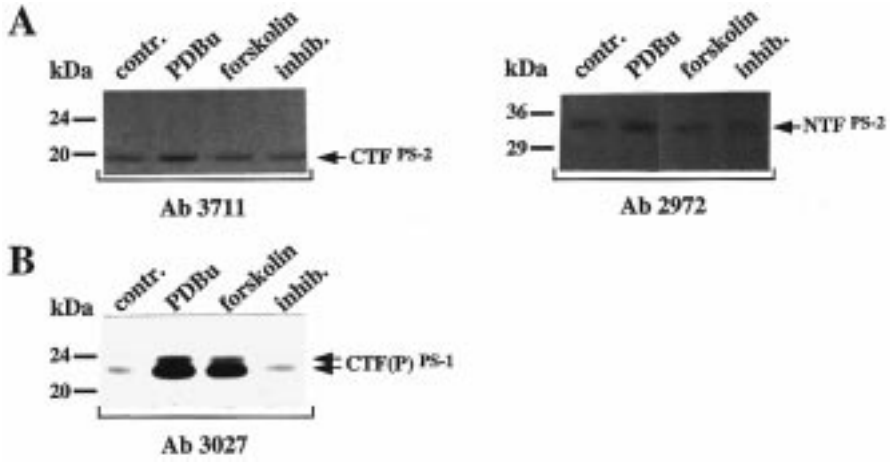

FIGURE 2: Differential phosphorylation of PS-1 and PS-2. Kidney 293 cells stably expressing PS-2 (A) or PS-1 (B) were incubated with [^{32}P]orthophosphate in the absence or presence of PDBu, forskolin, or a mixture of PKC inhibitor (GF109203X) and PKA inhibitor (KT5720), and the CTF and NTF were immunoprecipitated with the respective antibodies. ^{32}P-labeled fragments (marked by arrows) were detected by autoradiography. Phosphorylation of the PS-2 CTF and NTF is not affected by activation or inhibition of PKC or PKA. In contrast, phosphorylation of the PS-1 CTF strongly increases upon PDBu or forskolin treatment. The slightly more intensive band in lanes with PDBu (A and B) is due to experimental variability.

To identify the proteolytic processing products of PS-2, kidney 293 cells stably transfected with the PS-2 cDNA (*12*) were metabolically labeled with [^{35}S]methionine. Cell lysates were immunoprecipitated with antibody 3711 directed to the PS-2 loop or with antibody 2972 directed to the PS-2 N-terminus. Antibodies 3711 (data not shown) and 2972 (*13*) are specific for PS-2 and do not crossreact with PS-1. A ^{35}S-labeled polypeptide with an apparent molecular weight of about 18 kDa was immunoprecipitated with antibody 3711 representing the PS-2 CTF. Antibody 2972 identified a polypeptide of about 34 kDa representing the NTF of PS-2 (Figure 1A). These data indicate that stably expressed full-length PS-2 is proteolytically processed into an 18 kDa CTF and a 34 kDa NTF, which is consistent with previous reports (*13*, *32*).

To prove whether the proteolytic fragments of PS-2, like the PS-1 CTF, can be phosphorylated by PKC/PKA, cells expressing PS-2 were treated with PDBu (to activate PKC) or forskolin (to activate PKA). Neither PDBu nor forskolin had an effect on the electrophoretic mobility of the [^{35}S]methionine labeled PS-2 fragments in SDS gels (Figure 1A), whereas these compounds induced the characteristic decrease of gel mobility of the PS-1 CTF (Figure 1B; (*26*, *27*)).

