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Phosphorylation of Presenilin 1 at the Caspase Recognition Site Regulates Its Proteolytic Processing and the Progression of Apoptosis*

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The Alzheimer's disease-associated presenilin (PS) 1 is intimately involved in γ -secretase cleavage of β -amyloid precursor protein and other proteins. In addition, PS1 plays a role in β -catenin signaling and in the regulation of apoptosis. Here we demonstrate that phosphorylation of PS1 is regulated by two independent signaling pathways involving protein kinase (PK) A and PKC and that both kinases can directly phosphorylate the large hydrophilic domain of PS1 *in vitro* and in cultured cells. A phosphorylation site at serine residue 346 was identified that is selectively phosphorylated by PKC but not by PKA. This site is localized within a recognition motif for caspases, and phosphorylation strongly inhibits proteolytic processing of PS1 by caspase activity during apoptosis. Moreover, PS1 phosphorylation reduces the progression of apoptosis. Our data indicate that phosphorylation/dephosphorylation at the caspase recognition site provides a mechanism to reversibly regulate properties of PS1 in apoptosis.

Neuronal cell death underlies the pathogenesis of Alzheimer's disease, and it has been suggested that apoptotic mechanisms are involved in this process (1). Classical features of apoptosis, including DNA fragmentation, activation of caspases, and cleavage of poly(ADP-ribose) polymerase (PARP)¹ can be detected in Alzheimer's disease brains, and it has been shown that the amyloid β -peptide (A β), a major constituent of β -amyloid plaques, can induce apoptosis of neuronal cells (1).

A β derives from the larger β -amyloid precursor protein (β APP) by sequential cleavages mediated by β - and γ -secretases (2, 3). The presenilin (PS) 1 and PS2 proteins are critically involved in γ -secretase activity (4) and might represent members of a family of aspartic proteases that catalyze

cleavage of proteins within their transmembrane domains (5–7). Pathogenic mutations in the PS genes that cause familial forms of early onset Alzheimer's disease lead to increased production of the 42 amino acid form of A β that aggregates much faster than A β 40 to form insoluble fibrils (8). PS are multi-pass transmembrane proteins that undergo endoproteolytic processing to generate stable N-terminal (NTF) and C-terminal fragments (CTF) (9). The fragments assemble to a high molecular weight complex with other proteins including nicastrin (10), APH-1 a/b (11), and PEN-2 (12) that are also essential for γ -secretase activity and Notch signaling (13, 14).

In addition to the involvement in intramembranous proteolysis, other functions have been attributed to PS proteins, including the regulation of calcium homeostasis (15–17), cell adhesion (18, 19), and the subcellular trafficking of several membrane proteins, like TrkB, telencephalin, and β APP (20–22). Moreover, PS1 binds to β -catenin and negatively regulates Wnt signaling (23–25). Several lines of evidence indicate that PS proteins are also involved in apoptosis (1). Initially, a gene trap analysis for anti-apoptotic factors revealed a C-terminal sequence of PS2 (26). It was also demonstrated that inhibition of PS1 expression in cultured tumor cells or mice strains with spontaneous tumor development induces a higher rate of apoptosis (27). Both PS1 and PS2 are also substrates for caspases *in vitro* and in cultured cells (28–30), and the C-terminal cleavage product of PS2 has been shown to inhibit Fas-induced apoptosis (31). On the other hand, proapoptotic activities of PS proteins have also been reported (32–34). Notably, FAD-associated mutations in the PS genes have been shown to sensitize cells to several apoptotic stimuli (35–37).

In this study we investigated whether phosphorylation of PS1 modulates its role in apoptosis. PS1 was found to be phosphorylated at serine residue 346 by PKC. The phosphorylation at this site regulates the caspase-mediated cleavage of PS1 and inhibits the progression of apoptosis.

MATERIALS AND METHODS

cDNAs and Fusion Proteins—The phosphorylation site mutants of PS1 were generated by PCR techniques using appropriate oligonucleotides. The resulting PCR fragments were subcloned into the EcoRI/XhoI restriction sites of pcDNA3.1 containing a zeocine resistance gene (Invitrogen). The fusion proteins of the maltose-binding protein and the hydrophilic loop domain of PS1 have been described earlier (38).

Cell Culture and Transfection—Human embryonic kidney (HEK) 293 cells, green monkey kidney Cos-7 cells, human cervix carcinoma cells (HeLa), and human mammary carcinoma fibroblasts (MCF-7 cells) were cultured in Dulbecco's modified Eagle's medium with Glutamax (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). The HEK293 cell line stably overexpressing β APP₆₉₅ (39) and the MCF-7 cells stably expressing Fas (40) have been described previously. Transfection of cells with PS1 cDNAs was carried out using FuGENE 6 reagent (Roche Applied Science) according to the supplier's instruc-

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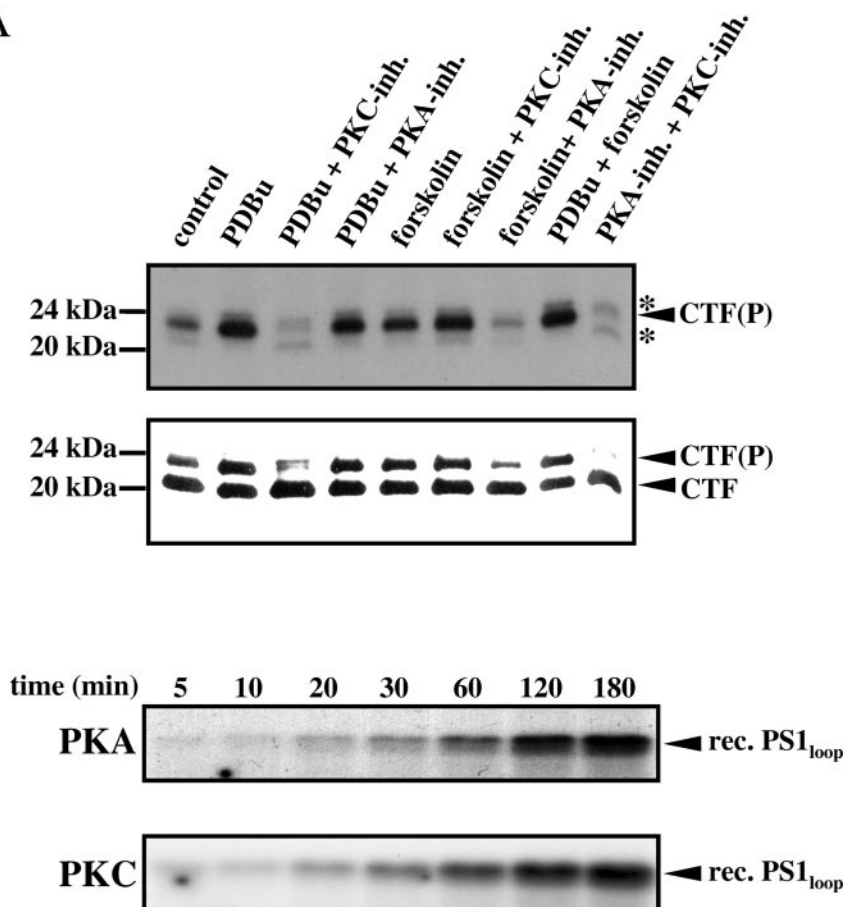
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¹ The abbreviations used are: PARP, poly(ADP-ribose) polymerase; β APP, β -amyloid precursor protein; PS, presenilin; NTF, N-terminal fragment; CTF, C-terminal fragment; HEK, human embryonic kidney; PKA, protein kinase A; PKC, protein kinase C; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PDBU, phorbol 12,13-dibutyrate; STS, staurosporine; OA, okadaic acid; WT, wild type; A β , amyloid β -peptide.

