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A Structural Switch of Presenilin 1 by Glycogen Synthase Kinase 3β-mediated Phosphorylation Regulates the Interaction with β-Catenin and Its Nuclear Signaling*

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Presenilins (PS) are critical components of the γ-secretase complex that mediates cleavage of type I membrane proteins including the β-amyloid precursor protein to generate the amyloid β-peptide. In addition, PS1 interacts with β-catenin and facilitates its metabolism. We demonstrate that phosphorylation of serines 353 and 357 by glycogen synthase kinase-3β (GSK3β) induces a structural change of the hydrophilic loop of PS1 that can also be mimicked by substitution of the phosphorylation sites by negatively charged amino acids *in vitro* and in cultured cells. The structural change of PS1 reduces the interaction with β-catenin leading to decreased phosphorylation and ubiquitination of β-catenin. The decreased interaction of PS1 with β-catenin leads to stabilization of β-catenin thereby increasing its nuclear signaling and the transcription of target genes, including c-*MYC*. Consistent with increased expression of c-*myc*, a PS1 mutant that mimics phosphorylated PS1 increased cell proliferation as compared with wild-type PS1. These results indicate a regulatory mechanism in which GSK3β-mediated phosphorylation induces a structural change of the hydrophilic loop of PS1 thereby negatively modulating the formation of a ternary complex between β-catenin, PS1, and GSK3β, which leads to stabilization of β-catenin.

Mutations in the two *PRESENILIN* (PS)[2] genes are associated with familial early onset Alzheimer disease. The genes encode two homologues of polytropic membrane proteins that have been shown to be critically involved in the generation of the amyloid β-peptide (1–3). PS1 and PS2 undergo endoproteolytic processing within a hydrophilic loop region between transmembrane domains 6 and 7 resulting in N- and C-terminal fragments (4–6). These assemble with nicastrin, aph-1, and pen-2 to form a catalytically active γ-secretase complex that catalyzes the intramembraneous cleavage of the β-amyloid precursor protein and other type I membrane proteins (1, 7). PS proteins have been localized to secretory and endocytic compartments including the endoplasmic reticulum, Golgi, and endosomal/lysosomal compartments (2, 8, 9). In addition, PS proteins are also located at the plasma membrane, where γ-secretase cleavage of β-amyloid precursor protein, Notch, and cadherins can occur (10–13).

In addition to their function in membrane protein proteolysis, PS proteins have been shown to be involved in the regulation of apoptosis (5, 14–17). PS1 is also implicated in β-catenin-dependent signaling and directly binds via a large hydrophilic loop domain to β-catenin (18–20). β-Catenin is a multifunctional protein initially identified as a mediator of the cadherin-dependent cell adhesion complex that links cadherins to the actin cytoskeleton (21). In addition, a cytosolic pool of β-catenin acts in the canonical Wnt signaling pathway (22, 23). In the absence of Wnt signal, free cytosolic β-catenin undergoes phosphorylation by casein kinase 1α and glycogen synthase kinase-3β (GSK3β) that associate in an axin-dependent multiprotein complex (22, 24, 25). The phosphorylation of β-catenin targets it for ubiquitination and degradation by the proteasome. Activation of Wnt receptors leads to an accumulation of β-catenin in the cytosol by inhibition of its phosphorylation (26). This pool of β-catenin can translocate to the nucleus where it associates with members of the T-cell factor/lymphoid enhancer factor-1 (Tcf/Lef) family and regulates the expression of target genes (23, 27).

Aberrant Wnt signaling has been shown to be associated with tumorigenesis (22, 27, 28). In accordance with this, mutations in β-catenin and other proteins of the Wnt signaling pathway are the major cause of colon carcinomas (27, 28). In these cells aberrantly high levels of β-catenin are observed that lead to increased nuclear signaling and the transcription of downstream genes. Of note, cells from *PS1* knock-out mice also show elevated levels of β-catenin and increased expression of β-catenin target genes including *CYCLIN D1* (29). Moreover, *PS1* knock-out mice with targeted expression of *PS1* in brain neurons develop skin cancer (30). It has been shown that PS1 can act as a scaffold protein, facilitating the phosphorylation of β-catenin by protein kinase A and GSK3β (25). Previous studies have demonstrated that GSK3β also phosphorylates PS1 *in*

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[2] The abbreviations used are: PS, presenilin; GSK3β, glycogen synthase kinase 3β; aa, amino acid; CTF, C-terminal fragment; GST, glutathione *S*-transferase; HEK293, human embryonic kidney 293; Lef, lymphoid enhancer factor-1; MBP, maltose-binding protein; PS, presenilin; Tcf, T-cell factor; PBS, phosphate-buffered saline; wt, wild type.

vitro and it has been proposed that the phosphorylation of PS1 increases binding of β-catenin (31). However, a functional role of this phosphorylation has not been demonstrated.

In this study we sought to investigate the function of PS1 phosphorylation by GSK3β. We found that phosphorylation of PS1 at the GSK3β phosphorylation sites induces a structural change in the large hydrophilic loop domain of PS1 that could be mimicked by introduction of negatively charged amino acids. This conformational change strongly decreases the association of PS1 with β-catenin leading to reduced phosphorylation and increased stabilization of β-catenin thereby enhancing its signaling to the nucleus.

EXPERIMENTAL PROCEDURES

cDNAs and Fusion Proteins—The phosphorylation site mutants of PS1 were generated by PCR techniques using appropriate oligonucleotides (sequences are available upon request). The resulting PCR fragments were subcloned into the EcoRI/XhoI restriction sites of pcDNA3.1 containing a Zeocin resistance gene (Invitrogen). All constructs were verified by sequencing of both strands. The fusion proteins of maltose-binding protein (MBP) and the hydrophilic loop domain of PS1 (amino acids (aa) 263–407) have been described earlier (32). Briefly, the coding region was amplified by PCR using the following primers: 5′-CCGAATTCTGTCCGAAAGGTCCA-3′ and 5′-CCGGATCCCTAGGTTGTGTTCCAGTC-3′. The resulting fragment was cloned into the EcoRI/BamHI restriction site of pMAL-c2 (New England Biolabs). Shorter versions of the hydrophilic loop domains (aa 298–380) were generated using the following primers: 5′-CCGGAATTCATGGCAGAAGGAG-3′, 5′-ACGCGTCGACCTATTTTACTCCCC-3′. The resulting fragments were cloned into the EcoRI/SalI restriction sites of pMAL-c2. A glutathione *S*-transferase (GST) fusion protein with β-catenin (aa 134–668) containing the binding region for PS1 was generated using the following primers: 5′-CGCGGATCCACCATGCAGTTGTCAATTTGATTAAC-3′, 5′-CCGCTCGAGTTACTGTGGCTTGTCCTCAGACA-3′. The resulting fragments were cloned into the BamHI/XhoI restriction sites of pGEX-5.1 (GE Healthcare). For the GST control protein the pGEX-5.1 vector was transformed. All fusion proteins were expressed in *Escherichia coli* DH5α. MBP and GST fusion proteins were purified on amylose- or GSH-resin, respectively, according to the supplier's instructions.

Antibodies—Polyclonal antibody 3109 was raised against the hydrophilic loop domain (aa 263–407) of PS1. The MBP fusion proteins were purified as described above and inoculated into rabbits (Eurogentec). The monoclonal antibody against PS1 hydrophilic loop, APS18, has been described previously (6). Anti-PS2-GST antibody (2972) was described previously (33). Antibodies against β-catenin (C2206) and phospho-specific anti-pSer^{33,37}-β-catenin (C4231) were obtained from Sigma. The anti-ubiquitin (A-100) antibody was obtained from Boston Biochem.

Cell Culture and Transfection—Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco's modified Eagle's medium with Glutamax (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). The HEK293 cell line stably overexpressing the β-amyloid precursor protein has been described previously (34). Transfections of cells with *PS1* cDNAs were carried out using FuGENE 6 (Roche) according to the supplier's instructions. Pools of stably expressing cells were generated by selection in 200 μg/ml Zeocin (Invitrogen). Cells were treated with the following compounds for the times indicated in the specific experiments: MG132 (5 μM, Sigma), okadaic acid (1 μM, Sigma), and GSK3β inhibitor VIII (Calbiochem, 10 μM, (35)). Dephosphorylation experiments were carried out with shrimp alkaline phosphatase as described (32).

