

Conserved Stress-protective Activity between Prion Protein and Shadoo*

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Vignesh Sakhivelu[‡], Ralf P. Seidel[§], Konstanze F. Winklhofer^{‡1}, and Jörg Tatzelt^{‡1,2}

From the [‡]Neurobiochemistry, Adolf-Butenandt-Institute, Ludwig-Maximilians-University Munich, D-80336 Munich and the [§]Max-Planck-Institute of Molecular Physiology, 44227 Dortmund, Germany

Shadoo (Sho) is a neuronally expressed glycoprotein of unknown function. Although there is no overall sequence homology to the cellular prion protein (PrP^C), both proteins contain a highly conserved internal hydrophobic domain (HD) and are tethered to the outer leaflet of the plasma membrane via a C-terminal glycosylphosphatidylinositol anchor. A previous study revealed that Sho can reduce toxicity of a PrP mutant devoid of the HD (PrP Δ HD). We have now studied the stress-protective activity of Sho in detail and identified domains involved in this activity. Like PrP^C, Sho protects cells against physiological stressors such as the excitotoxin glutamate. Moreover, both PrP^C and Sho required the N-terminal domain for this activity; the stress-protective capacity of PrP Δ N as well as Sho Δ N was significantly impaired. In both proteins, the HD promoted homodimer formation; however, deletion of the HD had different effects. Although Sho Δ HD lost its stress-protective activity, PrP Δ HD acquired a neurotoxic potential. Finally, we could show that the N-terminal domain of PrP^C could be functionally replaced by that of Sho, suggesting a similar function of the N termini of Sho and PrP^C. Our study reveals a conserved physiological activity between PrP^C and Sho to protect cells from stress-induced toxicity and suggests that Sho and PrP^C might act on similar signaling pathways.

Prion diseases in humans and other mammals are characterized by a conformational transition of the cellular prion protein (PrP^C)³ into an aberrantly folded isoform, designated scrapie prion protein (PrP^{Sc}). PrP^{Sc} can form amyloid plaques in the diseased brain and is the major constituent of infectious prions (reviewed in Refs. 1–4). Biogenesis of PrP^C is essential for the pathogenesis of prion diseases. Mice with a targeted disruption of the PrP gene (*Prnp*) are resistant to prion diseases and to the

propagation of infectious prions (5). Moreover, neuronal expression of PrP^C seems to be required to mediate neurotoxic effects of scrapie prions (6–9).

Structural studies with recombinantly expressed PrP revealed a large flexibly disordered N-terminal region, containing an octarepeat region, and a structured C-terminal domain (aa 121–231). This autonomously folding domain contains three α -helical regions and a short two-stranded β -sheet (10–12). The C-terminal domain is characterized by extensive co- and post-translational modifications, including two N-linked glycans with complex structure, a disulfide bridge, and a C-terminal glycosylphosphatidylinositol (GPI) anchor (reviewed in Ref. 13). Interestingly, the octarepeat region in the N-terminal domain as well as the C-terminal GPI anchor are dispensable for the generation of infectious prions (8, 14) but are required for the stress-protective activity of PrP^C (9, 15).

Although in the majority of prion diseases there is a correlation between the accumulation of PrP^{Sc}, formation of infectious prions, and neurodegeneration, there are some interesting exceptions. Transgenic mouse models revealed that several PrP mutants can induce neuronal cell death in the absence of infectious prion propagation (reviewed in Ref. 16). From one class of PrP mutants, it emerged that PrP^C can acquire a neurotoxic potential by deleting the internal HD (17–19). Similarly to PrP^C, PrP Δ HD is complex glycosylated and linked to the plasma membrane via a GPI anchor (20). Moreover, expression of PrP^C can completely prevent the neurotoxic activity of PrP Δ HD, suggesting that PrP^C and PrP Δ HD can induce neuroprotective or neurotoxic signaling via binding to the same signal-transducing protein(s) (9, 19). Indeed, numerous studies in transgenic animals and cultured cells are now supporting the idea that the physiological role of PrP^C is to protect neuronal cells against stress-induced cell death (reviewed in Ref. 21).

In the search for homologs/paralogs of PrP^C, a new gene was identified termed *Sprn*, encoding for a protein denoted Shadoo (Sho) (22). Sho is highly conserved from fish to mammals. The sequence homology between Sho and PrP is restricted to the internal hydrophobic domain; however, certain features, such as a N-terminal repeat region and a C-terminal GPI anchor, are conserved, suggesting that Sho and PrP may be functionally related. Experimental evidence for the post-translational modifications and cell surface localization of Sho was first presented for zebrafish Sho (23) and afterward also for mouse Sho (24). Moreover, it was demonstrated that Sho, similarly to PrP^C, can prevent neuronal cell death induced by the expression of PrP Δ HD mutants (24).

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¹ Both authors contributed equally to this work.

² To whom correspondence should be addressed: Ludwig-Maximilians-Universität München, Schillerstrasse 44, D-80336 Munich, Germany. Tel.: 49-89-2180-75442; Fax: 49-89-2180-75415; E-mail: Joerg.Tatzelt@med.uni-muenchen.de.

³ The abbreviations used are: PrP, prion protein; PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein; Sho, Shadoo; HD, hydrophobic domain; GPI, glycosylphosphatidylinositol; Endo H, endoglycosidase H; PNGase F, N-glycosidase F; PIPLC, phosphatidylinositol-specific phospholipase C; aa, amino acids; β -ME, 2-mercaptoethanol.

Shadoo and Neuroprotection

In this study, we analyzed the stress-protective activity of Sho in detail and defined domains required for this activity. Furthermore, the generation of a N-Sho·PrP-C chimeric protein revealed that the N terminus of Sho can restore the stress-protective activity of a PrP mutant devoid of the N-terminal domain (PrP Δ N).