A

FIG. 1. PKA and PKC independently regulate phosphorylation of PS1. A, HEK293 cells were incubated in phosphate-free medium for 1 h in the presence or absence of H-89 (inhibitor of PKA) or of GF109203X (inhibitor of PKC) as indicated prior to the addition of [32 P]orthophosphate. To activate PKA and PKC 5 μ M forskolin or 1 μ M PDBu, respectively, was added to the labeling medium (see "Materials and Methods"). After 2 h the cells were lysed, and PS1 CTF was immunoprecipitated with antibody 3027. Immunoprecipitates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The radiolabeled proteins were detected by autoradiography (upper panel). After exposure, PS1 CTFs were detected by immunoblotting with antibody 3027 on the same membrane (lower panel). Note that phosphorylated forms of PS1 CTF (CTF(P)) migrate slower in SDS gels as compared with unphosphorylated forms (CTF). The asterisks indicate weakly phosphorylated PS1 CTFs that might be phosphorylated by kinases distinct from PKA and PKC (see text). B, recombinant proteins representing amino acids 263–407 of PS1 (rec. PS1_{loop}) were incubated with PKA and PKC in the presence of [γ - 32 P]ATP for the time periods indicated. The reaction mixtures were separated by SDS-PAGE, and the radiolabeled proteins were detected by autoradiography.

B



tions. Single cell clones were generated by selection in 200 μ g/ml zeocine (Invitrogen).

Antibodies, Immunoprecipitation, and Immunoblotting—The antibodies 3027, 2953 (41), 3711 (38), APS18 (42), NT1 (43), and BI.HF5C (44), have been described previously. Monoclonal antibody against Fas was kindly provided by Drs. P. Krammer and M. E. Peter (German Cancer Research Center, Heidelberg, Germany), and polyclonal anti-PARP antibody was obtained from Roche Applied Science. The proteins were detected by Western immunoblotting using an enhanced chemiluminescence technique (Amersham Biosciences).

Phosphorylation of PS1—Phosphorylation of PS1 in cultured cells was carried out as described earlier (41). Protein kinase or phosphatase activities were modulated by the addition of the activators or inhibitors as indicated in the respective experiment. *In vitro* phosphorylation assays with purified PKA from bovine heart (kindly provided by Dr. V. Kinzel) or with rat brain PKC (Biomol) were carried out as described previously (38). Recombinant protein representing the loop region of PS1 (amino acids 263–407) was used as substrate. Phosphorylation reactions were started by the addition of 10 μ M [γ - 32 P]ATP and allowed to proceed for 20 min at 32 °C. To control the kinase activities, parallel phosphorylation reactions were carried out using histone (0.5 mg/ml; Sigma) as protein substrate. The reactions were stopped by the addition of SDS sample buffer.

Two-dimensional Phosphopeptide Mapping— 32 P-Labeled PS1 was analyzed by two-dimensional phosphopeptide mapping according to Boyle *et al.* (45) after digestion with trypsin (sequencing grade; Roche Applied Science). In some experiments synthetic peptides representing phosphorylated PS1 were added to the digest as indicated in the respective experiments. The peptides were dissolved in 300 μ l of pH 1.9 buffer (7.8% (v/v) glacial acetic acid, 2.5% (v/v) formic acid (88%)) and spotted onto a cellulose-coated TLC plate (Merck). After thin layer electrophoresis for 20 min at 1 kV (first dimension), chromatography with phosphochromatography buffer (37.5% (v/v) *n*-butanol, 25% (v/v) pyridine, 6.1% (v/v) glacial acetic acid) was carried out (second dimension). The radiolabeled peptides were detected by autoradiography. To visualize synthetic peptides, TLC plates were stained with ninhydrin.

In Vitro Cleavage by Caspases—One μ g of the respective fusion proteins or 5 μ g of synthetic peptides were incubated at 37 °C for 4 h in

25 μ l of cleavage assay buffer (20 mM Hepes, pH 7.2, 100 mM sodium chloride, 10 mM magnesium chloride, 1 mM EDTA, 0.1% CHAPS, 10% sucrose) in the presence or absence of 20 ng of recombinant active caspase-3 (PharMingen). The reactions were terminated by shock freezing in liquid nitrogen. Cleavage of fusion proteins was analyzed by Western immunoblotting, and cleavage of synthetic peptides was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry—Synthetic PS1 peptides, after incubation in the presence or absence of recombinant caspase-3, were desalted and purified by microbore reversed phase high pressure liquid chromatography. The peptides were eluted with an acetonitrile gradient in 0.1% trifluoroacetic acid on a 150 \times 1.0 mm Vydac C18 column. 0.5 μ l of selected peak fractions were applied onto the dried matrix spot. The matrix consisted of 13 mg of nitrocellulose (Bio-Rad) and 20 mg of α -cyano 4-hydroxycinnamic acid (Sigma) dissolved in 1 ml of acetone/isopropanol 2:3 (v/v). 0.5 μ l of the matrix solution was applied onto the sample target. The samples were analyzed with a Perseptive Biosystems (Framingham, MA) Voyager Elite delayed extraction time-of-flight reflectron mass spectrometer at an acceleration voltage of 20 kV. Calibration was internal to the samples with Des-Arg-Bradykinin (Sigma) and adrenocorticotrophic hormone (18–38) (Sigma).