Gel Electrophoresis and Western Blotting—Proteins were separated by SDS-PAGE with the appropriate acrylamide concentrations and transferred to nitrocellulose membranes by Western blotting techniques. Typically 25 μg of cell extract was loaded per well for Western immunoblotting. Proteins were detected using enhanced chemiluminescence techniques (GE Healthcare).

Immunoprecipitation—Specific proteins were immunoprecipitated with the respective antibodies, typically with 2–3 μl of serum or 1 μg of purified antibody. 20 μl of protein A-agarose (Zytomed) was used to bind the IgG over a 2–3-h shaking period at 4 °C. The bound protein-antibody complex was washed three times with wash buffer (50 mM Tris/HCl, pH 7.4, 500 mM NaCl, 2 mM EDTA, 0.2% Igepal).

Pull-down Experiments—Purified recombinant proteins were incubated at a concentration of 1 μg/ml (GST or GST-β-catenin) and 0.5 μg/ml (MBP or MBP-PS1 hydrophilic loop) with 10 μl of GSH-Sepharose in 1 ml of PBS for 2 h at 4 °C. After binding, the Sepharose was washed 4 times with PBS. Samples were subsequently separated by SDS-PAGE and proteins detected by Western immunoblotting.

Protein Purification—Chromatography was carried out using the Äkta FPLC system and appropriate columns from GE Healthcare. The short versions of the hydrophilic loop were expressed in *E. coli* as described above and the cleared homogenate was applied to anion exchange chromatography (ResourceQ column; buffer A, 20 mM Tris/HCl, pH 8.7; buffer B, 1 M NaCl in buffer A; gradient, 10–35% buffer B over 15 column volumes; flow rate, 6 ml/min). After concentration by ultrafiltration (Amicon Ultra, Millipore), fusion proteins were cleaved by treatment with factor Xa according to the supplier's instructions (New England Biolabs). The hydrophilic loop was separated from MBP by a second anion exchange chromatography (ResourceQ column, gradient: 7–28% buffer B over 25 column volumes, flow rate, 6 ml/min) and further purified to homogeneity by cation exchange chromatography (MonoS column: buffer A, 20 mM citrate buffer, pH 3.0; buffer B, buffer A with 1 M NaCl, gradient from 40 to 80% buffer B; flow rate, 1 ml/min).

Gel filtration was carried out using a Superdex 75 10/300 column. Proteins were applied in PBS (running buffer; flow rate, 0.75 ml/min, 20 μg of purified protein) and the elution monitored by UV absorbance.

In Vitro and in Vivo Phosphorylation of PS1—Phosphorylation of PS1 in cultured cells was carried out as described earlier (36). Briefly, a near confluent cell monolayer in a 21-cm^2 culture dish was incubated for 45 min in phosphate-free media (Sigma). The media was aspirated, and phos-

phate-free media was added, together with 0.125 mCi of [^{32}P]orthophosphate (Hartmann Analytical, Braunschweig, Germany). Cells were incubated for 1.5 h at 37 °C. The conditioned media was then aspirated, and cells were washed twice with ice-cold PBS and immediately lysed on ice with lysis buffer (see below). Cell lysates were centrifuged for 10 min at 16,000 × *g*, and supernatants were immunoprecipitated with specific anti-PS1 antibodies. Radiolabeled proteins were separated by SDS-PAGE as described above and detected by autoradiography/phosphorimaging.

In vitro phosphorylation assays with recombinant GSK3β (New England Biolabs) were carried out according to the manufacturer's instructions. Phosphorylation reactions were started by addition of 10 μM [γ-^{32}P]ATP and allowed to proceed for 20 min at 32 °C. Reactions were stopped by the addition of SDS sample buffer.

Pulse-Chase Experiments—Cells were starved with methionine-free medium for 1 h and subsequently pulsed for 20 min with [^{35}S]methionine containing medium (100 μCi/21-cm^2 dish). After washing the cells with medium containing unlabeled methionine, cells were chased for the indicated times. Cells were subsequently fractionated as described below and proteins were isolated by immunoprecipitation.

Cell Lysis and Fractionation—Cells were scraped off the culture dish and lysed for 30 min on ice (lysis buffer, 50 mM Tris/HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% Igepal, Complete protease inhibitor mixture (Roche)). The lysate was then cleared by centrifugation (16,000 × *g*, 4 °C, 30 min) and the supernatant removed. Protein estimations were carried out by bicinchoninic acid protein assay (Perbio).

In co-immunoprecipitation experiments cells were lysed in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl, 100 mM potassium fluoride, 25 mM β-glycerophosphate, 1 mM sodium orthovanadate, Complete protease inhibitor, and 1% digitonin.

For fractionation, cells were harvested and washed with PBS. Cells were then resuspended in hypotonic buffer (10 mM Tris/HCl, pH 7.4, 10 mM NaCl, 0.1 mM EGTA, 25 mM β-glycerophosphate, 1 mM dithiothreitol and Complete protease inhibitor mixture) and left on ice for 15 min. Cells were then disrupted by passing the suspension through a 23-gauge syringe needle for 15 cycles and centrifugation at 200 × *g* for 10 min at 4 °C, the supernatant contained cytosolic and membrane fraction (see below). The pellet, containing the nuclear fraction, was resuspended in HEPES buffer, pH 7.2, containing 25% glycerol, 400 mM NaCl, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and Complete protease inhibitor and agitated on ice for 20 min. The homogenate was centrifuged at 16,000 × *g* for 15 min at 4 °C and the supernatant, *i.e.* nuclear fraction, collected. Cytosolic and membrane fractions were separated by centrifugation (16,000 × *g*, 4 °C, 60 min). The supernatant, *i.e.* cytosolic fraction, was removed and the pellet, *i.e.* membrane fraction, was lysed in lysis buffer and centrifuged (16,000 × *g*, 4 °C, 15 min) to yield a debris-free sample.

Cell Proliferation—Cell proliferation was assessed by measuring the metabolic activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction (37). Cells were seeded at a concentration of 35,000 cells/ml and cultured for 3 days. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to a final concentration of 0.5 mg/ml and cells were left for 2 h. The precipitated formazan was dissolved overnight by addition of 10% SDS in 1 mM HCl and the absorbance measured at 570 nm.

Luciferase Reporter Assay—Luciferase reporter assays were carried out as described (38). Briefly, HEK293 cells were seeded at 1 × 10^5/well in six-well plates, cultured for 24 h, and transfected with 300 ng of the pTOPflash or pFOPflash luciferase reporter constructs and 500 ng of β-catenin plus either *PS1wt* or one of the *PS1* mutants. A constant amount of DNA was maintained by the addition of appropriate empty vector plasmid. Twenty-four h post-transfection the media was removed and the cells lysed for 5 min in 300 μl of Bright-Glo lysis buffer (Promega). The lysate was removed, vortexed, and briefly spun in a microcentrifuge. 150 μl of lysate supernatant was then combined with 150 μl of Bright-Glo luciferase assay reagent (Promega, half-life 30 min) and placed into a 24-well reader plate. Remaining lysate was used to check transfection efficiencies and expression levels by Western blotting. Luciferase activity was immediately measured in a Wallac 1450 Microbeta Trilux luminometer (PerkinElmer Life Sciences).

Data Analysis and Statistics—In metabolic labeling experiments, band intensities were analyzed with a phosphorimager (Fuji, FLA2000) and the Fuji Image Gauge 3.0 Software. For enhanced chemiluminescence detection, signals were measured and analyzed using an ECL imager (ChemiDoc™ XRS, Bio-Rad) and the Quantity One software package (Bio-Rad). Statistical analysis was carried out using Student's *t* test or one-way analysis of variance with a Newman-Keuls multiple comparison post-test (GraphPad Prism, GraphPad Software). Significance values are as noted in the legends for Figs. 3–6.

RESULTS

GSK3β Phosphorylates the PS1 Hydrophilic Loop at Positions 353 and 357—The hydrophilic loop of PS1 contains two serine residues at positions 353 and 357 and it has been shown that synthetic peptides representing amino acids 349–361 can be phosphorylated by GSK3β *in vitro* (31) (Fig. 1*A*). We verified phosphorylation at these sites using a recombinant hydrophilic loop domain (aa 263–403) of PS1 and purified GSK3β (Fig. 1*B*). Mutation analysis showed that mutation of serine residues 353 and 357 to alanine strongly reduced the incorporation of [^{32}P]. A S353A/S357A double mutation reduced the phosphorylation even more (Fig. 1*C*), whereas mutations at positions 310, 313, or 324 had little if any effect on GSK3β-dependent phosphorylation (Fig. 1*B*). However, residual phosphorylation was also observed in the double mutant, indicating additional phosphorylation sites of GSK3β.