To directly test the phosphorylation of PS-2 proteolytic fragments in vivo, cells were labeled with [^{32}P]orthophosphate. As described above, the CTF and NTF of PS-2 were immunoprecipitated with antibodies 3711 and 2972, respectively. Both, the CTF and the NTF of PS-2 were radiolabeled, indicating that they are phosphorylated in vivo (Figure 2 A). Phosphorylation of the PS-2 NTF is consistent with our previous finding that full-length PS-2 is phosphorylated at its N-terminus (*19*). Interestingly, phosphorylation of the PS-2 CTF is independent of PKC and PKA activities, since no increase in phosphate incorporation was observed after PDBu or forskolin treatment (Figure 2A). In addition, inhibitors of PKC (GF109203X) and of PKA (KT5720) do not alter phosphorylation of the PS-2 CTF (Figure 2A). In a parallel experiment, phosphorylation of the PS-1 CTF was strongly increased upon stimulation of PKC and PKA (Figure

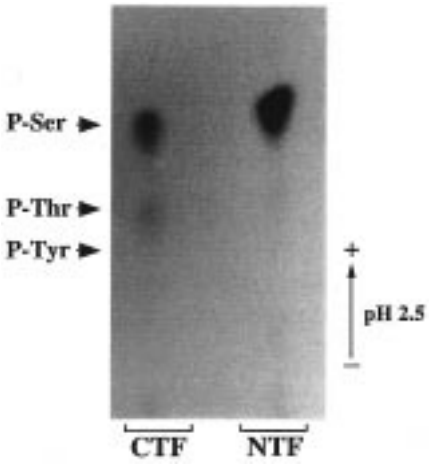

FIGURE 3: The PS-2 CTF and NTF are phosphorylated predominantly on serine. In vivo ^{32}P-phosphorylated CTFs and NTFs were isolated by immunoprecipitation and subjected to one-dimensional phosphoamino acid analysis. Phosphorylated amino acids were detected by autoradiography. The mobility of ninhydrin-stained standards is indicated. The PS-2 CTF is phosphorylated predominantly on serine and to a minor extent on threonine residues, while the NTF is phosphorylated solely on serine residues.

2B), indicating that the CTFs of PS-1 and PS-2 are phosphorylated by distinct mechanisms involving different protein kinases.

To analyze which amino acids of the PS-2 fragments were phosphorylated, in vivo ^{32}P-phosphorylated CTF and NTF were isolated by immunoprecipitation and subjected to phosphoamino acid analysis. The NTF of PS-2 appears to be phosphorylated exclusively on serine residues (Figure 3), which is consistent with our previous findings for fl-PS-2 (*19*). The PS-2 CTF is phosphorylated predominantly on serine residues and to a minor extent on threonine residues (Figure 3). Therefore, the results in Figures 2 and 3 indicate that phosphorylation of PS-2 (NTFs and CTFs) is catalyzed exclusively by serine/threonine protein kinases, which are independent of phorbol ester and intracellular cAMP levels.

The PS-2 Loop Is Phosphorylated in Vitro by Protein Kinases CK-1 and CK-2. Recently, we demonstrated that the N-terminus of PS-2 can be phosphorylated in vitro by protein kinases CK-1 and CK-2 (*19*). The large hydrophilic loop domain of PS-2, which is present in the CTF after endoproteolytic cleavage, also contains several potential phosphorylation sites for CK-1 and CK-2 with consensus sequences D/E-X-X-S*/T* and S*/T*-X-X-D/E, respectively (*33*) (Figure 4A). To identify protein kinases capable of phosphorylating the loop domain of PS-2, we carried out in vitro assays with several purified protein kinases (for details see under Materials and Methods). As demonstrated in Figure 4B, the recombinant loop domain of PS-2 can be readily phosphorylated in vitro by CK-1 and the catalytic α-subunit of CK-2. In contrast, PKA and PKC did not phosphorylate the PS-2 loop in vitro (Figure 4B, upper panel), which is consistent with the data from the in vivo phosphorylation analysis described above (Figures 1 and 2).