Induction and Analysis of Apoptosis—HeLa, HEK293, or Cos-7 cells were treated with 1 μ M staurosporine (STS) for the time points indicated. Apoptosis of MCF-7 cells overexpressing Fas was induced by incubation with monoclonal anti-Fas antibody (2 μ g/ml). Progression of apoptosis was monitored by the following parameters: (a) Cleavage of poly(ADP-ribose) polymerase (PARP) was analyzed by immunoblotting (46). (b) Early changes in membrane permeability were detected by cell staining with 1 μ g/ml Hoechst 33342 (Sigma) (47). The accumulation of the dye in apoptotic cells was analyzed using an inverted fluorescence microscope (Leica DMIL, Wetzlar, Germany) equipped with a band pass excitation filter of 340–380 nm and a long pass emission filter of 425 nm. (c) Caspase-3 activity was measured as described previously (48). In brief, the cells were exposed to 1 μ M STS for 2 or 6 h, respectively. After determination of cell numbers, the cells were homogenized in 750 μ l of caspase-3 assay buffer (10 mM Hepes/KOH, pH 7.4, 2 mM EDTA,

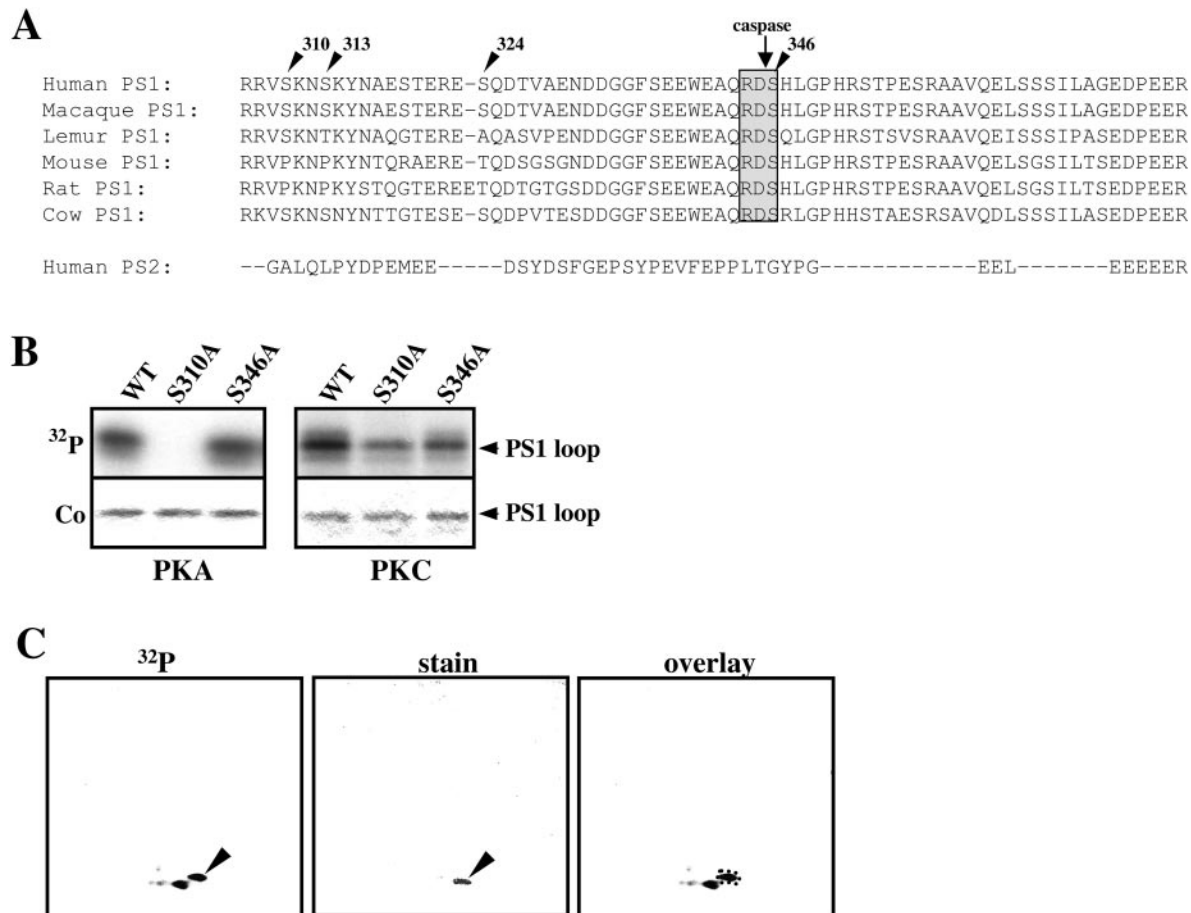


FIG. 2. PS1 is phosphorylated at Ser³⁴⁶ by PKC. *A*, alignment of PS1 loop amino acid sequences from several mammalian species. Potential phosphorylation sites within the minimal consensus sequence for PKC ((R/K)XS) are indicated by arrowheads. The amino acid sequence of human PS1 also contains a recognition motif for PKA (RRX(S/T)) at Ser³¹⁰. Note that the recognition motif at Ser³⁴⁶ is conserved in all mammalian species (boxed). The cleavage site for caspase is indicated by an arrow. *B*, recombinant proteins representing amino acids 263–407 of PS1 WT, S310A and S346A were incubated with PKA or PKC in the presence of [³²P]ATP for 20 min. The radiolabeled proteins were detected by autoradiography (upper panels), and the total proteins were visualized by Coomassie staining (lower panels). Note that PKA-mediated phosphorylation of PS1 containing the S310A mutation is completely abolished. *C*, HEK293 cells were labeled with [³²P]orthophosphate in the presence of PDBu, and PS1 CTF was isolated by immunoprecipitation with antibody 3027 and SDS-PAGE. After blotting to polyvinylidene difluoride membrane, radiolabeled PS1 CTF was digested with trypsin in the presence of the synthetic phosphopeptide DS(p)HLGPLR, which represents a tryptic digestion product of PS1 (amino acids 345–352). After two-dimensional separation of the digestion products, radiolabeled peptides were detected by autoradiography (left panel). The synthetic peptide was visualized by staining the plate with ninhydrin (middle panel). Overlay of the autoradiogram with the stained TLC plate demonstrated co-migration of one *in vivo* phosphorylated peptide with the synthetic peptide (indicated by dashed circle (right panel)).

0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml pepstatin A, 20 μ g/ml leupeptin, 10 μ g/ml aprotinin). The homogenate was centrifuged at 100,000 $\times g$ at 4 $^{\circ}$ C, and the supernatant was incubated with the fluorogenic caspase-3 tetrapeptide-substrate Ac-DEVD-amino-4-methylcoumarin (Calbiochem, Bad Soden, Germany) at a final concentration of 20 μ M. The cleavage of the caspase-3 substrate was followed by determination of emission at 460 nm after excitation at 390 nm using a fluorescence plate reader.

RESULTS

Phosphorylation of PS1 by PKA and PKC—Previously, we and others have shown that phosphorylation of the PS1 CTF increases upon activation of PKC or PKA (41, 49). To investigate whether PKC and PKA independently regulate phosphorylation of PS1, we pharmacologically activated or inactivated these kinases in HEK293 cells during incubation with [³²P]orthophosphate. Activation of PKC with phorbol 12,13-dibutyrate (PDBu) results in strong increase of phosphate incorporation into the PS1 CTF (Fig. 1A, upper panel). Consistent with previous results (41, 49), phosphorylation of the PS1 CTF induces a slower migration in SDS gels resulting in an apparent molecular mass shift from \sim 20 to \sim 23 kDa (Fig. 1A, lower panel). Cell treatment with the PKC inhibitor GF109203X se-

lectively suppresses the PDBu-induced phosphorylation of PS1 CTF. In contrast, the PKA inhibitor H-89 had no significant effect on PDBu induced phosphorylation, indicating that PDBu selectively increases PKC-dependent phosphorylation of PS1. Activation of PKA by cell treatment with forskolin also increases phosphorylation of PS1. In contrast to the PDBu-induced phosphorylation, the forskolin-induced phosphorylation of PS1 is strongly suppressed by the PKA inhibitor H-89 but not by the PKC inhibitor GF109203X (Fig. 1A). These results demonstrate that phosphorylation of the PS1 CTF can be regulated by two independent signaling pathways involving PKA or PKC, respectively.