To determine whether phosphorylation at these sites also occurred in cultured cells, we stably expressed PS1-wt and the PS1-S353D/S357D mutant in HEK293 cells and analyzed [^{32}P]phosphate incorporation. Consistent with previous results, PS1-wt showed incorporation of [^{32}P] into its C-terminal fragment (*CTF-P*; Fig. 1*D*, *top panel*) (32, 36). In contrast, phosphorylation of PS1-S353D/S357D was markedly reduced (Fig. 1*D*). Notably, the S353D/S357D mutant, which should mimic phosphorylated PS1 showed very similar migration as the *in vivo* phosphorylated CTF of PS1-wt (Fig. 1*D*, also see Fig.

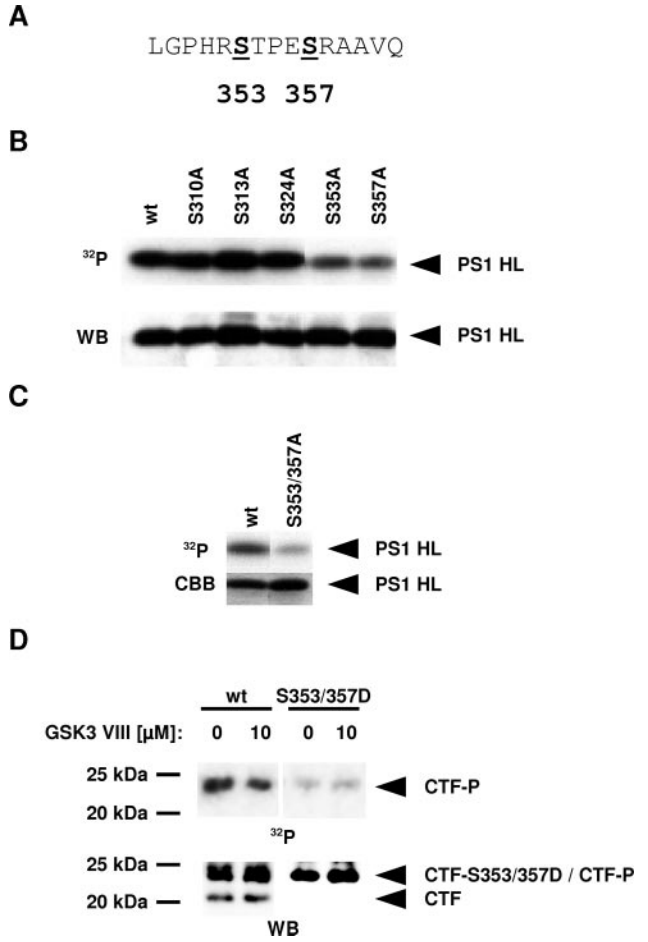

FIGURE 1. **Phosphorylation of presenilin 1 hydrophilic loop by GSK3β.** *A*, sequence of PS1 adjacent to amino acid residues 353 and 357. The sequence contains the recognition motif for GSK3β: (S,T)*XXX*(S,T). *B* and *C*, recombinant PS1 hydrophilic loop domains (PS1 HL) are phosphorylated *in vitro* by GSK3β. The domains that harbor phosphorylation site mutations as indicated. *Upper panels*, autoradiograph of ^{32}P-labeled hydrophilic loop mutants (^{32}P). *Lower panels*, Western immunoblotting (*WB*) using APS18 antibody (*B*) or Coomassie Brilliant Blue (*CBB*) stain (*C*) as loading control. *D*, *in vivo* phosphorylation of PS1 CTFs. HEK293 cells stably expressing PS1-wt or PS1-S353/357D mutant were labeled with [^{32}P]orthophosphate for 1½ h in the presence or absence of GSK3β inhibitor VIII (Calbiochem). PS1 CTFs were immunoprecipitated with antibody 3109 and Western blotted. Incorporated [^{32}P]phosphate was detected by phosphorimaging (*^{32}P*). Subsequently, total PS1 CTFs were detected by probing the membrane with the APS18 antibody (*WB*). Phosphorylated PS1 CTFs (*CTF-P*) showed reduced mobility compared with not phosphorylated PS1 CTFs (*CTF*). One representative experiment is shown.

2*B*). The weak phosphorylation of the S353D/S357D mutant is consistent with additional phosphorylation sites in the PS1 CTF besides Ser^{353} and Ser^{357} (15, 31, 32). Cell treatment with a GSK3β inhibitor decreased the phosphorylation of PS1-wt, but had little effect on that of PS1-S353D/S357D, indicating an involvement of GSK3β in the *in vivo* phosphorylation of PS1 at Ser^{353} and Ser^{357} (Fig. 1*D*).

Phosphorylation of Ser^{353} and Ser^{357} Induces a Structural Change of the Hydrophilic Loop Domain of PS1—To prove the different migration behavior of phosphorylated and non-phosphorylated PS1 CTF (Fig. 1*D*), cells were incubated in the presence or absence of okadaic acidic that inhibits protein dephosphorylation by protein phosphatases 1 and 2A. Okadaic acid treatment resulted in an increase in the slower migrating PS1 CTF (Fig. 2*A*). Treatment of isolated PS1 CTF with shrimp alkaline phosphatase reversed this effect, demonstrating that phosphorylation of the PS1 CTF reduces the migration in SDS gels (Fig. 2*A*). As shown in Fig. 1*D*, CTFs of the PS1-S353D/S357D mutant showed identical migration as the *in vivo* phosphorylated CTF of PS1-wt. These data indicate that the S353D/S357D mutant mimics the migration characteristics of phosphorylated PS1 CTF (Fig. 2*B*). In contrast, alanine mutations at these sites increased the migration of the PS1 CTF (Fig. 2*B*). Next, we analyzed the migration of recombinant purified loop domains of PS1-wt and the S353D/S357D mutant. As observed for the cellular PS1 CTFs, the purified loop domain of the S353D/S357D mutant showed slower migration as compared with the wt protein (Fig. 2*C*). To address the question whether this change in migration is due to a structural change or merely a difference in the charge, we analyzed migration in SDS gels with increasing concentrations of urea. Whereas the PS1-wt hydrophilic loop did not change its migration, the PS1-S353D/S357D mutant showed urea concentration dependent mobility (Fig. 2*D*). At 4 M urea, the mutant co-migrated with the wt loop, indicating that denaturation abolished the migrational differences. We also analyzed the retention of wt and S353D/S357D mutant loop by size exclusion chromatography (Fig. 2*E*). Here, the PS1-S353D/S357D mutant loop (*middle panel*) had a reduced retention time compared with the wild-type loop (*top panel*). The two different variants of the hydrophilic loop could still be separated when a mixture was applied to the column (*bottom panel*). Because the molecular masses of the two hydrophilic loops only differ by 56 Da, these results also indicate a structural difference between wt and phosphomimicking mutant. This is reflected by differences in the calculated Stokes radii of 2.4 and 2.9 nm for the wt and S353D/S357D mutant, respectively. Together, these data indicate that phosphorylation of PS1 CTF induced a structural change of the hydrophilic loop domain that can be mimicked by the S353D/S357D mutant.