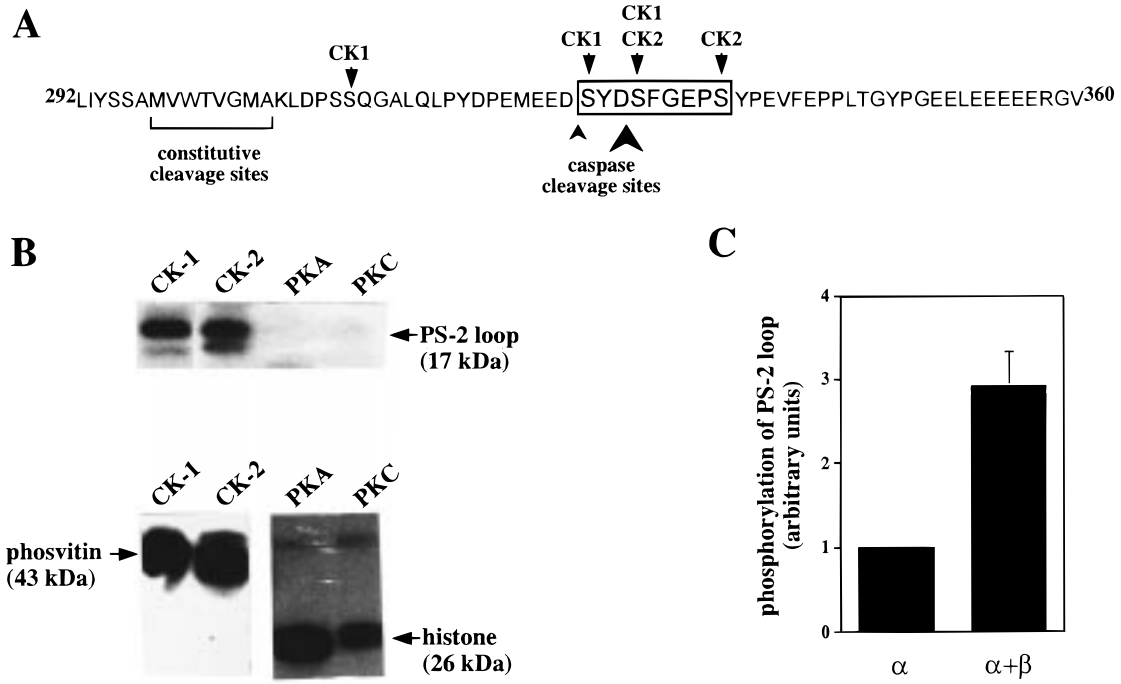

FIGURE 4: In vitro phosphorylation of the PS-2 loop domain by protein kinases CK-1 and CK-2. (A) The amino acid sequence (292−360) of the PS-2 loop contains several potential phosphorylation sites for CK-1 (D/E-X-X-S*/T*) and CK-2 (S*/T*-X-X-D/E). The sites for constitutive endoproteolytic cleavage and for caspase-3 cleavage are also indicated (*24*, *25*). The amino acid sequence which was deleted for mapping the in vivo phosphorylation sites is boxed. (B) In vitro phosphorylation of recombinant PS-2 loop using several protein kinases (CK-1, CK-2, PKA, and PKC). Purified PS-2 loop (50 ng) was incubated with 20 μM [γ-^{32}P]ATP in the presence of the respective kinase for 15 min at 32 °C. To control protein kinase activities, phosvitin (as a substrate for CK-1 and CK-2) or histone (as a substrate for PKA and PKC) was phosphorylated in vitro (lower panel). ^{32}P-labeled protein was detected by autoradiography. The PS-2 loop is detected as a doublet band (marked by an arrow), presumably due to proteolysis during the preparation. The recombinant loop is phosphorylated by CK-1 and CK-2, but not by PKA or PKC. (C) Stimulation of PS-2 loop phosphorylation upon association of CK-2 α-subunit and β-subunit. Equimolar amounts of CK-2 α- and β-subunits or α-subunit alone were preincubated on ice for 15 min in assay buffer including recombinant PS-2 loop. Phosphorylation reaction was carried out as described for part B. PS-2 loop phosphorylation was quantified by phosphorimaging of dried gels. Values represent means of two independent experiments.