The hydrophilic loop region between TM6 and TM7 of PS1 contains several consensus recognition sites for these kinases (Fig. 2A). We therefore tested whether both kinases can phosphorylate this domain *in vitro* using a recombinant protein that represents amino acids 263–407 of PS1 as a substrate. Both PKA and PKC readily phosphorylate the recombinant PS1 sequence in a time-dependent manner (Fig. 1B), indicating that the hydrophilic loop region of PS1 is a substrate for both kinases.

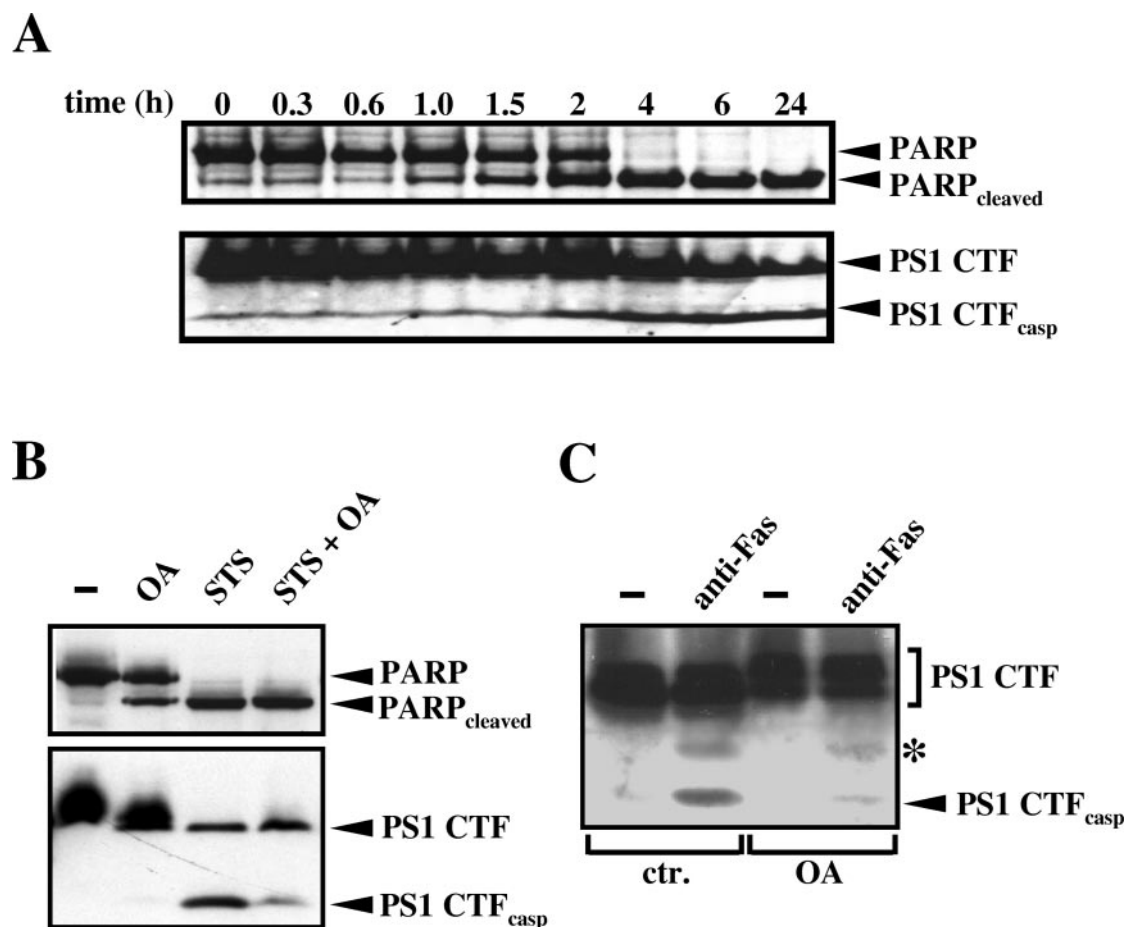


FIG. 3. Cleavage of PS1 CTF during staurosporine induced apoptosis is inhibited by phosphorylation. **A**, HeLa cells were incubated with 1 μ M STS for the time periods indicated. Endogenous PARP (upper panel) and PS1 CTF (lower panel) from cell lysates were detected by Western immunoblotting. Both PARP and PS1 CTF are processed upon staurosporine treatment to yield smaller proteins indicated as PARP_{cleaved} and PS1 CTF_{casp}. **B**, HeLa cells were incubated for 16 h in the presence or absence of STS and/or 1 μ M OA as indicated. Cleavage of PARP and PS1 CTF was analyzed as described above. **C**, MCF-7 cells stably expressing Fas were incubated with (anti-Fas) or without (–) monoclonal anti-Fas antibody in the absence (ctr.) or presence of 1 μ M OA. A minor cleavage product of PS1 in anti-Fas antibody-treated cells that is not significantly affected by OA treatment is indicated by an asterisk.

It has been demonstrated that PS1 is predominantly phosphorylated on serine residue(s) (41). A computer-assisted data base search revealed that Ser³⁴⁶ is conserved in PS1 of mammalian species but not in PS2 (Fig. 2A). This residue is preceded by an arginine residue at the –2 position and therefore fulfills the minimal primary sequence requirements for phosphorylation by PKC (Fig. 2A). Importantly Ser³⁴⁶ is located at the caspase recognition site of PS1 that is cleaved during apoptosis by caspase activity (28, 29). In addition, a potential phosphorylation site for PKA (RRXS) was identified at Ser³¹⁰ (Fig. 2A).

To test whether Ser³¹⁰ and Ser³⁴⁶ represent phosphorylation sites for PKA and/or PKC *in vitro*, we substituted these residues by alanine residues and used the respective recombinant proteins representing amino acids 263–407 of PS1 as substrates. PKA-mediated phosphorylation of PS1 is completely inhibited by mutation of Ser³¹⁰ but not affected by the S346A mutant (Fig. 2B), indicating that Ser³¹⁰ is the sole PKA phosphorylation site within the hydrophilic loop domain of PS1. The PKC-mediated phosphorylation is inhibited with both mutants, indicating that PKC can phosphorylate at least two distinct sites within this region *in vitro* (Fig. 2B). Because Ser³⁴⁶, in contrast to Ser³¹⁰, is fully conserved between mammalian species, we analyzed phosphorylation of Ser³⁴⁶ in more detail.