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—All standard chemicals and reagents were purchased from Sigma if not otherwise noted. The following antibodies were used: mouse monoclonal anti-PrP 3F4 antibody (25), mouse monoclonal anti-V5 tag antibody (Invitrogen), mouse monoclonal anti-HA tag antibody (Covance), anti-active caspase-3 polyclonal antibody (Promega), Cy3-conjugated anti-rabbit IgG antibody (Dianova), and Cy3-conjugated anti-mouse IgG antibody (Dianova). For the generation of a rabbit polyclonal anti-Sho antibody, recombinant human full-length Sho, expressed in and purified from *Escherichia coli*, was used for the immunization of rabbits (Eurogentec, Seraing, Belgium). The mounting medium Mowiol (Calbiochem) was supplemented with 4',6-diamidino-2-phenylindole (DAPI).

Plasmid Construction—The cloning of PrP, PrP Δ N (Δ 28–89), PrP Δ HD (Δ 113–133), and PrPS131C was described previously (20, 26, 27). The human Sho gene was prepared by ligation-based strategy for chemical gene synthesis using the Sloning building block technology (28) (Sloning, Puchheim, Germany) and cloned into a pcDNA 3.1/Zeo (+). A V5 tag (5'-GGT AAA CCG ATA CCG AAC CCG CTC CTC GGT CTC GAT TCG ACG-3') or HA tag (5'-TAC CCA TAC GAT GTT CCA GAT TAC GCT-3') was inserted between amino acids 124 and 125. Sho was used as a template to introduce the following deletions and mutations by standard PCR cloning techniques: Sho Δ N (aa 30–56 deleted), Sho Δ HD (aa 68–89 deleted), ShoS87C (serine at aa 87 replaced with cysteine), and Sho Δ N,S87C (aa 30–56 deleted; serine at aa 87 replaced with cysteine). All amino acid numbers refer to the human Shadoo sequence (GenBankTM association number NM_001012508). The chimeric N-Sho·PrP-C (Sho 1–63 and PrP 89–254) was generated by PCR and cloned into pcDNA 3.1/Zeo (+) vector.

Cell Culture, Transfection, Protein Deglycosylation, Phospholipase C, Tunicamycin, Brefeldin A Treatment, and Western Blotting—Human neuroblastoma cells (SH-SY5Y) were cultured as described earlier (20). Cultured cells were transfected with Lipofectamine Plus (Invitrogen) according to the manufacturer's instruction. After 24 h, cells were washed with cold PBS, scraped off the plate, pelleted, and lysed in cold detergent buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS). Endo H (New England Biolabs) and PNGase F (Roche Applied Science) digestions were done according to the manufacturer's instruction. To inhibit N-linked glycosylation or transport to the plasma membrane, transfected cells were grown in the presence of tunicamycin (0.5 μ g/ml) or brefeldin A (1 μ g/ml), respectively. 24 h later, cells were analyzed. For phosphatidylinositol-specific phospholipase C (PIPLC) treatment, cells were washed twice with cold PBS. PIPLC (1 units/ml) was added to the cells for 3 h at 4 °C. The medium was collected, and cells were washed twice with cold PBS and lysed in cold detergent

buffer. Proteins present in the medium were precipitated by trichloroacetic acid (TCA), boiled in Laemmli sample buffer, and analyzed by Western blotting as described earlier (29).

Apoptosis Assay—As described earlier (27), SH-SY5Y cells were grown on coverslips. 24 h after transfection, the cells were incubated with glutamate (500 μ M) for 3 h. The cells were then fixed, and activated caspase-3 was detected by indirect immunofluorescence using an anti-active caspase-3 antibody. To detect cells undergoing apoptosis, the number of activated caspase-3-positive cells out of at least 1100 transfected cells was determined using a Zeiss Axioskop 2 Plus microscope (Carl Zeiss, Göttingen, Germany). Quantifications were based on triplicates of at least three independent experiments.

Co-immunoprecipitation—To analyze formation of a mixed PrP/Sho dimer, SH-SY5Y cells were co-transfected with PrPS131C and ShoS87C. At 24 h after transfection, the cells were harvested and lysed in ice-cold detergent lysis buffer (0.5% Triton X-100, 0.5% sodium deoxycholate in PBS) supplemented with protease inhibitors.

Pre-cleared lysates were incubated with α V5 antibody overnight at 4 °C. The immunocomplex was precipitated with protein A-Sepharose beads and analyzed by Western blotting. To analyze formation of Sho trans-dimers, separate dishes of SH-SY5Y cells were transfected with either ShoS87C-V5 or ShoS87C-HA. 3 h after transfection, cells were extensively washed, trypsinized, mixed together, and seeded in one cell culture dish. 24 h later, the cells were harvested and analyzed as described above.

Indirect Immunofluorescence Microscopy—Transiently transfected SH-SY5Y cells were grown on glass coverslips and fixed 24 h after transfection with 3% paraformaldehyde for 20 min. Fixed cells were incubated with primary antibody for 45 min at 37 °C in PBS containing 1% BSA. After extensive washing with cold PBS, incubation with the Cy3-conjugated secondary antibody followed at 37 °C for 30 min. Cells were mounted onto glass slides and examined by fluorescence microscopy.

Statistical Analysis—Data were expressed as means \pm S.E. Statistical analysis was carried out using Student's *t* test. *p* values are as follows: *, *p* < 0.05, **, *p* < 0.005, ***, *p* < 0.0005.

RESULTS

Sho Mutants Devoid of the N-terminal Domain or the Internal Hydrophobic Domain Are Complex Glycosylated and Targeted to the Outer Leaflet of the Plasma Membrane via a GPI Anchor—In a previous study, we have shown that zebrafish Sho expressed in mammalian cells is a complex glycosylated, GPI-anchored protein present at the outer leaflet of the plasma membrane (23). For a functional characterization of mammalian Sho, we cloned the human homolog and several mutants as outlined in Fig. 1A. The design of the Sho mutants was based on PrP mutants that have been characterized previously. PrP Δ N, a mutant lacking large parts of the intrinsically disordered N terminus (Δ aa 27–89), can promote propagation of infectious prions (14) but shows an impaired neuroprotective activity (9, 15). PrP Δ HD lacks the highly conserved internal hydrophobic domain (Δ aa 113–133) and is characterized by a neurotoxic potential that can be blocked by the co-expression of wild type (WT) PrP^C (9, 17–19). Importantly, these PrP mutants are