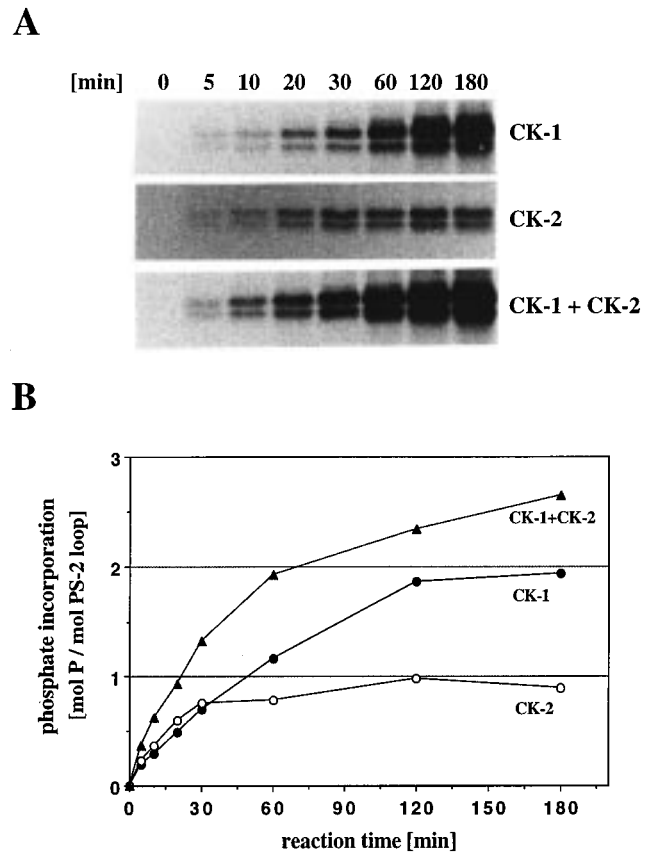

FIGURE 5: The PS-2 loop contains three different in vitro phosphorylation sites. (A) Purified PS-2 loop (50 ng) was incubated with 250 μM [γ-^{32}P]ATP in the presence of CK-1, CK-2, or a mixture of both kinases. Phosphorylation reactions were terminated at the time points indicated by the addition of SDS-sample buffer. (B) Time course of the PS-2 loop phosphorylation. The phosphorylation reaction was carried out as described for part A with CK-1 (●), CK-2 (○), or a CK-1/CK-2 mixture (▲). Quantification by phosphor-imaging revealed that CK-1 incorporates 2 mol of phosphate/mol of substrate, while CK-2 incorporates 1 mol of phosphate/ mol of substrate. The phosphorylation of the PS-2 loop by CK-1 and CK-2 is additive, indicating that CK-1 and CK-2 phosphorylate different sites.

Protein kinase activities were monitored by the phosphorylation of known protein substrates for the respective protein kinases (histone for PKA and PKC and phosvitin for CK-1 and CK-2; Figure 4B, lower panel).

Phosphorylation of most protein substrates of CK-2 is enhanced when the catalytic α-subunit of CK-2 is associated with the regulatory β-subunit (*34*, *35*). The phosphorylation of the PS-2 loop domain by CK-2 was therefore tested in the absence or presence of the regulatory β-subunit. The phosphorylation of the PS-2 loop increased about 3-fold when the CK-2 catalytic α-subunit was allowed to associate with the regulatory β-subunit as compared to the phosphorylation by α-subunit alone (Figure 4C). Thus, the PS-2 loop shares a characteristic feature typical for many protein substrates of CK-2 (*36*, *37*).

To assess how many sites are phosphorylated within the PS-2 loop by each kinase, the purified PS-2 loop domain was incubated with CK-1, CK-2, or a mixture of both kinases in the presence of [γ-^{32}P]ATP for various periods of time up to 3 h. Phosphorylation of the PS-2 loop increases with reaction time and reaches saturated levels after about 0.5−1 h (CK-2) and 1−2 h (CK-1), respectively (Figure 5A,B).