To assess whether Ser³⁴⁶ is an *in vivo* phosphorylation site, HEK293 cells were labeled with [³²P]orthophosphate in the presence of PDBu to activate PKC, and PS1 CTF was isolated

by immunoprecipitation. Two-dimensional phosphopeptide mapping was carried out in the presence of a synthetic phosphopeptide representing a tryptic digestion product of PS1 in a phosphorylated state (D(p)SHLGPLR; amino acids 345–352 of PS1). ³²P-Labeled peptides were detected by autoradiography (Fig. 2B, left panel), and the synthetic phosphopeptide was visualized by staining with ninhydrin (Fig. 2B, middle panel). The overlay of both images reveals co-migration of a ³²P-labeled tryptic digestion product of *in vivo* phosphorylated PS1 with the synthetic phosphopeptide (Fig. 2B, right panel), indicating that Ser³⁴⁶ is phosphorylated in cultured cells. The additional ³²P-labeled spot might represent another tryptic peptide containing another phosphorylation site (e.g. Ser³¹⁰, Ser³⁵³, or Ser³⁵⁷) (50) or a partially digested product phosphorylated at Ser³⁴⁶.

Phosphorylation at Ser³⁴⁶ Inhibits Caspase-mediated Cleavage—Ser³⁴⁶ is located in the P-1' position to the cleavage site for caspases that cleave PS1 after Asp³⁴⁵ during apoptosis (28–30). To analyze the effect of phosphorylation of PS1 on the proteolytic processing by caspases during apoptosis, we first used HeLa cells, a cell type that reveals several apoptotic features including cleavage of endogenous PARP and endogenous PS1 CTF (46). After induction of apoptosis by treatment with STS, cleavage of PARP and endogenous PS1 was analyzed by Western immunoblotting. In untreated cells mainly full-length PARP and only very low amounts of cleavage products are detected (Fig. 3A, upper panel). The amount of the charac-

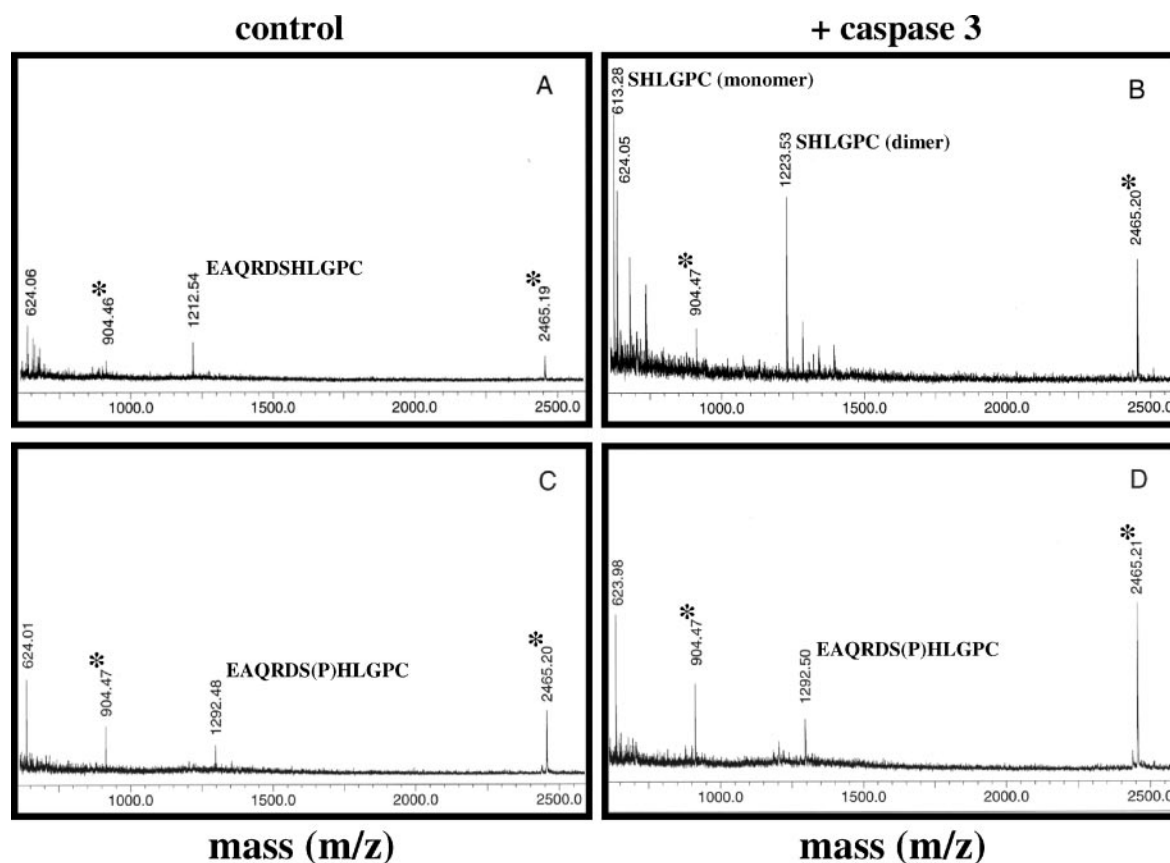


FIG. 4. **Phosphorylation at Ser³⁴⁶ inhibits cleavage by caspase-3 *in vitro*.** Synthetic peptides EAQRDShLGPC (A and B) and EAQRDS(p)HLGPC (C and D) representing amino acid sequence 341–350 of PS1 with unphosphorylated and phosphorylated Ser³⁴⁶, respectively, were incubated in the absence (A and C) or presence (B and D) of recombinant caspase-3 for 4 h at 37 °C. The samples were subjected to MADLI-TOF MS. The unphosphorylated peptide ($MH^+ = 1212.54$ Da) is readily cleaved by caspase-3, as indicated by the presence of the monomeric ($MH^+ = 613.28$ Da) and dimeric ($MH^+ = 1223.53$ Da) C-terminal cleavage products (B). In contrast the phosphorylated peptide ($MH^+ = 1292.48$ Da) is resistant against caspase-3 activity (C and D). *, adrenocorticotrophic hormone ($MH^+ = 2465.20$ Da) and Des-Arg-bradykinin ($MH^+ = 904.47$ Da) were added as internal standards. The extraneous peak ($m/z \sim 624.0$) could not be attributed to a sequence related to synthetic peptide.

teristic cleavage product of PARP increases in a time-dependent manner upon treatment with STS. After 4 h of treatment with staurosporine, PARP is almost completely converted (Fig. 3A, upper panel). STS treatment also induces cleavage of the endogenous PS1 CTF with similar kinetics, resulting in the generation of a smaller fragment (PS1 CTF_{casp}; Fig. 3A, lower panel). In contrast to PARP, the PS1 CTF is not fully converted, even after 24 h of treatment with STS. These results are consistent with previous studies, indicating that some fractions of PS1 are resistant to caspase activity even after long treatment with apoptotic stimuli (28–30). To investigate the role of PS1 phosphorylation on its turnover during apoptosis, the cells were incubated with okadaic acid (OA) that inhibits protein dephosphorylation by protein phosphatases 1 and 2A. Very low amounts of cleavage products of PARP and the PS1 CTF are detected in untreated cells (Fig. 3B). Cell treatment with OA alone induces cleavage of PARP to some extent, indicating that this reagent lead to caspase activation. However, cleavage of PS1 CTF is hardly detectable under these conditions. STS has a much stronger effect on induction of apoptosis and leads to almost complete conversion of PARP and of significant portions of PS1 CTF to yield CTF_{casp}. Notably, the presence of OA during staurosporine-induced apoptosis reduces cleavage of the PS1 CTF, whereas cleavage of PARP is not inhibited (Fig. 3B). These data suggest that phosphorylated PS1 might be protected against caspase-mediated cleavage during apoptosis of HeLa cells. Very similar results were also obtained with Cos-7 cells (data not shown).