Phosphorylation of the PS1 Hydrophilic Loop Decreases the Interaction with β-Catenin—β-Catenin has been shown to interact with PS1 (18, 39). However, others have suggested that PS1 does not bind directly to β-catenin (40). To analyze a direct interaction, we performed pull-down assays with recombinant purified fusion proteins. A fusion protein consisting of the MBP-PS1-hydrophilic loop (aa 298–380) could be pulled down by GST-β-catenin (aa 134–668), demonstrating a direct interaction of both proteins (Fig. 3*A*). To investigate whether the phosphorylation of PS1 CTF reduces the interaction with β-catenin in cultured cells, we carried out co-immunoprecipitation experiments. Precipitation of β-catenin with anti-β-catenin antibodies led to co-precipitation of the PS1 CTF (Fig. 3*B*, *first* and *second lanes*). However, the signal was strongly reduced compared with a co-precipitation with an antibody directed against the N terminus of PS1 (PS1 NTF; Fig. 3*B*, *third* and *fourth lanes*), indicating that only a fraction of β-catenin is bound to PS1 CTF. Interestingly, β-catenin preferentially co-precipitated the non-phosphorylated, lower migrating form of PS1 CTF (Fig. 3, *B* and *C*). As compared with precipitation with the PS1 NTF antibody, co-precipitation of the phosphorylated PS1 CTF with a β-catenin antibody was reduced by about 40%

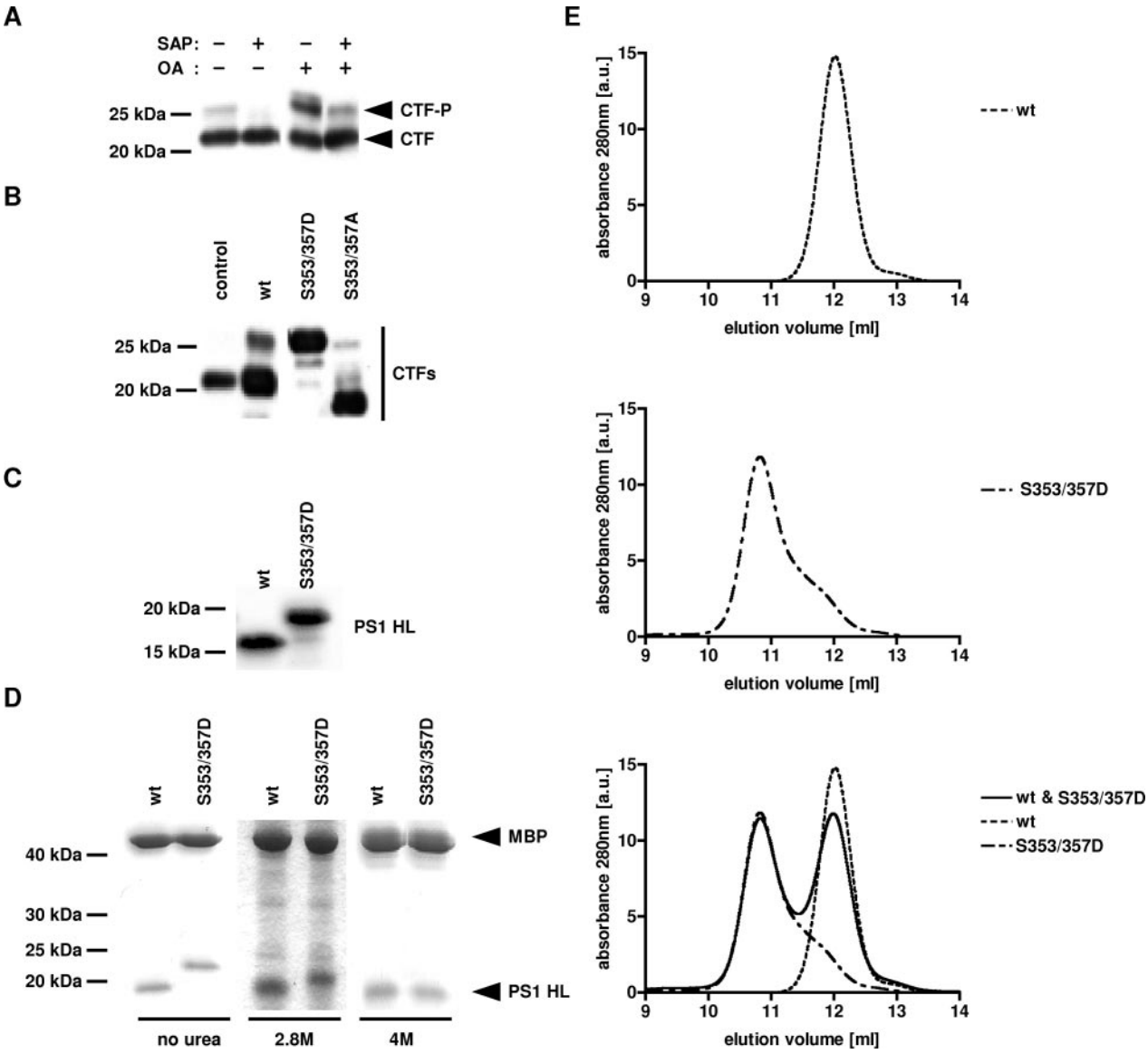

FIGURE 2. **Phosphorylation of PS1 hydrophilic loop induces a structural change.** *A*, HEK293 cells stably expressing PS1-wt were incubated in the presence (+) or absence (−) of okadaic acid (*OA*). PS1 was immunoprecipitated and treated with (+) or without (−) shrimp alkaline phosphatase (*SAP*) and detected by Western immunoblotting with APS18 antibody. *B*, detection of different PS1 CTFs by Western immunoblotting. Mutations are indicated: *control*, non-transfected (endogenous PS1-wt CTF). *C*, Coomassie Brilliant Blue stain of purified recombinant PS1 hydrophilic loop (PS1 HL, aa 298–380) separated on 15% SDS-PAGE. *D*, urea abolishes migration differences between PS1-S353D/S357D and PS1-wt hydrophilic loops. Purified MBP-PS1 hydrophilic loop fusion proteins (5 μg) were cleaved by factor Xa and separated by SDS-PAGE at the indicated urea concentrations. Whereas the migration of the PS1 hydrophilic loop domains (PS1 HL) was changed by urea, MBP did not show a changed migration. *E*, gel filtration of purified recombinant PS1-wt (20 μg, wt in *upper panel*) and PS1-S353/357D (20 μg, S353/357D in *middle panel*) hydrophilic loop domains on a Sephadex 75 size exclusion column. Note that both proteins could still be separated when applied together (*lower panel*, S353D/S357D and wt profiles are shown as overlays).

(Fig. 3, *B* and *C*). Similar results were obtained from co-precipitation studies with endogenous PS1 (data not shown). We also carried out co-immunoprecipitation experiments from total cell lysates of HEK293 cells expressing wt or mutant PS1 (Fig. 3, *D* and *E*). The amount of co-immunoprecipitated β-catenin was significantly reduced with the PS1-S353D/S357D mutant, as compared with PS1-wt (Fig. 3, *D* and *E*). Notably, PS1-S353A/S357A, which also shows a structural change (see Fig. 2*B*), also reduced the interaction with β-catenin. These results indicate that Ser^{353} and Ser^{357} are critical for the conformation dependent interaction of PS1 and β-catenin. Because the S353A/S357A mutant also decreases this interaction, this variant could not be used to mimic a non-phosphorylated form of PS1. For further functional analysis of PS1 phosphorylation, we therefore compared the PS1-S353D/S357D mutant with PS1-wt.

Phosphorylation of PS1 Hydrophilic Loop Decreases the Phosphorylation and Ubiquitination of β-Catenin—PS1 facilitates β-catenin degradation in cells by coupling the paired phosphorylation of β-catenin (25). Because the phosphorylation of PS1 at serine residues 353 and 357 reduces β-catenin binding, we analyzed the phosphorylation and ubiquitination of β-catenin in cells expressing PS1-wt or -S353D/S357D. In cytosolic fractions of PS1-wt expressing cells, two β-catenin bands were detected. Whereas the lower band corresponds to the recorded molecular mass of 94 kDa (Fig. 4*A*), the upper band (*β-catenin** in Fig. 4*A*) migrates at