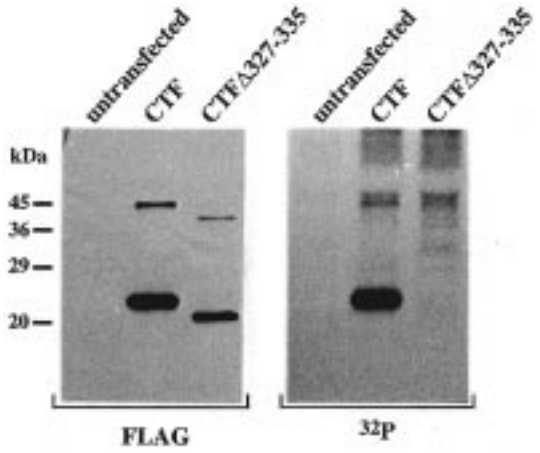

FIGURE 6: The PS-2 CTF is phosphorylated in vivo within a cluster of CK recognition sites close to the caspase-3 cleavage site. COS-7 cells were transiently transfected with cDNAs encoding the PS-2 CTF or the deletion mutant PS-2 CTFΔ327−335 (for details see Materials and Methods) both tagged with FLAG epitopes at their C-termini. Cells were labeled with [^{32}P]orthophosphate for 2 h. After immunoprecipitation of cell lysates with antibody 3711, precipitates were separated by SDS−PAGE and proteins transferred to PVDF membrane. Radiolabeled proteins were detected by autoradiography, and expression of the respective FLAG tagged PS-2 CTF constructs was analyzed by ECL using anti-FLAG antibody M2 on the identical membrane. Although both proteins are strongly expressed, only the wt PS-2 CTF is phosphorylated. The weak bands at 45 and 42 kDa represent dimeric forms of the PS-2 CTF wt and the CTFΔ327−335, respectively.

Quantitative analysis revealed that CK-1 incorporates about 1.9 mol of phosphate/mol of substrate, whereas CK-2 incorporates about 1 mol/mol. When the PS-2 loop was phosphorylated by a mixture of CK-1 and CK-2, phosphate incorporation reached 2.7 mol/mol of substrate (Figure 5A,B). These data indicate that CK-2 phosphorylates the PS-2 loop at a single site, while CK-1 phosphorylates two different sites. Since phosphorylation by CK-1 and CK-2 is additive, we propose that three different sites are phosphorylated by CKs in vitro within the hydrophilic loop domain of PS-2.

In Vivo Phosphorylation of the PS-2 CTF Occurs Adjacent to Caspase Cleavage Sites. As shown in Figure 4A, three potential recognition sites for CK-1 and CK-2 cluster in an acidic region between amino acids 327 and 335. To prove whether phosphorylation occurs within this sequence motif in vivo, we deleted amino acids 327−335 in a cDNA construct encoding the wt PS-2 CTF. This deletion removes the potential phosphorylation sites at serines 327 (for CK-1), 330 (for CK-1 and CK-2) and 335 (for CK-2). COS-7 cells were transiently transfected with cDNA constructs encoding the PS-2 CTF wt or the deletion mutant (PS-2 CTFΔ327−335), containing a FLAG epitope at their C-termini to discriminate exogenous from endogenous proteins. Expression of the transfected cDNA constructs was analyzed by immunoblotting with a monoclonal anti-FLAG antibody. In cells transfected with the wt PS-2 CTF cDNA construct a polypeptide of 23 kDa was detected (Figure 6). The increased molecular weight as compared to the endogenous PS-2 CTF (20 kDa, see Figures 1 and 2) is due to the FLAG epitope at the C-terminus. Cells transfected with PS-2 CTFΔ327−335 express a slightly smaller polypeptide of 20 kDa, presumably due to the lack of the nine deleted amino acids (Figure 6). These data demonstrate that both cDNA

constructs were expressed by COS-7 cells. Immunofluorescence analysis of transiently transfected cells revealed that the overexpressed PS-2 CTF wt and CTFΔ327−335 are located within the ER and Golgi apparatus (data not shown). Therefore these exogenous proteins are localized within the same subcellular compartments like the wt PS-1 and PS-2 proteins (*17*−*20*). After in vivo labeling with [^{32}P]orthophosphate, cell lysates were immunoprecipitated with antibody 3711, and phosphorylation of both PS-2 CTFs was analyzed by autoradiography. The exogenously expressed PS-2 CTF is strongly phosphorylated (Figure 6), which is consistent with the phosphorylation of the endogenous PS-2 CTF (see Figure 2). In contrast, phosphorylation of PS-2 CTFΔ327−335 lacking the potential CK phosphorylation sites was strongly reduced (Figure 6). Since the deletion removes three potential CK phosphorylation sites, these data strongly suggest that CK type protein kinases phosphorylate the PS-2 CTF in vivo.