To test another experimental paradigm of apoptosis, MCF-7

cells stably overexpressing Fas were incubated in the presence or absence of cross-linking monoclonal anti-Fas antibody (Fig. 3C). In untreated MCF-7 cells, very little if any PS1 CTF_{casp} is detected, whereas robust amounts of endogenous PS1 CTF are present. Cell treatment with anti-Fas antibody for 6 h results in the accumulation of the characteristic PS1 CTF_{casp}, indicating processing of PS1 CTF by caspase activity during Fas-mediated apoptosis (Fig. 3C). The presence of OA significantly reduces the generation of PS1 CTF_{casp} during Fas-mediated apoptosis (Fig. 3C). Taken together, these data indicate that cleavage of PS1 by caspases during apoptosis is very likely to be controlled by phosphorylation. Because this effect is observed in different cell types and different experimental paradigms of apoptosis, the inhibition of caspase-mediated cleavage of PS1 by phosphorylation appears to be a general phenomenon. However, OA itself is a modulator of apoptosis by interference with other dephosphorylation events (51). These data therefore do not rule out the possibility that the observed inhibition of caspase-mediated cleavage of PS1 is due to indirect effects of OA.

To prove that phosphorylation of Ser³⁴⁶ indeed inhibits caspase-mediated cleavage, we used synthetic peptides that represent amino acids 341–350 of PS1 and contain Ser³⁴⁶ in unphosphorylated or phosphorylated state. The *in vitro* cleavage of these peptides by recombinant caspase-3 was analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Caspase-3 efficiently cleaves the unphosphorylated peptide ($MH^+ = 1212.54$) after Asp³⁴⁵, as indicated by the detection of monomeric ($MH^+ = 613.28$) and dimeric

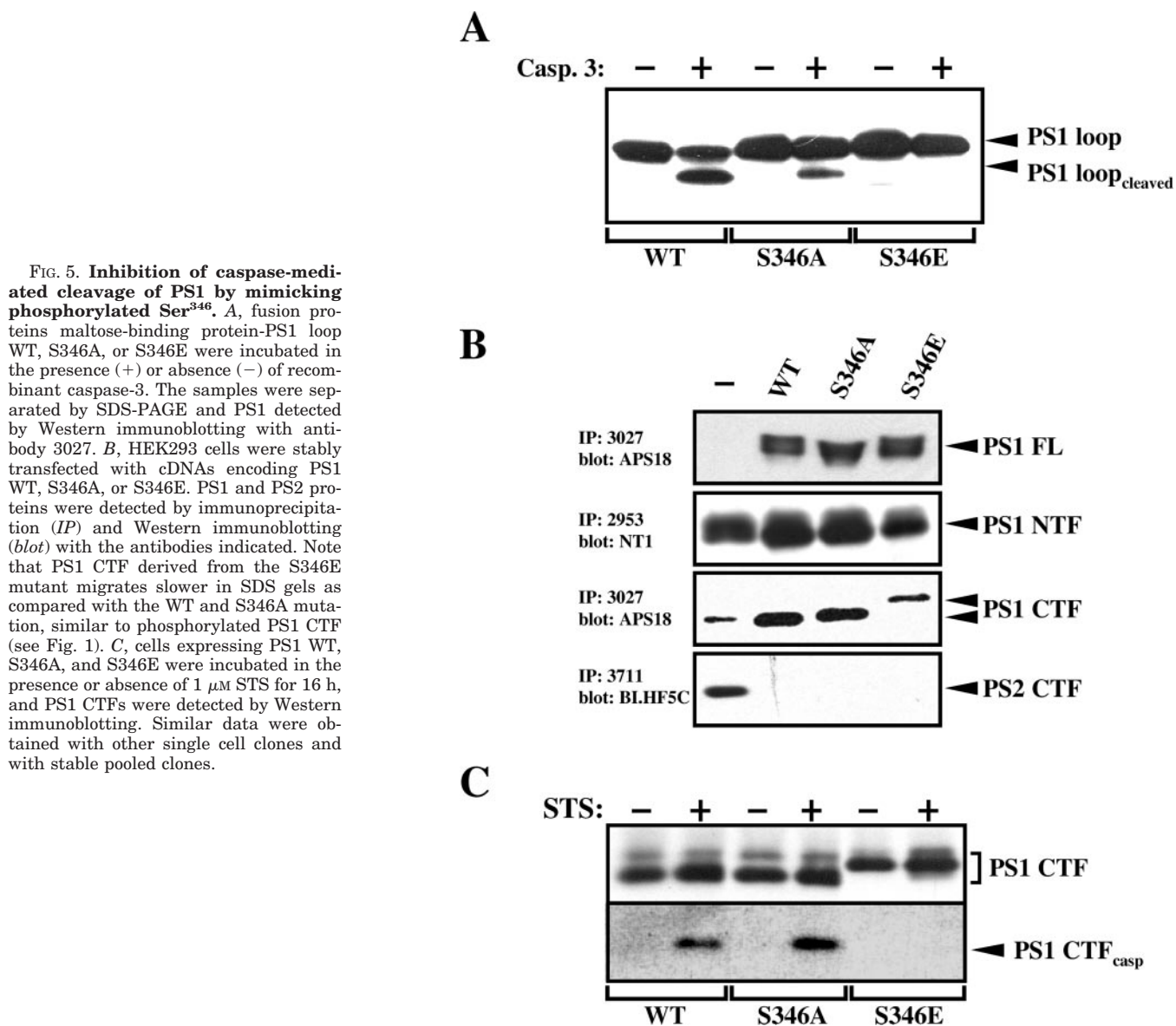


FIG. 5. Inhibition of caspase-mediated cleavage of PS1 by mimicking phosphorylated Ser³⁴⁶. *A*, fusion proteins maltose-binding protein-PS1 loop WT, S346A, or S346E were incubated in the presence (+) or absence (-) of recombinant caspase-3. The samples were separated by SDS-PAGE and PS1 detected by Western immunoblotting with antibody 3027. *B*, HEK293 cells were stably transfected with cDNAs encoding PS1 WT, S346A, or S346E. PS1 and PS2 proteins were detected by immunoprecipitation (IP) and Western immunoblotting (blot) with the antibodies indicated. Note that PS1 CTF derived from the S346E mutant migrates slower in SDS gels as compared with the WT and S346A mutation, similar to phosphorylated PS1 CTF (see Fig. 1). *C*, cells expressing PS1 WT, S346A, and S346E were incubated in the presence or absence of 1 μ M STS for 16 h, and PS1 CTFs were detected by Western immunoblotting. Similar data were obtained with other single cell clones and with stable pooled clones.