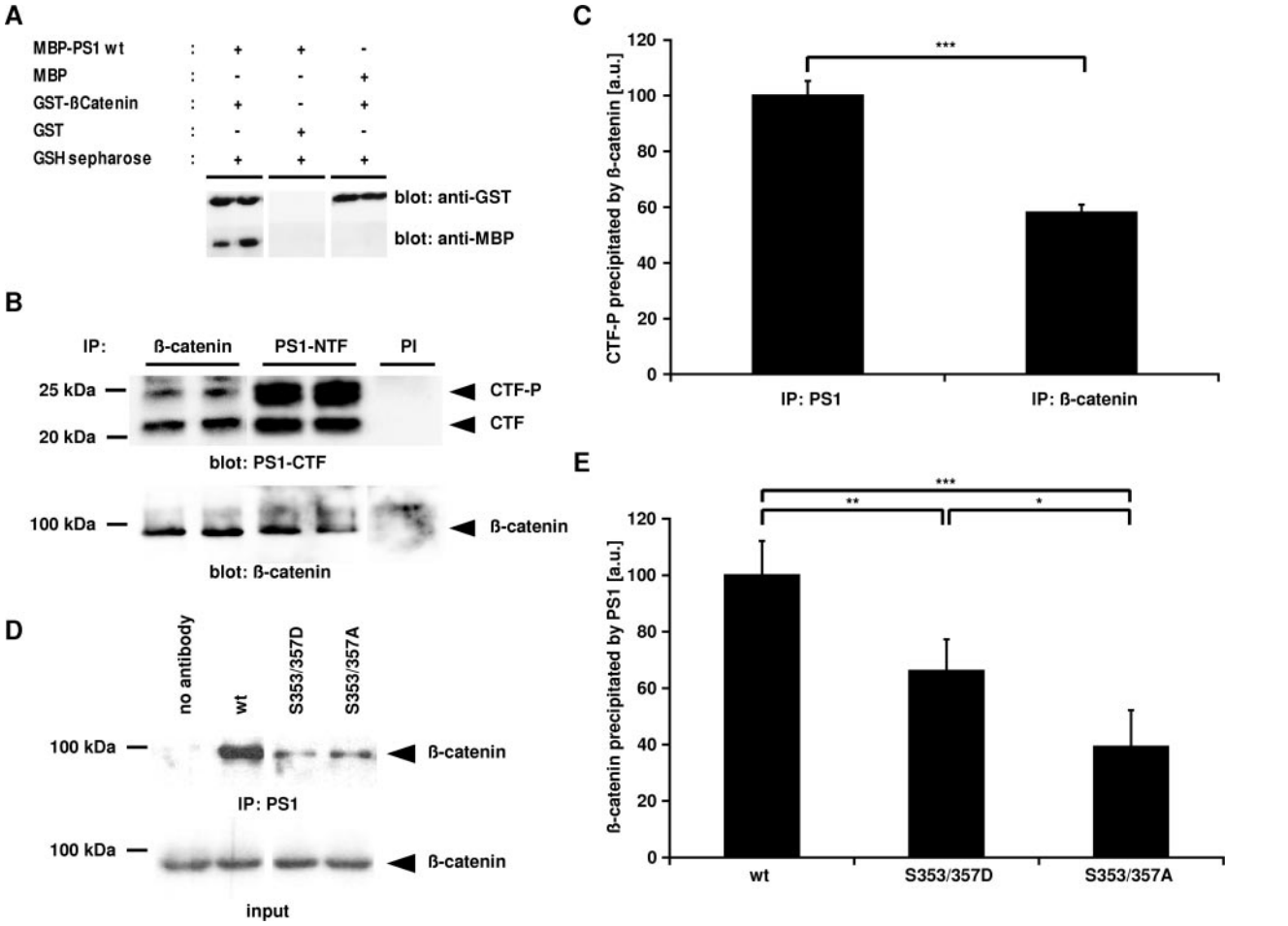

FIGURE 3. **Phosphorylation of PS1 hydrophilic loop reduces binding to β-catenin.** *A*, pull-down of PS1 hydrophilic loop by β-catenin. Purified fusion proteins GST-β-catenin (0.5 μg, aa 134–668) and MBP-PS1-wt (1 μg, aa 298–380) were incubated and precipitated with GSH-Sepharose (see "Experimental Procedures"). Specific fusion proteins were detected by Western immunoblotting with anti-MBP antibodies (3109, raised against MBP-PS1-HL) or anti-GST antibodies (2972, raised against GST-PS2-NTF). *B*, co-immunoprecipitation of PS1 by β-catenin. HEK293 cells stably expressing PS1-wt were lysed and PS1 was precipitated by anti-β-catenin (β-catenin), anti-PS1-NTF (PS1-NTF), or preimmune serum (*PI*). Co-precipitated PS1-CTF was analyzed by Western immunoblotting with APS18 (*top panel*). β-Catenin was detected as a control (*bottom panel*). *C*, quantification of co-immunoprecipitation experiments and statistical analysis by *t* test ($n = 3$). *D*, co-immunoprecipitation of β-catenin from total cell lysate by PS1. HEK293 cells stably expressing PS1-wt, -S353D/S357D or -S353A/S357A were lysed and PS1 was immunoprecipitated by APS18 antibody. Co-precipitated β-catenin was analyzed by Western immunoblotting. *E*, quantification of co-immunoprecipitation experiments and statistical analysis by analysis of variance ($n = 4$). Significance values are as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

about 105 kDa. As compared with PS1-wt cells, PS1-S353D/S357D mutant cells revealed less cytosolic β-catenin migrating at a higher molecular weight, whereas the lower migrating form was slightly increased (Fig. 4*A*). The relative amount of the upper band changed from 50% of total cytosolic β-catenin in PS1-wt to 30% in PS1-S353D/S357D expressing cells (Fig. 4*B*).

To characterize the different β-catenin variants in more detail, we immunoprecipitated β-catenin from the cytosolic fraction of cells treated with MG132. As described in Fig. 4*A*, the pan-β-catenin antibody detects two bands of about 94 and 105 kDa (Fig. 4*C*, *lanes 1* and *2*). A phosphorylation state-specific antibody, which selectively recognizes β-catenin phosphorylated at Ser^{33}/Ser^{37}, detected a main band with an apparent molecular mass of 105 kDa but not the 94-kDa form (Fig. 4*C*, *third* and *fourth lanes*). The upper band was also detected by an anti-ubiquitin antibody (Fig. 4*C*, *fifth* and *sixth lanes*). Furthermore, samples run on low percentage SDS gels showed laddering and a smear typical for polyubiquitinated proteins (Fig. 4*D*, *first* and *second lanes*). Indeed, probing with an anti-ubiquitin antibody indicated ubiquitination of the higher migrating β-catenin variants (Fig. 4*D*, *third* and *fourth lanes*), indicating that the higher migrating bands represent phosphorylated and/or ubiquitinated β-catenin. Further analysis showed that these variants are significantly reduced in the cytosolic and membrane fractions by about 25 and 20%, respectively, in PS1-S353D/S357D compared with PS1-wt expressing cells (Fig. 4, *E* and *F*). Together, these data indicate that phosphorylation of PS1 reduced phosphorylation and ubiquitination of β-catenin.

Phosphorylation of PS1 Hydrophilic Loop Increased Nuclear Levels of β-Catenin—To analyze the time-dependent phosphorylation/ubiquitination of β-catenin, we performed pulse-chase experiments with cells expressing PS1-wt or PS1-S353D/S357D. After pulse labeling, β-catenin was immunoprecipitated from isolated membranes and analyzed by phosphorimaging. The major 94-kDa form of β-catenin was almost unchanged over a 3-h period (Fig. 5*A*). In PS1-wt expressing cells, a time dependent increase of the 105-