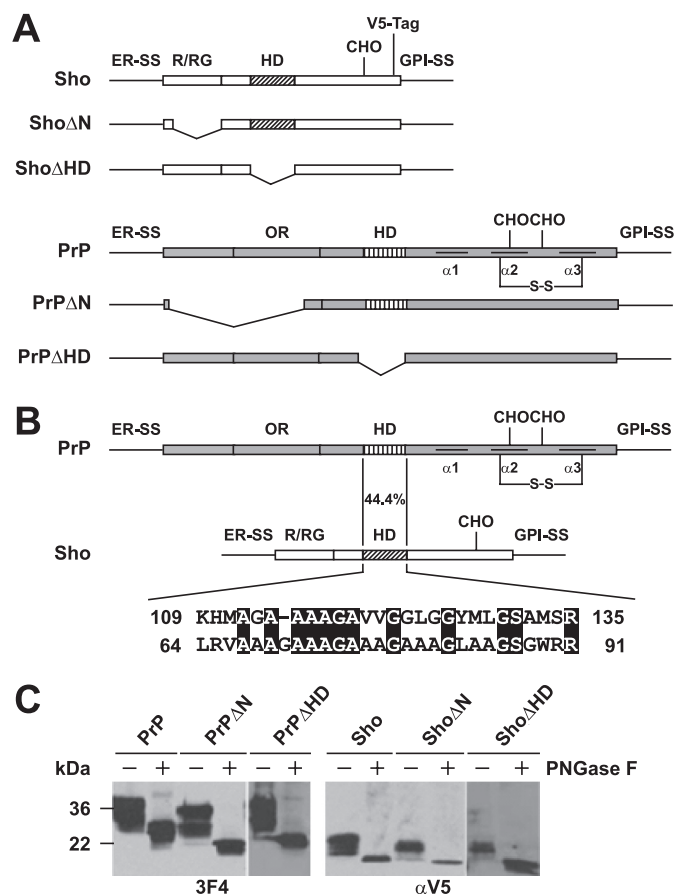


FIGURE 1. Deletion of the N-terminal or the hydrophobic domain does not interfere with biogenesis of Sho. *A*, schematic presentation of the constructs used. ER-SS, endoplasmic reticulum signal sequence; R/RG, arginine- and glycine-rich basic repeats; CHO, N-linked glycosylation acceptor site; S-S, disulfide bridge; GPI-SS, glycosylphosphatidylinositol anchor signal sequence; OR, octarepeat region; α , α -helical region. *B*, sequence alignment of the hydrophobic domains of PrP and Sho. Conserved amino acids are shaded. *C*, Sho and its mutants are complex glycosylated. SH-SY5Y cells were transiently transfected with the constructs indicated. Total cell lysates were treated with PNGase F (+) or left untreated (-), and PrP or Sho were detected by Western blotting.

complex glycosylated and targeted to the outer surface of the plasma membrane via a GPI anchor (20).

First, we expressed the different Sho constructs and the corresponding PrP mutants in human SH-SY5Y cells to analyze the co- and post-translational modifications. To evaluate the modification with N-linked glycans, cell lysates were treated with PNGase F to remove all N-linked glycans. The apparent shift in electrophoretic mobility after PNGase F digestion indicated that all constructs are modified with N-linked glycans (Fig. 1C). The glycans were resistant to Endo H digestion (data not shown), indicating that all constructs are modified with N-linked glycans of complex structure.

To test for a C-terminal GPI anchor, live cells were incubated with PIPLC to release GPI-anchored proteins from the cell surface. Indeed, after PIPLC treatment, the relative amount of the PrP constructs in the cell lysates decreased concomitantly with the appearance of the respective proteins in the cell culture supernatant (Fig. 2A). Similarly, PIPLC treatment reduced the relative amount of the Sho constructs present in the cell lysates. In parallel, Sho and Sho Δ N were found in the supernatant of PIPLC-treated cells (Fig. 2A). Curiously, we never detected sig-

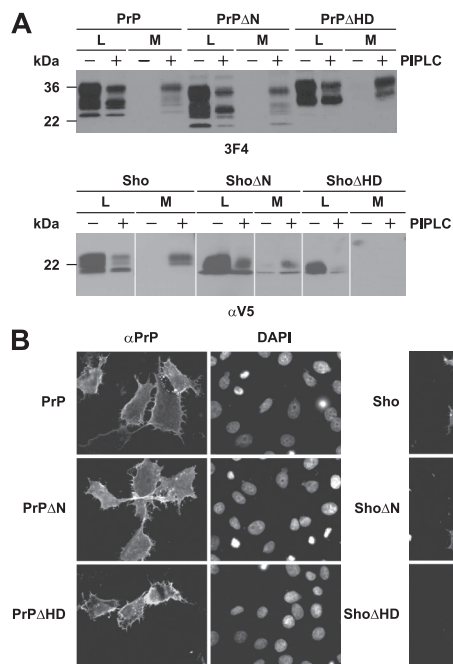


FIGURE 2. Sho and its mutants are tethered to the outer leaflet of the plasma membrane via a GPI anchor. *A*, Sho and the different mutants contain a C-terminal GPI anchor. Transiently transfected cells were incubated at 4 °C for 3 h with PIPLC to release GPI-anchored proteins from the cell surface (PIPLC+) or mock-treated (PIPLC-). PrP or Sho present in the cell lysates (L) or the cell culture supernatant (M) was analyzed by Western blotting. *B*, Sho and the different mutants are present at the outside of the plasma membrane. SH-SY5Y cells grown on coverslips were transiently transfected, and localization of the constructs indicated was analyzed by indirect immunofluorescence of non-permeabilized cells. Nuclei were stained with DAPI.

nificant amounts of Sho Δ HD in the cell culture medium after PIPLC treatment, using neither the V5 nor our newly generated polyclonal anti-Sho antisera. So far, we have not been able to unravel the underlying mechanisms for this phenomenon. However, when we performed indirect immunofluorescence of non-permeabilized cells, we were able to demonstrate localization of all constructs, including Sho Δ HD, at the outer surface of the plasma membrane (Fig. 2B). In sum, these data revealed that wild type Sho as well as Sho mutants devoid of the N-terminal domain or the internal HD are complex glycosylated and tethered to the outer leaflet of the plasma membrane via a GPI anchor.