(MH⁺ = 1223.53) forms of the C-terminal cleavage product SHLGPC (Fig. 4, *A* and *B*). In contrast, the phosphorylated peptide is resistant against caspase-3 activity (Fig. 4, *C* and *D*), demonstrating that phosphorylation at Ser³⁴⁶ inhibits caspase-mediated cleavage of PS1.

We next tested whether the effect of phosphorylation can be mimicked by substitution of Ser³⁴⁶ by a negatively charged amino acid. Fusion proteins containing the hydrophilic loop domain (amino acids 263–407) of PS1 WT or the S346A or S346E substitutions were used as substrates for *in vitro* caspase assays. Both the PS1 WT and the S346A mutation are readily cleaved by caspase-3 *in vitro* (Fig. 5*A*). In contrast, substitution of Ser³⁴⁶ by a glutamate residue strongly inhibits caspase-3-mediated cleavage of PS1 CTF, thereby mimicking the effect of phosphorylation at this site. By using the phosphorylation site mutants we were able to investigate the effect of PS1 phosphorylation in living cells without further pharmacological modulation of kinase/phosphatase activities that might cause unspecific side effects.

HEK293 cells were stably transfected with cDNAs of human PS1 WT, PS1 S346A, or PS1 S346E, and the respective proteins were detected by Western immunoblotting. Consistent with previous reports (9), endogenous PS1 is predominantly de-

tected as an ~30-kDa NTF and an ~20-kDa CTF, whereas the full-length protein is hardly detectable (Fig. 5*B*). We also detected endogenous PS2 CTF in untransfected cells. Overexpression of PS1 variants leads to the detection of full-length proteins and of the respective NTFs and CTFs, indicating that the S346A and S346E variants are proteolytically processed like the PS1 WT protein. Moreover, all variants replace endogenous PS proteins (Fig. 5*B*), indicating incorporation into the characteristic PS containing high molecular weight protein complexes (9, 42). Notably, substitution of Ser³⁴⁶ by glutamate induces a shift in the apparent molecular mass of the PS1 CTF in SDS gels, very similar to that observed for PS1 WT upon cell treatment with PDBu (Fig. 5*B*; compare with Fig. 1). These data indicate that introduction of a negatively charged amino acid has a similar effect on the conformation of PS1 CTF as phosphorylation.

To test the caspase-mediated cleavage of PS1 WT, S346A, or S346E, the cells were incubated in the presence or absence of STS. Very little if any PS1 CTF_{casp} is detected in all untreated cell lines (Fig. 5*C*). STS treatment leads to the accumulation of CTF_{casp} derived from both PS1 WT and the S346A mutant (Fig. 5*C*). In contrast, the generation of PS1 CTF_{casp} is strongly inhibited in cells expressing the PS1 S346E mutant, indicating

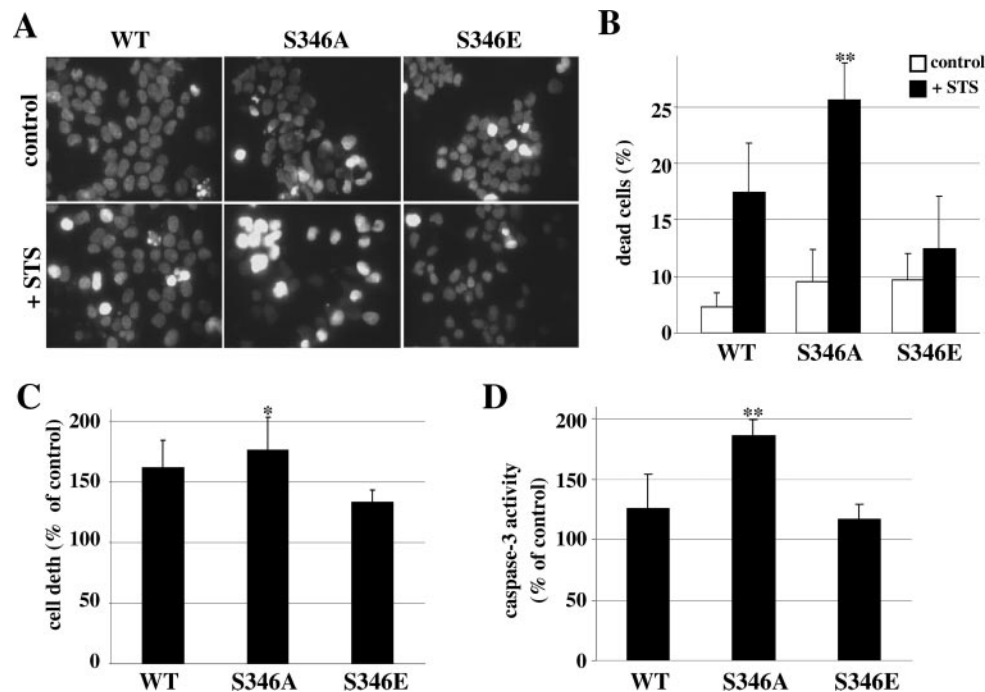


FIG. 6. Phosphorylation of PS1 inhibits progression of apoptosis and the activation of caspase-3.0. *A* and *B*, HEK293 cells stably expressing PS1 WT, S346A, or S346E were incubated for 2 h in the absence or presence of 1 μ M STS and then stained with Hoechst 33342. Photomicrographs (*A*) are representative for three independent experiments that were quantified in the graph (*B*). *C*, quantification of cell death by STS treatment as determined by trypan blue exclusion (see “Materials and Methods”). The values represent the means \pm S.D. of four independent experiments and are expressed as increases in positive cells as compared with untreated cells. *, $p < 0.025$. *D*, measurement of caspase-3 activity was carried out as described under “Materials and Methods.” The values represent the means \pm S.D. of 4–6 independent experiments. **, $p < 0.001$. Highly similar data were obtained in experiments with independent single cell clones and with stable mixed clones.

protection of this mutant against caspase-mediated cleavage during apoptosis. Mutations of the phosphorylation site at Ser³¹⁰ do not inhibit caspase-mediated cleavage during STS-induced apoptosis (data not shown). Taken together, these data provide evidence that phosphorylation of PS1 at Ser³⁴⁶, as mimicked by introduction of negatively charged glutamate residue, strongly inhibits proteolytic processing of PS1 CTF by caspase activity during apoptosis of cultured cells.