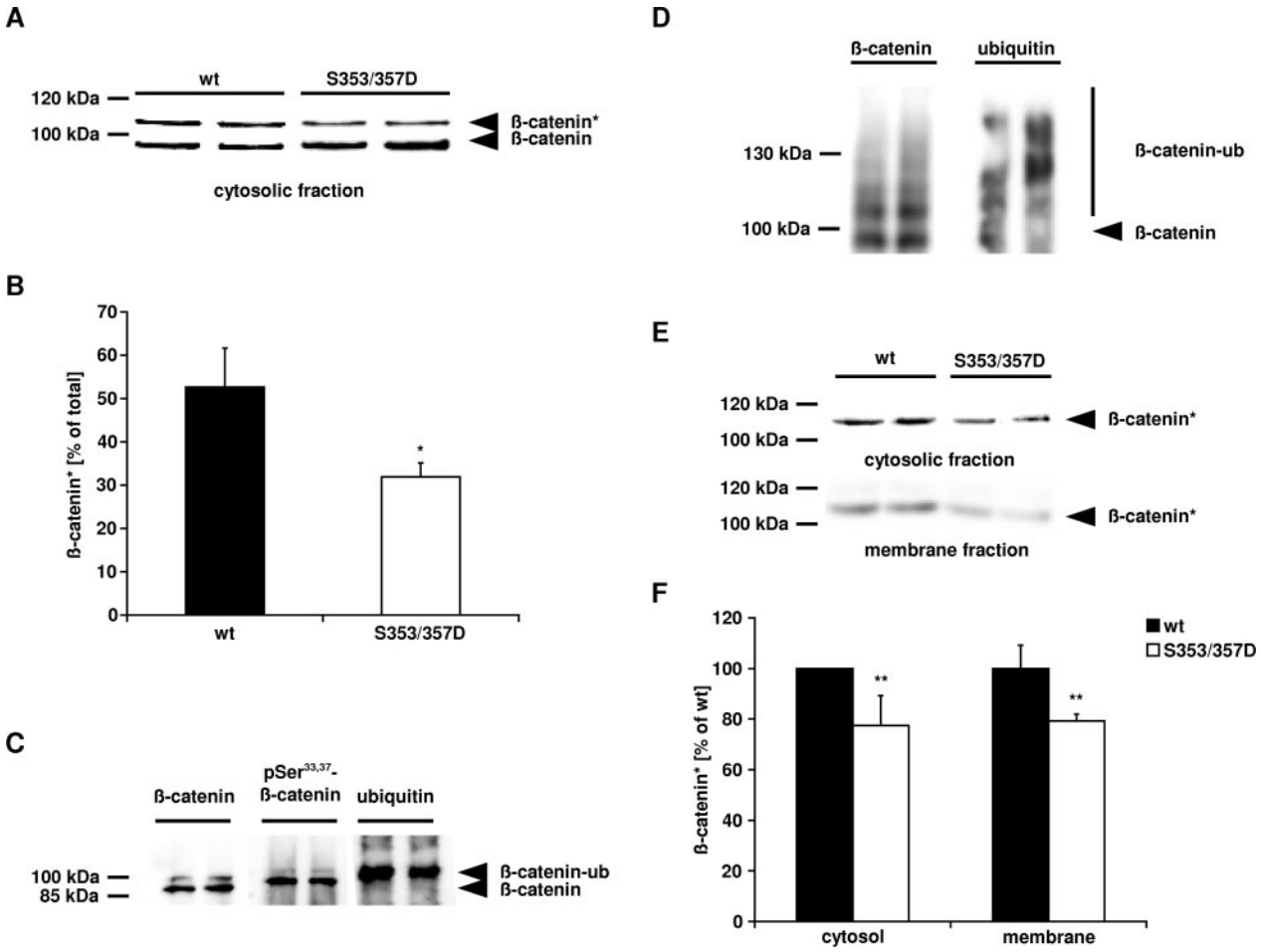

FIGURE 4. **PS1 phosphorylation reduces β-catenin degradation.** *A*, cytosolic β-catenin levels in PS1-wt and PS1-S353D/S357D mutant cells. Cytosolic fractions (see "Experimental Procedures") were analyzed by Western immunoblotting using an anti-β-catenin antibody (C2206). *β-Catenin** represents a high molecular weight species of β-catenin. *B*, quantification of cytosolic β-catenin* levels in *A* as the percentage of total β-catenin. Statistical analysis was carried out using a Student's *t* test ($n = 3$). *C*, characterization of the observed β-catenin isoforms. HEK293 cells stably expressing PS1-wt were treated with 10 μM MG132 for 16 h and β-catenin was immunoprecipitation from the cytosolic fraction with an anti-β-catenin antibody (C2206). The specific isoforms were detected by anti-β-catenin (C2206), anti-$pSer^{33,37}$-β-catenin (C4231), and an anti-ubiquitin antibody (A-100) as indicated. Each detection was carried out in duplicate. *D*, stabilization of β-catenin in HEK293 cells stably expressing PS1-wt. Cells were treated with 5 μM MG132 for 16 h. Cytosolic fractions were isolated, β-catenin immunoprecipitated, and analyzed by Western immunoblotting with an anti-β-catenin (C2206) or anti-ubiquitin antibody (A-100). Duplicate samples are shown. *E*, cytosolic and membrane $pSer^{33,37}$-β-catenin in PS1-wt and PS1-S353D/S357D mutant cells were analyzed by Western immunoblotting using an anti-$pSer^{33,37}$-β-catenin (C4231) antibody. *F*, quantification of cytosolic $pSer^{33,37}$-β-catenin levels, statistical analysis was carried out using a Student's *t* test ($n = 4$). Significance values are as follows: *, $p < 0.05$; **, $p < 0.01$.

kDa β-catenin was observed, indicating phosphorylation and/or ubiquitination (Fig. 5, *A* and *B*). This conversion was strongly decreased in PS1-S353D/S357D expressing cells (Fig. 5, *A* and *B*). As in PS1-wt expressing cells, the membrane-associated pool of β-catenin was stable over the 3-h chase period. Interestingly, significant differences were observed after a 6-h chase. About 60% of β-catenin was turned over in PS1-wt expressing cells (Fig. 5, *C* and *D*). In contrast, very little decrease of membrane-associated β-catenin was detected in PS1-S353D/S357D expressing cells (20%, Fig. 5, *C* and *D*). These data indicate that phosphorylated PS1 decreased the phosphorylation and degradation of the membrane-associated pool of β-catenin. In contrast to the slow turnover of membrane-associated β-catenin, cytosolic β-catenin was efficiently turned over after 6 h (Fig. 5, *E* and *F*). However, cytosolic levels of β-catenin were higher in PS1-S353D/S357D (10%) as compared with PS1-wt expressing cells (6%, Fig. 5, *E* and *F*). In further experiments, cells stably expressing wt or mutant PS1 were treated with cycloheximide that inhibits protein *de novo* synthesis. In PS1-wt expressing cells, cytosolic β-catenin was turned over rapidly ($t_{½} = 24$ min), whereas in cells expressing the PS1-S353D/S357D mutant the turnover of β-catenin was prolonged ($t_{½} = 65$ min) (data not shown).

Stabilized cytosolic β-catenin could translocate to the nucleus and mediate transcription of target genes. Consistent with stabilization of the membrane-associated and the cytosolic pool of β-catenin, we also observed increased levels of β-catenin in the nuclear fraction of PS1-S353D/S357D as compared with PS1-wt expressing cells (Fig. 5, *G* and *H*).

Phosphorylation of the PS1 Hydrophilic Loop Negatively Modulates Signaling of β-Catenin—To determine whether the increase of β-catenin in the nucleus has an effect on signaling activity via the Tcf/Lef transcription pathway, TOPflash reporter assays were carried out. As compared with non-transfected cells, expression of PS1-wt reduced the reporter signal, which is consistent with previous studies (29, 38). Notably, PS1-