Sho Protects Cells from Apoptotic Cell Death Induced by Excitotoxic Stress or the Expression of a Neurotoxic PrP Mutant—To evaluate the stress-protective capacity of Sho, we employed two different stress paradigms, exposition of cells to the excitotoxic glutamate and expression of the neurotoxic PrP mutant PrP Δ HD. To identify cells undergoing apoptosis, transiently transfected SH-SY5Y cells were analyzed by indirect immunofluorescence using an antibody specific for activated caspase-3. Please note that SH-SY5Y cells are characterized by low levels of endogenous PrP^C (Fig. 3A, right panel, pcDNA, 3F4). Consistent with previous findings (9), PrP^C was able to protect cells against excitotoxic cell death, whereas the deletion of the intrinsically disordered N-terminal domain lead to a loss of this activity (Fig. 3A). Similarly to PrP^C, full-length Sho displayed a stress-protective capacity, which was abolished by the deletion of the N-terminal domain (Fig. 3A). These findings indicate

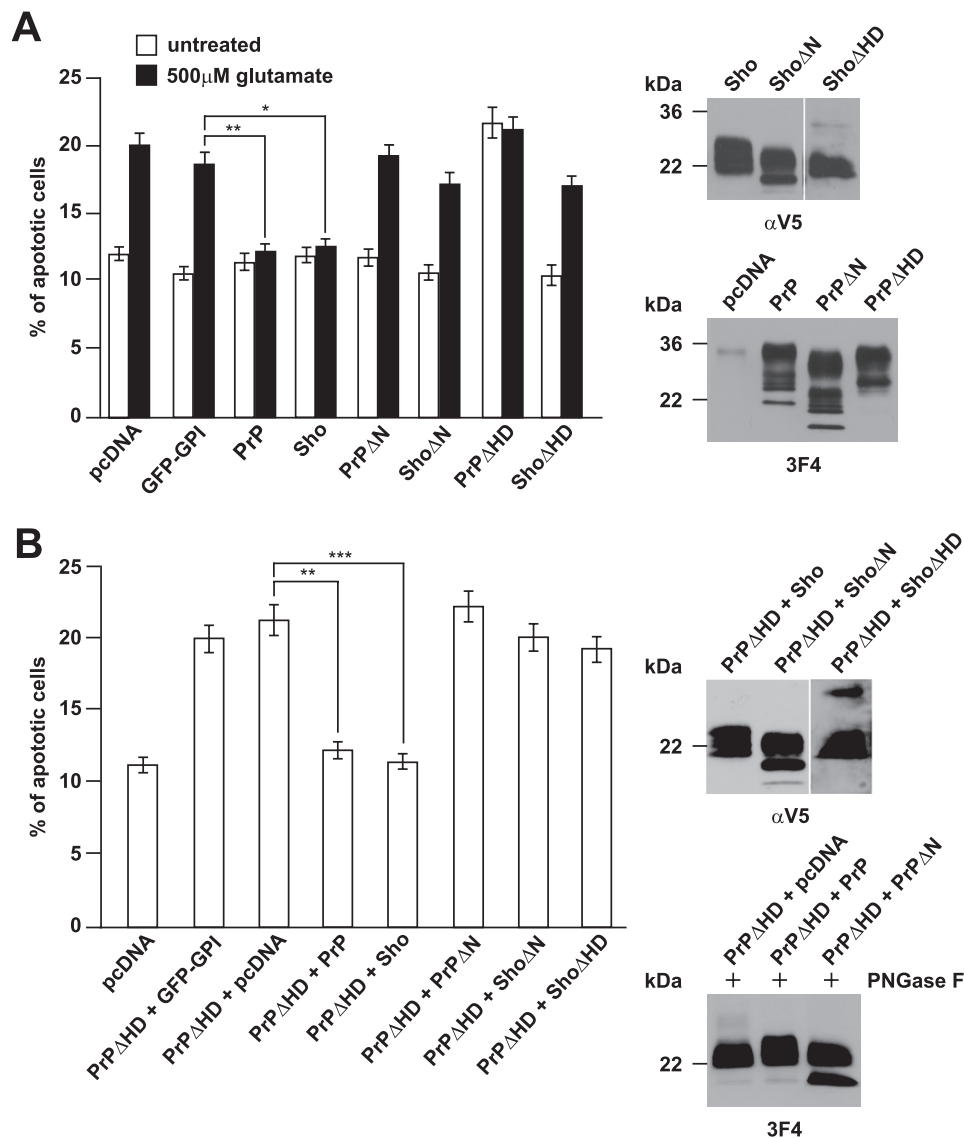


FIGURE 3. The N-terminal and the internal hydrophobic domain are necessary for the stress-protective activity of Sho. *A*, Sho protects against stress-induced apoptosis. SH-SY5Y cells expressing the constructs indicated were stressed with glutamate (500 μ M) for 3 h at 37 °C, fixed, and permeabilized, and activation of caspase-3 was analyzed by indirect immunofluorescence. To detect cells undergoing apoptosis, the number of activated caspase-3-positive cells out of at least 1100 transfected cells was determined in at least three independent experiments. The percentage of apoptotic cells among transfected cells is shown. Expression levels were analyzed by immunoblotting (*right panel*). *B*, expression of Sho interferes with toxic effects of PrP Δ HD. SH-SY5Y cells were transiently co-transfected with PrP Δ HD and the constructs indicated. Apoptotic cell death was determined as described under *A*. Expression levels were analyzed by immunoblotting (*right panel*). To specifically detect PrP Δ N, cell lysates were treated with PNGase F prior to the Western blot analysis (3F4). *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.0005$.

that deletion of the N-terminal domain of PrP or Sho had similar consequences. However, deletion of the internal HD had distinct effects. As previously documented in transgenic mice and cell culture models (9, 17–19), PrP Δ HD acquires a toxic activity (Fig. 3A). Sho Δ HD lost its stress-protective activity; however, in contrast to PrP Δ HD it did not induce cell death (Fig. 3A).