Phosphorylation of PS1 Regulates the Progression of Apoptosis—We took advantage of these cell lines stably overexpressing PS1 WT or the phosphorylation site mutants to assess the effect of PS1 phosphorylation on apoptosis. The cells were incubated in the presence or absence of STS and then stained with Hoechst 33342 to selectively detect cells with permeabilized membranes caused by early apoptosis (47). In untreated cultures low numbers of positive cells are detected (Fig. 6, *A* and *B*). STS treatment increases the number of bright nuclei in PS1 WT-expressing cells and to a stronger extent in cells expressing PS1 S346A, indicating the induction of apoptosis. In contrast, the cells expressing the PS1 S346E mutant are less sensitive to the apoptotic stimulus, as indicated by the lower number of bright nuclei after STS treatment (Fig. 6, *A* and *B*). Very similar data were also obtained when the increase in cell death by STS treatment was determined by trypan blue exclusion (Fig. 6*C*). These data indicate that phosphorylation of PS1 at Ser³⁴⁶ decreases the susceptibility of cells to apoptotic stimuli.

The progression of apoptosis critically depends on the activation of caspases. We therefore tested whether the observed differences in apoptotic cell death that depend on the phosphorylation state of PS1 are associated with different levels of active caspase-3. Upon treatment with STS for 2 h, caspase-3 activity increases in cells expressing PS1 WT (Fig. 6*D*). Highest levels of caspase-3 activity are detected in cells expressing PS1 S346A that can not be phosphorylated at this site. Importantly, only a slight increase in caspase-3 activity is detected in PS1

S346E-expressing cells after STS treatment, indicating that phosphorylation of PS1, as mimicked by the S346E mutation, decreases the activation of caspase-3.

DISCUSSION

We demonstrate that phosphorylation of the PS1 CTF at Ser³⁴⁶ inhibits its caspase-mediated cleavage and modulates the progression of apoptosis. Ser³⁴⁶ is phosphorylated by PKC *in vitro* and in cultured cells, whereas PKA exclusively phosphorylates Ser³¹⁰. However, phosphorylation of Ser³¹⁰ does not inhibit the caspase-mediated cleavage of PS1 (data not shown), and the biological function of this phosphorylation event remains to be determined in further experiments.

In previous studies, phosphorylation of PS1 at serine residues 353, 354, and 357 has also been reported (50, 52). These sites are phosphorylated by GSK3 β or CDK5 *in vitro*. Our data are in line with these findings, because two-dimensional phosphopeptide mapping and *in vitro* phosphorylation experiments revealed additional phosphorylation sites in PS1 beside Ser³⁴⁶ (Fig. 2). We also find that S346A and S310A mutants can still be phosphorylated by GSK3 β *in vitro* (data not shown). Using synthetic peptides as substrates, we demonstrate that phosphorylation of Ser³⁴⁶ strongly inhibits caspase-3-mediated cleavage of PS1. Treatment of cells with OA that inhibits dephosphorylation of PS1 also reduces its cleavage in several cell types including HeLa, Cos-7, and MCF-7 cells and upon distinct apoptotic stimuli, indicating a general role of PS1 phosphorylation in the regulation of caspase-mediated cleavage during apoptosis. However, OA affects multiple signaling events and also interferes with regulatory pathways in apoptosis (51). To circumvent indirect effects of OA treatment in analyzing the function of PS1 phosphorylation at Ser³⁴⁶, we generated cell lines stably expressing PS1 mutants that mimic phosphorylated or unphosphorylated variants. This strategy allowed us to investigate the biological effect of phosphoryla-

tion without additional pharmacological manipulation of kinase or phosphatase activities. Indeed, the substitution of Ser³⁴⁶ by negatively charged amino acids inhibits cleavage of PS1 *in vitro* and in cultured cells, thereby mimicking the effect of phosphorylation.

The observed mechanism of cleavage inhibition of PS1 is highly similar to that of PS2 (46). In contrast to PS1, which was found to be phosphorylated at its caspase cleavage site by PKC, PS2 is phosphorylated by the second messenger-independent casein kinases 1 or 2 (53), indicating separate regulatory pathways for PS1 and PS2 (38). However, further studies are needed to reveal the role of the distinct regulatory pathways that regulate phosphorylation of PS1 and PS2.

It has been demonstrated that protein phosphorylation is involved in the regulation of apoptosis (51), and anti-apoptotic functions for PKC have been reported. Activation of (54, 55) PKC inhibits apoptosis in several cell types (51, 56). Moreover, PKC is up-regulated in a wide range of cancers (51, 57). However, also pro-apoptotic activities of PKC have been reported, and it is assumed that the different functions of this kinase in apoptosis might be exerted by distinct isoenzymes (51). In this study phosphorylation of PS1 is strongly inhibited by GF109203X, which predominantly targets PKC α and PKC β I (58). Notably, these isoforms can inhibit apoptosis in a number of cell lines and are up-regulated in a large number of cancers (51). The molecular targets involved in the PKC-mediated inhibition of apoptosis are not fully defined. It has been shown that PKC α can phosphorylate Bcl-2 (59), which might increase the anti-apoptotic functions of this protein. Our study indicates that the anti-apoptotic function of PKC might also involve phosphorylation of PS1.

It has been suggested that the PS1 CTF derived by caspase-mediated cleavage (CTF_{casp}) promotes apoptosis (29, 30), allowing a positive feedback regulation of caspase-mediated cleavage of PS1. As demonstrated in this study, PKC-mediated phosphorylation of PS1 strongly reduces the generation of CTF_{casp} and might thereby retard the progression of apoptosis by inhibiting this feedback regulation. We also found that cells expressing PS1 S346E have decreased caspase-3 activity in early stages of apoptosis, suggesting that PS1 phosphorylation interferes with the activation of this or an upstream caspase. It should be noted that PS1 phosphorylation does not completely inhibit the progression of STS-induced apoptosis. Rather, PS1 might be a modulator involved in the initiation phase of apoptosis that is regulated by phosphorylation/dephosphorylation.

Previous studies have shown that the large hydrophilic domain of PS1, in which we localized phosphorylation, is dispensable for γ -secretase activity (60). In line with this, we did not observe a significant effect of PS1 phosphorylation at Ser³⁴⁶ on the γ -secretory processing of β APP (data not shown). It has also been shown that the PS1 loop binds β -catenin and regulates Wnt signaling (24, 61). The binding region of PS1 for β -catenin is localized between amino acids 340–371 (62). Although Ser³⁴⁶ is located within this binding domain, phosphorylation of PS1 does not significantly affect its binding to β -catenin (data not shown). Thus, phosphorylation at Ser³⁴⁶ appears to be mainly responsible for regulation of the apoptotic properties of PS1 independent of its other biological functions, like in the γ -secretase cleavage of β APP and the regulation of Wnt signaling.

Despite the large number of proteins that become cleaved during apoptosis by caspase activity, only some of them have been shown to be protected by phosphorylation, including Bid (63), I κ B (64), PS2 (46), and the PS2-interacting protein calseinin (65). Phosphorylation of proteins at caspase recognition sites provides a mechanism to regulate their proteolytic cleavage and at least in some cases their functions in apoptosis.

Changes in the phosphorylation and/or dephosphorylation of PS1 might therefore contribute to the pathogenesis of Alzheimer's disease by altering the susceptibility of brain neurons to apoptotic stimuli.

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**Phosphorylation of Presenilin 1 at the Caspase Recognition Site Regulates Its
Proteolytic Processing and the Progression of Apoptosis**

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