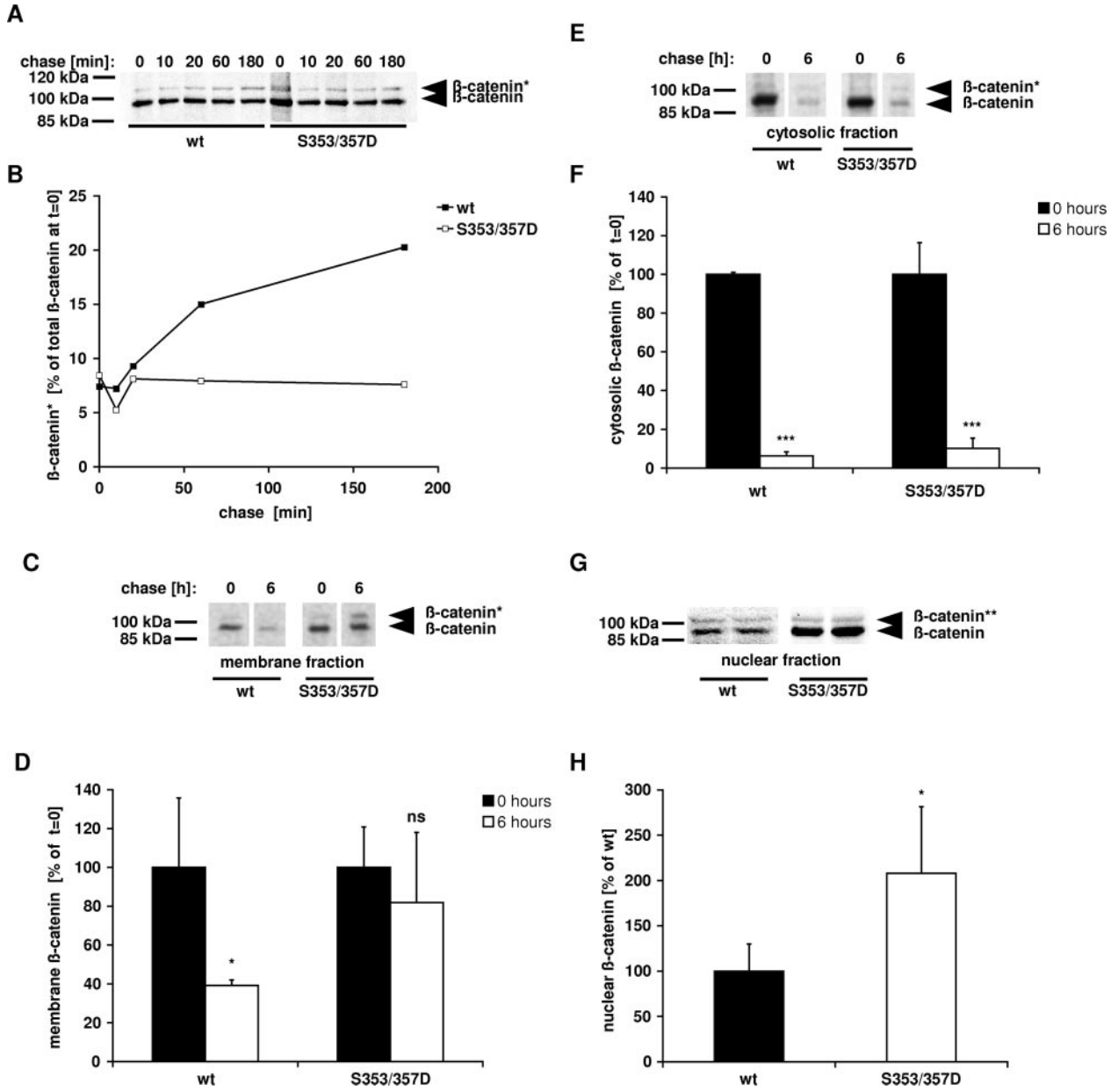

FIGURE 5. **PS1 phosphorylation decreases β-catenin turnover.** *A*, short term pulse-chase of β-catenin. Cells expressing PS1-wt or PS1-S353D/S357D were starved with methionine-free medium for 60 min and pulsed with [^{35}S]methionine containing medium for 20 min. After replacing the medium with unlabeled methionine, cells were chased for the indicated times. Membrane fractions were prepared, lysed, and β-catenin immunoprecipitated (C2206). Labeled β-catenin was analyzed by Western blotting and subsequent phosphorimaging. The generation of the high molecular weight species of β-catenin (*β-catenin**) as a fraction of total β-catenin at the start of the chase is depicted in *B* ($n = 2$). *C–F*, long term pulse-chase of β-catenin. Same procedures as described in *A* for longer chase times. Cytosolic (*C*) and membrane (*E*) fractions were prepared, lysed, and β-catenin immunoprecipitated (C2206). Labeled β-catenin was analyzed by Western blotting and subsequent phosphorimaging. *D* and *F*, quantification of total β-catenin as the percentage of β-catenin at the beginning of the chase ($t = 0$, $n = 3$). *G*, β-catenin translocation into the nucleus. HEK293 cells stably expressing PS1-wt or PS1-S353D/S357D were subjected to cell fractionation and the nuclear fraction was analyzed by Western immunoblotting (C2206). The high molecular weight form observed (*β-catenin***) could represent a phosphorylated β-catenin described recently (47, 48). *H*, quantification of β-catenin as the ratio of nuclear β-catenin to total β-catenin, normalized to PS-1wt cells. Statistical analysis were carried out using a Student's *t* test ($n = 5$). Significance values are as follows: *ns*, not significant; *, $p < 0.05$; ***, $p < 0.001$.

S353D/S357D did not significantly reduce the reporter signal (Fig. 6*A*). Very similar data were also obtained when cell signaling was stimulated by transfection with β-catenin (Fig. 6*B*).

Because c-*MYC* is a known target gene of the Tcf/Lef transcription pathway (41, 42), we next analyzed its expression in PS1-wt and PS1-S353/357D mutant expressing cells. Whereas expression of PS1-wt decreased the cellular levels of c-myc as compared with non-transfected cells, this reduction was much less pronounced by expression of PS1-S353D/S357D (Fig. 6*C*). Consistent with decreased expression of c-myc, cells overexpressing PS1-wt showed decreased proliferation as compared with non-transfected cells (Fig. 6*D*). Cells overexpressing PS1-S353D/S357D showed intermediate proliferation rates, indicating a reduced ability to regulate cell proliferation.

DISCUSSION

PS1 has been shown to bind β-catenin (18, 20, 39) and facilitate its degradation and therefore serves a function compara-

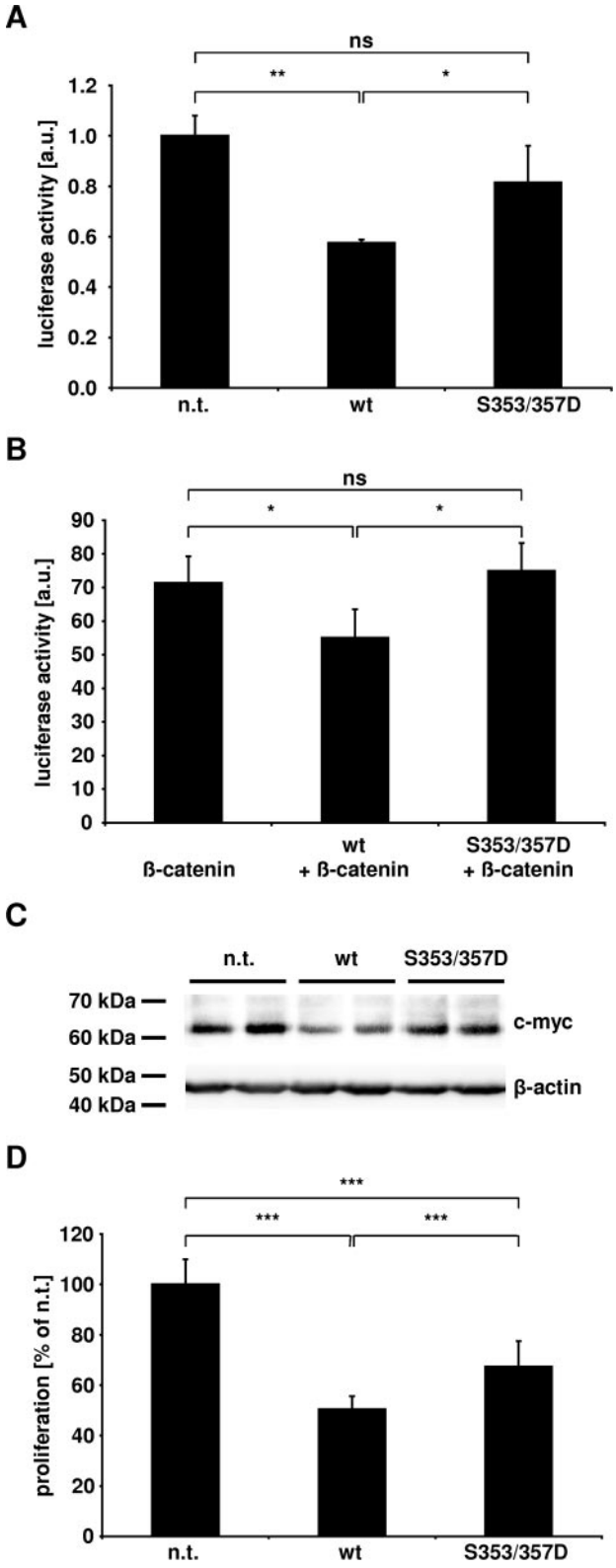

FIGURE 6. **PS1 phosphorylation positively modulates β-catenin signaling.** *A*, TOPflash reporter assays were carried out with HEK293 cells transiently transfected with PS1-wt, PS1-S353D/S357D, or non-transfected (*n.t.*). *B*, as *A* with co-transfection of β-catenin. Values represent fold-stimulation over non-transfected cells (*n.t.* = 1, not shown). *C*, detection of c-myc and β-actin in stably transfected HEK293 cells by Western immunoblotting. *D*, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide proliferation assay of stably transfected HEK293 cells. Statistical analyses were carried out by analysis of variance ($n = 3$ (*A* and *B*), $n = 16$ (*D*)). Significance values are as follows: *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ble with the adenomatous polyposis coli-axin scaffolding complex involved in the Wnt signaling pathway (25). It has also been shown that GSK3β could phosphorylate synthetic peptides representing a PS1 sequence that contains Ser^{353} and Ser^{357} (31), and that GSK3β is associated with PS1/β-catenin to form a ternary complex (43). We now show that the phosphorylation of PS1 by GSK3β at serines 353 and 357, which are highly conserved in PS1 of different mammalian species (31, 32), has significant effects on the structure of the large hydrophilic loop domain of the PS1 CTF, as indicated by reduced mobility in SDS-PAGE and retention in gel filtration experiments. The structural change of PS1 induced by phosphorylation could be mimicked by a PS1-S353D/S357D double mutation that allowed the functional analysis in cultured cells without the need for pharmacological manipulation with protein kinase and phosphatase modulators. Of note, the S353D/S357D mutation did not inhibit the endoproteolytic processing of PS1 and exerts normal γ-secretase activity as indicated by unaltered production of Aβ (data not shown).