In the next step, we employed expression of PrP Δ HD as a toxic insult. As expected from previous studies, toxic effects of PrP Δ HD expression were alleviated by the co-expression of PrP^C (Fig. 3B). Corroborating earlier results with primary neurons (24), Sho was also able to suppress toxicity induced by PrP Δ HD (Fig. 3B). However, neither PrP Δ N nor Sho Δ N prevented apoptosis in cells expressing PrP Δ HD. Similarly,

Sho Δ HD could not interfere with the toxic effects of PrP Δ HD (Fig. 3B).

The Hydrophobic Domain Mediates Homodimerization of Sho—In a previous study, we could show that the HD mediates dimerization of PrP^C and is part of the dimer interface (9). Because the HDs of Sho and PrP are highly homologous (44.4%, Fig. 1B), we tested whether the HD of Sho has a similar activity. To stabilize a possible Sho dimer, we replaced serine 87 with cysteine (Fig. 4A). In case the HD is part of a dimer interface, cysteine 87 could form an intermolecular disulfide bond, which is stable in SDS buffers under non-reducing conditions. Thus, dimer formation can be analyzed by SDS-PAGE followed by Western blotting by preparing protein lysates with sample buffer devoid of reducing agents, such as 2-mercaptoethanol

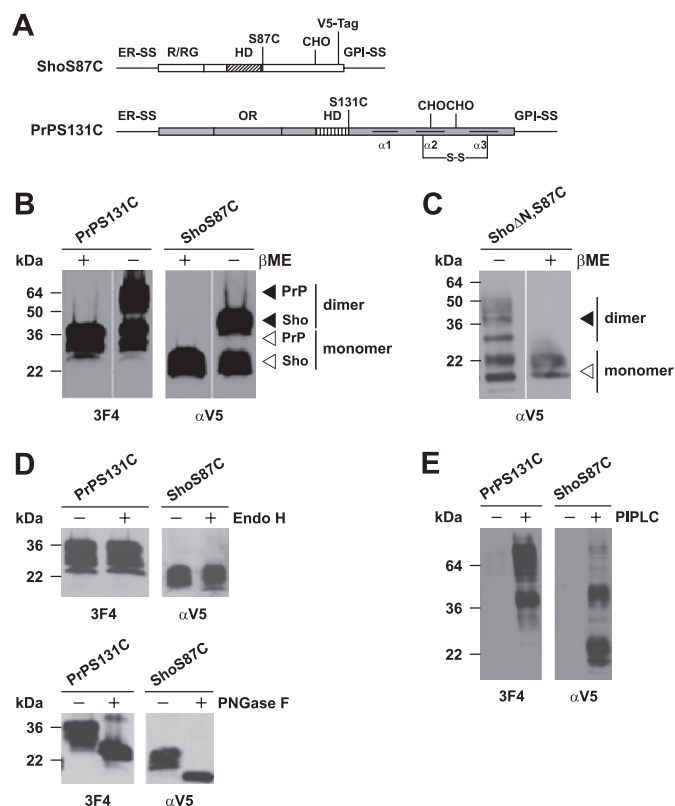


FIGURE 4. The internal hydrophobic domain promotes homodimerization of Sho. *A*, schematic presentation of the mutants used. *ER-SS*, endoplasmic reticulum signal sequence; *R/RG*, arginine- and glycine-rich basic repeats; *CHO*, *N*-linked glycosylation acceptor site; *S-S*, disulfide bridge; *GPI-SS*, glycosylphosphatidylinositol anchor signal sequence; *OR*, octarepeat region; α , α -helical region. *CHO*, *N*-linked glycosylation acceptor site. *B*, the hydrophobic domain is part of the dimer interface. SH-SY5Y cells were transiently transfected with PrPS131C or ShoS87C, and cell lysates were analyzed under reducing (+ β ME) or non-reducing (– β ME) conditions. *C*, the *N*-terminal domain is dispensable for dimer formation. Cells transiently expressing Sho Δ N,S87C were lysed and analyzed by Western blotting under either reducing (+ β ME) or non-reducing (– β ME) conditions. *D*, cysteine mutants are complex glycosylated. SH-SY5Y cells expressing the constructs indicated were lysed, and lysates were treated with Endo H (+) or PNGase F (+) or left untreated (–) prior to a Western blot analysis under reducing conditions. *E*, cysteine mutants are attached to the outer leaflet of the plasma membrane via a GPI anchor. Transiently transfected cells were incubated at 4 °C for 3 h with PIPLC to release GPI-anchored proteins from the cell surface (*PIPLC* +) or mock-treated (*PIPLC* –). PrP or Sho present in the cell culture supernatant was analyzed by Western blotting under non-reducing conditions.

(β -ME) or dithiothreitol (DTT). SH-SY5Y cells were transiently transfected with ShoS87C. As a control, PrPS131C was analyzed in parallel. Protein lysates were boiled in Laemmli sample buffer with or without 2-mercaptoethanol and analyzed by Western blotting. In line with earlier results, PrP dimers were detected in lysates prepared from PrPS131C-expressing SH-SY5Y cells when reducing agents were omitted from the sample buffer (Fig. 4*B*). Similarly, in extracts from cells expressing ShoS87C, a slower migrating species appeared with a molecular mass indicative of a Sho dimer (Fig. 4*B*). In the presence of 2-mercaptoethanol, the migration pattern of ShoS87C was identical to that of wild type Sho, indicating that the introduced cysteine residue has induced the formation of an intermolecular disulfide bond. To assess the role of the *N*-terminal domain in dimer formation, we deleted residues 30–56. As shown in Fig. 4*C*, Sho Δ N,S87C formed dimers similarly to

ShoS87C. Treatment of cell lysates with Endo H or PNGase F revealed that ShoS87C is modified with *N*-linked glycans of complex structure, similarly to wild type Sho (Fig. 4*D*). In addition, both ShoS87C and PrPS131C were released from the cell surface of live cells after incubation with PIPLC, revealing the presence of a *C*-terminal GPI anchor (Fig. 4*E*).