DISCUSSION

In the present study we analyzed the phosphorylation of the proteolytic processing products of the Alzheimer's disease associated PS-2 protein. The homologous PS-1 and PS-2 proteins undergo endoproteolytic cleavage within their large hydrophilic loop, resulting in the generation of stable NTFs and CTFs (*13*, *21*, *22*, *32*). Recently, it was shown that the CTF of PS-1 is phosphorylated in vivo. Phosphorylation of the PS-1 CTF can be induced by treatment of cells with phorbol ester (PDBu), by increasing the intracellular cAMP level (forskolin), or by activation of muscarinic receptors (*26*, *27*).

Here we found that both the NTF and the CTF of PS-2 occur as phosphorylated polypeptides in vivo. This was observed in stably transfected kidney 293 cells as well as in transiently transfected COS-7 cells (data not shown). Interestingly, neither treatment of cells with PDBu nor treatment with forskolin increased phosphate incorporation into the PS-2 CTF. In addition, selective inhibitors of PKC and PKA had no effect on its phosphorylation state. Therefore, phosphorylation of the PS-2 CTF is independent of the activity of PKC and PKA. This clearly differs from the phosphorylation of the PS-1 CTF, which was shown to be increased by treatment of cells with phorbol ester or forskolin (*26*, *27*) (see Figures 1 and 2). PKC-mediated phosphorylation of the PS-1 CTF occurs after proteolytic cleavage of the full-length precursor (*27*). In contrast to PS-1, PS-2 can be phosphorylated as a full-length protein within the N-terminal domain (*19*). Therefore, the phosphorylated PS-2 NTF detected in vivo may result from cleavage of a phosphorylated full-length precursor.

As reported previously, the loop domain of PS-1 contains several potential phosphorylation sites for PKC and PKA (*1*, *27*). In contrast, no potential PKC/PKA phosphorylation site is present within the loop domain of PS-2 (Figure 4). However, the PS-2 loop bears several consensus sequences for protein kinases CK-1 and CK-2 (see Figure 4). Using in vitro phosphorylation assays, we demonstrate that the PS-2 loop can be phosphorylated by both kinases, CK-1 and CK-2. PKC and PKA were not effective in phosphorylating PS-2 both in vitro and in vivo. Analysis of the phosphorylation of exogenously expressed proteins representing the PS-2 CTF either containing or lacking three potential phosphorylation sites for CKs demonstrates that the PS-2 CTF is phosphorylated between amino acids 327 and 335 in vivo. Our results therefore strongly suggest that CK type protein kinases, but not PKA or PKC, are involved in the in vivo phosphorylation of PS-2 CTF.

Protein kinases CK-1 and CK-2 have been shown to be involved in many cellular processes, including signal transduction, transcription, and protein synthesis (*36*, *38*, *39*). Both kinases seem to be localized predominantly within the nucleus (*37*, *40*), but are also found in the cytoplasm (*41*, *42*) where they can associate with the cytoskeleton, transport vesicles, and the endoplasmic reticulum (*38*, *43*, *44*). PS proteins (*17*−*20*) and the exogenously overexpressed PS-2 CTFs (data not shown) have been localized to the ER and may therefore be phosphorylated by ER-associated CK-1 and/or CK-2 in vivo.

Although the functional consequences of phosphorylation are unknown, it should be noted that the potential phosphorylation sites for CK-1 and CK-2 cluster adjacent to the recently identified alternative cleavage sites of PS-2 for proteases of the caspase superfamily (*24*, *25*). Notably, two out of three potential phosphor acceptor sites are located directly adjacent to the aspartate residues 326 and 329 required at the P1 position for substrate recognition by caspases (*45*). The third potential phosphorylation site is only five amino acids C-terminal to the caspase cleavage site. Therefore, the three potential phosphorylation sites cluster in the vicinity of the sites for caspase cleavage. Since our in vitro study revealed three different phosphorylation sites for CKs, phosphorylation of these three sites might regulate caspase-mediated cleavage of the PS-2 CTF. The caspase cleavage site of PS-1 at aspartate 345 (*23*) is also directly followed by a potential phosphorylation site at serine 346 (*1*, *27*). It will therefore be of great interest to test whether phosphorylation of the respective sites regulates death substrate cleavage of the two homologous PS proteins.

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