The hydrophilic loop domain could interact with β-catenin (39). However, Serban *et al.* (40) suggested a ternary complex of β-catenin, PS1, and E-cadherin in which E-cadherin is essential for the binding of PS1 and β-catenin. As shown here, purified recombinant fusion proteins of the PS1 hydrophilic loop and β-catenin bind directly in the absence of E-cadherin. However, the interaction of PS1 and β-catenin might be modulated by E-cadherin. The functional investigation of PS1 phosphorylation in cultured cells revealed that it negatively regulates the interaction with β-catenin by changing the structure of its hydrophilic loop domain.

Pulse-chase experiments revealed that phosphorylation of PS1 reduces the phosphorylation and ubiquitination of membrane-associated β-catenin, resulting in its stabilization. Moreover, we also observed stabilization of the cytosolic β-catenin pool. This is in accordance to findings where cells expressing a PS1 variant lacking the β-catenin binding site but exerting γ-secretase activity show decreased turnover of β-catenin (29, 30). How could the phosphorylation of membrane-tethered PS1 affect cytosolic β-catenin levels? The decreased binding of β-catenin to phosphorylated PS1 could uncouple the paired phosphorylation of β-catenin and thereby decrease the turnover of membrane-associated β-catenin. The stabilized β-catenin could dissociate from the membrane leading to increased levels of β-catenin in the cytosolic and nuclear fraction. Indeed, we observed increased cytosolic and nuclear pools of β-catenin at steady state, whereas the membrane-associated pool was not significantly increased (not shown). In addition, PS1 might also affect the metabolism of cytosolic β-catenin indirectly by regulating the levels of GSK3β in the cytosol or other signal transduction pathways (see also Ref. 38).

Cells expressing PS1-S353D/S357D showed increased transcription of the TOPflash promoter compared with cells expressing PS1-wt. In addition, PS1-S353D/S357D expressing cells revealed higher levels of endogenous c-myc, and increased

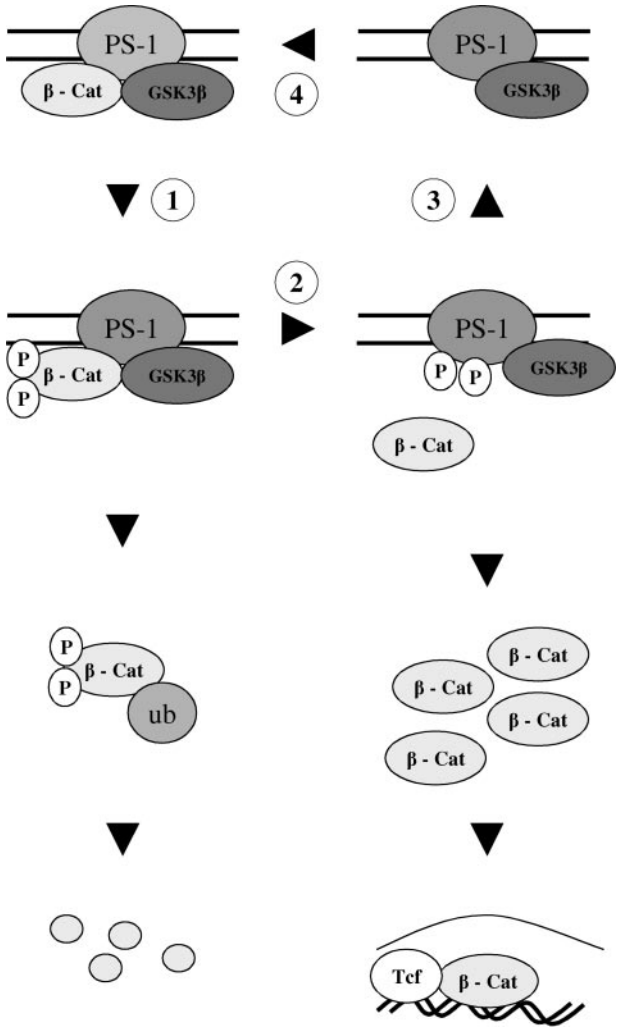

FIGURE 7. **Model for phosphorylation dependent β-catenin signaling.** PS1, β-catenin, and GSK3β form a ternary complex that aids the phosphorylation of β-catenin and its subsequent degradation by the proteasome (1). PS1 hydrophilic loop can be phosphorylated by GSK3β after the release of β-catenin and a structural change is induced that precludes the binding of free β-catenin (2). This leads to a stabilization of β-catenin and increased nuclear signaling via Tcf/Lef-regulated transcription. PS1 can be de-phosphorylated (3) and the ternary complex re-established leading to the binding of free β-catenin (4).

cell proliferation as compared with cells expressing PS1-wt. Together, these data indicate that the metabolism of β-catenin and its nuclear signaling can be regulated by phosphorylation-dependent binding to PS1. In accordance with our findings, PS1-deficient fibroblasts show increased *CYCLIN D1* expression and cell proliferation (29). Moreover, mice that only express PS1 in neurons develop skin tumors (30). The lack or loss of function of PS1 may therefore lead to aberrant cell proliferation and tumorigenesis. However, β-catenin signaling in development and tumorigenesis might be mainly regulated via the Wnt/adenomatous polyposis coli/Axin pathway (27, 28) and further studies are necessary to elucidate the contribution of PS1-phosphorylation dependent regulation of β-catenin metabolism in these processes.

Moreover, PS1 could affect β-catenin signaling by an additional mechanism. Because PS1 is the proteolytic active component of the γ-secretase complex, the cleavage of its substrates N- and E-cadherin can also liberate β-catenin from cellular membranes (12, 13, 44). It has also been shown recently that ADAM10 induced shedding of N-cadherin increased c-*MYC*, c-*JUN*, and *CYCLIN D1* expression, likely by subsequent cleavage of N-cadherin and liberation of β-catenin from the membrane (12). Thus PS1 might exert a dual function in β-catenin regulation, either by direct association with β-catenin via the hydrophilic loop domain and facilitation of β-catenin phosphorylation, or by cleaving cadherins and liberation of bound β-catenin from the membrane.

Recently, the role of Wnt signaling and β-catenin in the regulation of synaptic differentiation and organization has been highlighted (45,46). Because PS1 is recruited to cadherin/catenin-dependent cell-cell adhesion contacts in synapses (10), the regulation and modulation of synaptic function and plasticity via the proteolytic activity of γ-secretase has been proposed (10, 44). In addition, GSK3β-mediated phosphorylation of PS1 could modulate synapse function via β-catenin in a γ-secretase independent manner.

The following model is consistent with the observed PS1 phosphorylation state-dependent metabolism and signaling of β-catenin (Fig. 7). β-Catenin and GSK3β are recruited to the non-phosphorylated hydrophilic loop domain of PS1. This ternary complex allows efficient phosphorylation of β-catenin by GSK3β and subsequent release into the cytoplasm where it undergoes ubiquitination and degradation. Dissociation of β-catenin then allows phosphorylation of PS1 by GSK3β, which induces a structural change that inhibits further binding of β-catenin. Thus, phosphorylation of PS1 would be an efficient switch-off mechanism of PS1-dependent degradation of β-catenin. To test this model, further research could focus on the time-dependent association and dissociation as well as the coordinated phosphorylation of PS1 and β-catenin by GSK3β at cellular membranes.

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A Structural Switch of Presenilin 1 by Glycogen Synthase Kinase 3β-mediated Phosphorylation Regulates the Interaction with β-Catenin and Its Nuclear Signaling

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