Next we tested whether dimer formation required *N*-linked glycosylation and occurs during transit through the secretory pathway. Transiently transfected cells expressing either PrPS131C or ShoS87C were cultivated in the presence of tunicamycin or brefeldin A, and dimer formation was analyzed by Western blotting. Indeed, homodimers of PrP or Sho could be detected under both conditions (Fig. 5*A*). These data indicated that dimer formation can obviously occur as soon as the proteins enter the endoplasmic reticulum lumen. However, we also addressed the possibility that dimer formation occurs *in trans* at the plasma membrane between Sho molecules located on adjacent cells. For this purpose, we generated a ShoS87C construct with a *C*-terminal HA tag instead of the V5 tag. Separately transfected cells expressing either ShoS87C-V5 or ShoS87C-HA were mixed and co-cultivated for an additional 24 h. The density was chosen to allow cell-cell contact. The co-immunoprecipitation analysis did not indicate the formation of trans-dimers (Fig. 5*B*). However, we cannot exclude the possibility that Sho trans-dimers can form *in vivo* under certain conditions.

The HDs of Sho and PrP are highly similar; thus, we analyzed whether they might promote formation of a mixed PrP/Sho dimer. Transiently transfected cells expressing both PrPS131C and ShoS87C were lysed, and Sho immunopurified with the V5 antibody. The immunopellet was then analyzed by Western blotting. Sho homodimers were efficiently detected with this approach; however, we were not able to show the formation of mixed PrP/Sho dimers (Fig. 5*C*).

The N-terminal Domain of Sho Can Restore Stress-protective Activity of PrP Δ N—The functional characterization of Sho mutants presented above revealed a critical role of the *N*-terminal domain for the stress-protective activity of Sho. Similarly, PrP Δ N lacks a stress-protective activity in cell culture and animal models (Fig. 3) (9, 15). To test for the intriguing possibility of a conserved function of the *N*-terminal domains of PrP^C and Sho, we fused the *N*-terminal domain of Sho to PrP Δ N (Fig. 6*A*). Expression and post-translational modifications of *N*-Sho·PrP-C were similar to that of wild type PrP or wild type Sho; the chimeric protein was complex glycosylated and tethered to the outer leaflet of the plasma membrane via a GPI anchor (Fig. 6, *B* and *C*). We then compared the stress-protective activity of *N*-Sho·PrP-C to that of PrP Δ N. As described above, we used two different toxic conditions: 1) expression of the neurotoxic PrP mutant PrP Δ H_D and 2) treatment of cells with the excitotoxin glutamate. Indeed, these experiments revealed that the *N*-terminal domain of Sho can restore the stress-protective capacity of PrP Δ N. Similarly to full-length Sho or full-length PrP, *N*-Sho·PrP-C interfered with apoptotic cell death of SH-SY5Y cells expressing PrP Δ H_D (Fig. 6*D*) or exposed to glutamate (Fig. 6*E*).

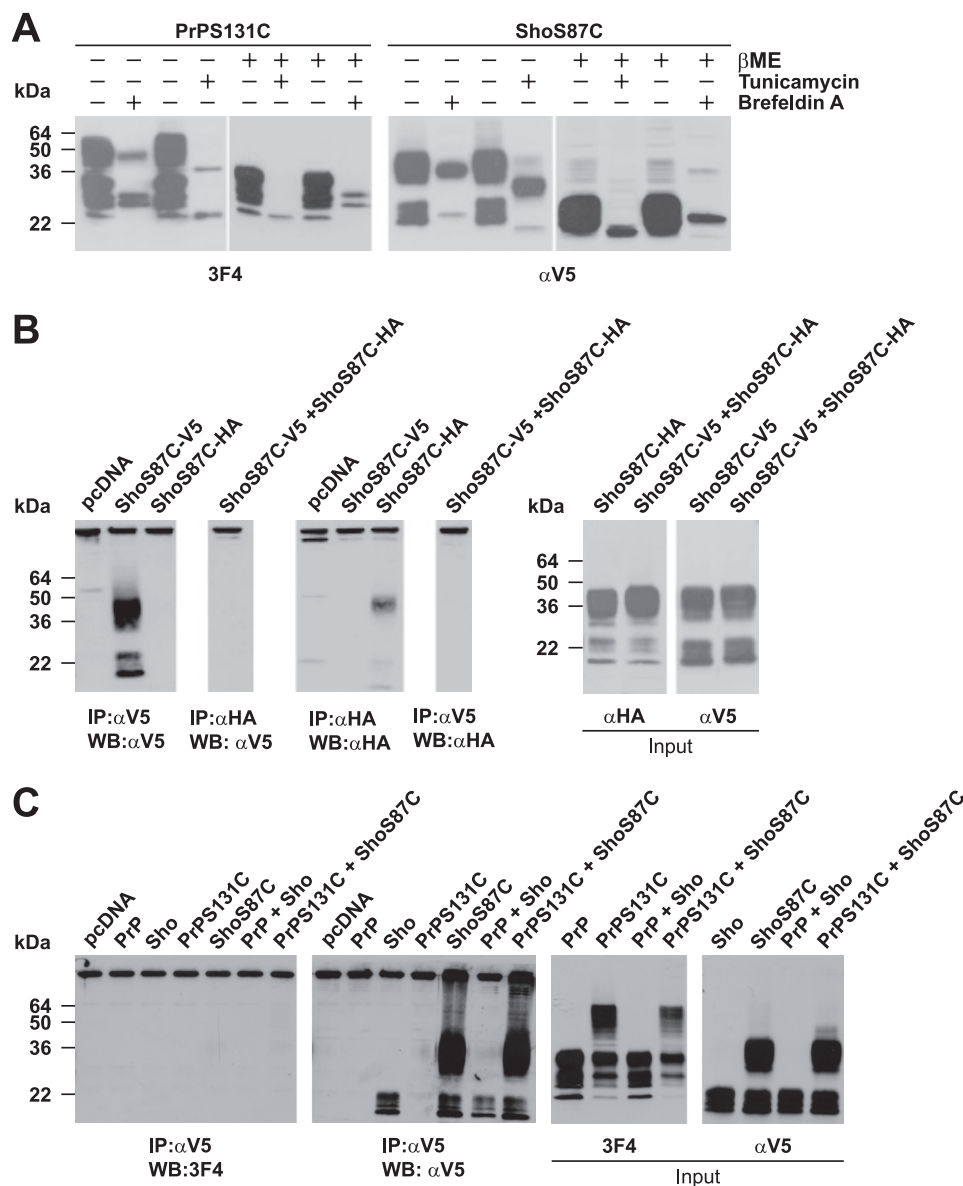


FIGURE 5. PrP and Sho homodimers are formed within the secretory pathway independently of N-linked glycosylation. A, transiently transfected SH-SY5Y cells were grown in the presence of tunicamycin or brefeldin A overnight. Protein extracts were prepared and analyzed under reducing (+ β ME) or non-reducing condition (– β ME) by immunoblotting. B, no evidence for the formation of Sho trans-dimers. Separately transfected cells expressing either ShoS87C-HA or ShoS87C-V5 were mixed and co-cultivated for 24 h. Cells were lysed, and Sho was immunoprecipitated (IP) under non-reducing conditions with an anti-HA or -V5 antibody. The immunopellet was analyzed by Western blotting (WB) using the anti-HA or -V5 antibody. Western blot analysis of the input is shown in the right panel. C, no evidence for the formation of a mixed PrP/Sho dimer. Transiently transfected cells co-expressing PrPS131C and ShoS87C-V5 were lysed, and Sho was immunoprecipitated under non-reducing conditions using the anti-V5 antibody. The immunopellet was then analyzed by Western blotting using the anti-PrP antibody 3F4 or the anti-V5 antibody. Sho and PrP present in the lysates prior to the immunoprecipitation were analyzed by immunoblotting (right panel, Input).

DISCUSSION

Sho is a highly conserved protein found from fish to humans. Although it has no overall sequence homology to PrP, both proteins contain a conserved internal hydrophobic domain and a C-terminal GPI anchor, two elements important for physiological and toxic activities of PrP. Our study presents experimental evidence that Sho and PrP are characterized by a conserved activity to protect cells against stress-induced cell death and suggests that both proteins might act on similar signaling pathways.

The Stress-protective Activity of Sho Is Dependent on the N-terminal Domain and the Internal Hydrophobic Domain—The first finding of our study was that the stress-protective activity of Sho is not restricted to counteracting the toxic effects

of PrP Δ HD, an artificial PrP mutant devoid of the internal HD. We employed glutamate as a physiologically relevant stressor to show that Sho can efficiently protect cells against excitotoxin-induced cell death. Deletion mutants revealed that the stress-protective activity of Sho and PrP seems to be dependent on similar domains, in particular the N-terminal and the internal HD. Sho Δ N and Sho Δ HD displayed a reduced stress-protective activity but are complex glycosylated and attached to the outer leaflet of the plasma membrane via a GPI anchor, indicating that the impaired activity is not due to incorrect cellular trafficking.

What could be the role of the N-terminal domain and the internal HD in promoting protective signaling? The N-terminal

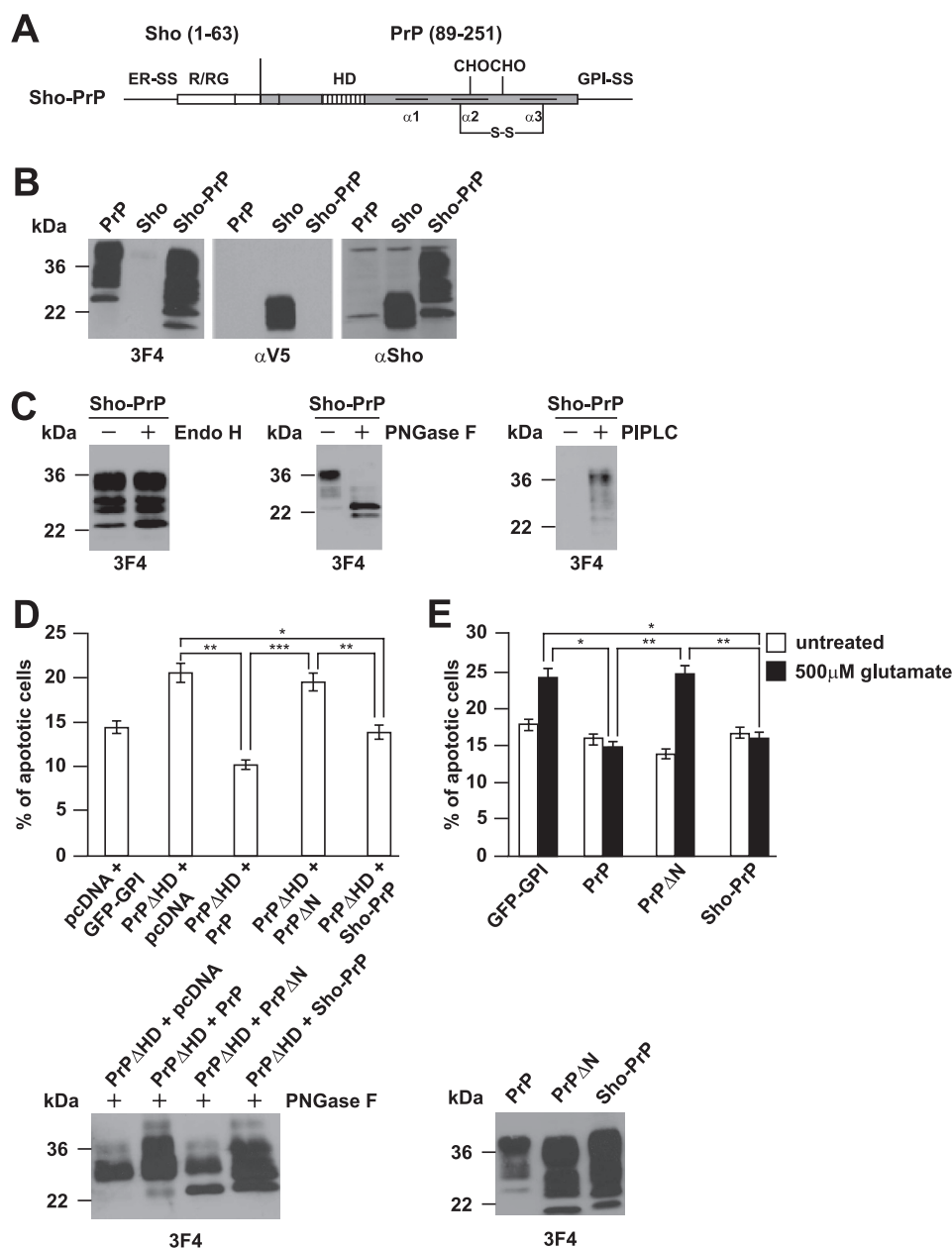


FIGURE 6. The N-terminal domain of Sho can functionally replace that of PrP. *A*, schematic presentation of the chimeric protein N-Sho-PrP-C (*Sho-PrP*). *ER-SS*, endoplasmic reticulum signal sequence; *R/RG*, arginine- and glycine-rich basic repeats; *S-S*, disulfide bridge; *GPI-SS*, glycosylphosphatidylinositol anchor signal sequence; α , α -helical region. *CHO*, N-linked glycosylation acceptor site. *B*, *Sho-PrP* is efficiently expressed in SH-SY5Y cells. Cell lysates from transiently transfected cells were analyzed by Western blotting. *C*, *Sho-PrP* is complex glycosylated and GPI-anchored. Lysates prepared from transiently transfected SH-SY5Y cells were treated with Endo H or PNGase F to analyze N-linked glycosylation. In parallel, transfected cells were incubated at 4 °C for 3 h with PIPLC to release GPI-anchored proteins from the cell surface (*PIPLC* +) or mock-treated (*PIPLC* -). *Sho-PrP* present in the cell culture supernatant was analyzed by Western blotting. *D*, expression of *Sho-PrP* interferes with toxic effects of PrP Δ HD. SH-SY5Y cells were transiently transfected with PrP Δ HD or PrP Δ HD and the constructs indicated. Cells undergoing apoptosis were analyzed as described in the legend for Fig. 3. The percentage of apoptotic cells among transfected cells is shown. Expression levels were analyzed by immunoblotting (*lower panel*). *E*, *Sho-PrP* protects against stress-induced apoptosis. SH-SY5Y cells expressing the constructs indicated were stressed with glutamate (500 μ M) for 3 h at 37 °C. Apoptotic cell death was determined as described in the legend for Fig. 3. Expression levels were analyzed by immunoblotting (*lower panel*). *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.0005$.

domain of PrP is intrinsically disordered, and it has been shown that intrinsically disordered domains are often involved in protein-protein interactions (30). Thus, it would be an attractive idea to assume that the N-terminal domains of PrP^C and Sho mediate interaction with a yet unknown co-receptor required for intracellular signal transmission. Interestingly, a circular dichroism analysis of recombinant Sho indicated that the whole protein might be unstructured (24). The HD is the only domain with significant sequence homologies between Sho and PrP^C.

Indeed, the HD promoted dimerization of both Sho and PrP^C and was part of the dimer interface. In this context, it is worth mentioning that dimerization is a common feature of many cell surface receptors; therefore, it can be speculated that dimer formation is involved in signal transmission of PrP^C- and Sho-dependent pathways. Notably, formation of both PrP and Sho homodimers is not dependent on N-linked glycosylation and already occurs during transit through the secretory pathway. Similarly, PrP and Sho mutants devoid of the N-terminal

domain are still capable of forming homodimers; however, they have an impaired stress-protective activity.

Distinct Activity of PrP Δ HD—Studies in transgenic mice revealed the unexpected finding that PrP can acquire a neurotoxic potential simply by deleting the internal hydrophobic domain (17–19). Importantly, the neurotoxic potential of PrP Δ HD is independent of the propagation of infectious prions, a phenomenon also seen for other neurotoxic PrP mutants (reviewed in Ref. 16). Although the underlying mechanisms of PrP Δ HD-induced toxicity are still elusive, co-expression of wild type PrP^C completely prevents toxic effects of PrP Δ HD. Based on this intriguing observation, it has been hypothesized that the stress-protective signaling of PrP^C and the neurotoxic signaling of PrP Δ HD are transmitted through a common co-receptor, which remains to be identified (9, 17–19). Interestingly, co-transfection experiments with PrP-deficient cerebellar granule neurons indicated that Sho has a PrP^C-like activity to alleviate toxic effects of PrP Δ HD expression (24). We have been able to recapitulate the toxic activity of PrP Δ HD expression in our cell culture model and to demonstrate the protective activity of PrP and Sho against PrP Δ HD-induced toxicity. In addition, we could show that Sho Δ HD lost its ability to protect against stress-induced cell death; however, Sho Δ HD did not acquire a toxic activity, at least not under the experimental conditions tested. Thus, it appears that Sho and PrP share a stress-protective activity; however, the ability to adopt a toxic conformation seems to be specific for PrP. In this context, it is tempting to speculate that this ability might be linked to the unique feature of PrP^C to convert into PrP^{Sc}.

The N-terminal Domain of Sho Can Functionally Replace That of PrP—Our study emphasized a similar activity of Sho and PrP to protect cells against excitotoxin- and PrP Δ HD-induced toxicity and revealed a crucial role for the N-terminal domain proximal to the HD. Both PrP Δ N and Sho Δ N showed a significantly reduced stress-protective activity. To address the possibility that both proteins interact with similar co-factors through their N termini, we fused the N-terminal domain of Sho to PrP Δ N. The chimeric protein N-Sho-PrP-C had a stress-protective activity similar to that of WT Sho or WT PrP, indicating that the N-terminal domain of Sho could functionally replace that of PrP. There is neither sequence homology between the N-terminal domains of Sho and PrP nor an indication that the N-terminal domain of Sho is able to bind copper. The only similarity seems to be that both domains are intrinsically disordered. Based on the idea that intrinsically disordered domains are involved in protein-protein interactions, it appears plausible to assume that the N-terminal domain of PrP and Sho mediates interaction with the same co-receptor, required for stress-protective signaling. Further studies are now required to identify the putative co-receptor and signaling pathway(s) activated by PrP and Sho.

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