

Mapping the interaction site of prion protein and Sho

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Abstract The cellular prion protein (PrP^C) is a highly conserved protein among mammals and is considered to have important cellular functions. Despite decades of intensive research, however, the physiological function of PrP^C remains unclear. Sho (Shadoo, shadow of prion protein) and PrP^C have similar N-terminals, which suggests that the two proteins share biological functions. Using truncation mutants of both proteins and yeast two-hybrid analysis, with validation by co-immunoprecipitation and surface plasmon resonance (SPR), we have identified an interaction between Sho 61–77 and PrP^C 108–126 domains. This indicates that Sho may play a role in the physiological function of PrP^C and prion pathogenesis.

Keywords Prion protein · Sho · Yeast two-hybrid · Surface plasmon resonance · Co-immunoprecipitation · Interaction

Introduction

Prion diseases are infectious neurodegenerative diseases of mammals that include bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goat, and Creutzfeldt-Jakob disease (CJD) in humans. Prion diseases have been subjected to much recent attention due to the epizootics of BSE and the subsequent emergence of its human form, variant CJD (vCJD). A central event in prion diseases is the

conversion of the normal cellular form of the prion protein (PrP^C) into a misfolded pathological amyloid form called the scrapie isoform (PrP^{Sc}). The molecular mechanism of the conversion still remains unclear. There has been some speculation about a host chaperone protein, named ‘protein X’, involved in conversion of PrP^C into PrP^{Sc} [1, 2]. PrP^{Sc} has several structural and biochemical differences from PrP^C, including a high percentage of β -sheet, partial resistance to proteolysis, insolubility in detergents, and a propensity to polymerize into amyloid-like fibrils [3]. PrP^C is absolutely required for disease progression, as PrP^C knockout (Prnp^{0/0}) mice do not succumb to disease and do not propagate infectivity following intracerebral challenge with infectious prions [4, 5].

The biological function of PrP^C remains largely unknown. However, the many lines of mice lacking PrP^C that have been generated by homologous recombination develop normally, without severe pathologies in later life [6]. There are suggestions that PrP^C may be involved in one or more of the following: neurotransmitter metabolism, immune cell activation [7, 8], cell adhesion, signal transduction, copper metabolism, antioxidant activity, or programmed cell death [9].

PrP^C is attached to the outer cell membrane by a glycosylphosphatidylinositol (GPI) anchor and is predominantly expressed in cells of the central nervous system (CNS) and several other cell types [10]. Investigators have hypothesized that PrP^C exerts its function via interaction with other cell-surface components. A number of proteins with a high affinity for PrP^C have been identified by different methods such as yeast two-hybrid, co-immunoprecipitation, and cross-linking experiments. These include membrane proteins (receptors, enzymes, caveolin-1, Na–K-ATPase, and a potassium channel), cytoplasmic proteins (components of the cytoskeleton, heat-shock

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proteins, and adaptor proteins involved in signaling), and even the nuclear protein CBP70 [6, 11, 12]. Although the identification of numerous candidate binding partners for PrP^C has hinted at possible cellular roles, none of them could serve as the chaperone ‘protein X’.

Efforts have been made to identify proteins interacting with PrP^C that could provide new insights into its physiological functions and pathological role. There is therefore a growing interest in identifying proteins that could have an influence on the susceptibility of animals to prion diseases. Another avenue of research into the biology of PrP^C has recently been opened up with the discovery of two paralogs, Doppel [13] and Sho [14], although several lines of evidence argue against the involvement of the former in prion disease [12, 15, 16]. Doppel is a testis-expressed protein which appears to have a critical role in the proper functioning of the male reproductive system [13, 17]. *SPRN*, the gene encoding Sho, has been identified by comparative gene analysis. This gene and its encoded protein have several characteristics suggestive of a role in prion biology [18]. Since PrP^C and Sho share many common features, we investigated whether PrP^C interacts with Sho.

The yeast two-hybrid system is a powerful tool to detect protein–protein interactions that might lead to the identification of PrP^C-interacting proteins. Here, we used this approach to demonstrate that PrP^C interacts with Sho, suggesting that Sho may play a role in the physiological function of PrP^C and prion pathogenesis.

Materials and methods

Construction of plasmids

The gene encoding mature mouse PrP^C (23–231) was amplified by polymerase chain reaction (PCR) using mouse genomic DNA as a template, and then cloned into the pSos vector (Stratagene) via *Bam*HI and *Sal*I restriction sites, yielding pSos/PrP^C (23–231) as bait plasmid. The mouse Sho (25–122) cDNA fragment was amplified from mouse genomic DNA and cloned into the pMyr vector (Stratagene) by *Eco*RI and *Sal*I sites to get pMyr/Sho(25–122). To delineate the regions involved in the association of PrP^C and Sho, a series of constructs expressing amino acid residues 23–90, 23–108, 23–126, 23–130, 23–142, 23–153, 23–159, 23–163, 23–178, 23–192, 23–198, 126–231, and 108–231 of PrP^C and 25–61, 25–77, 25–85, 25–100, 25–112, 77–122 and 61–122 of Sho were generated to get pSos/PrP^C(23–90), pSos/PrP^C(23–108), pSos/PrP^C(23–126), pSos/PrP^C(23–130), pSos/PrP^C(23–142), pSos/PrP^C(23–153), pSos/PrP^C(23–159), pSos/PrP^C(23–163), pSos/PrP^C(23–178), pSos/PrP^C(23–192), pSos/PrP^C(23–198), pSos/PrP^C(126–231),

pSos/PrP^C(108–231), pMyr/Sho(25–61), pMyr/Sho(25–77), pMyr/Sho(25–85), pMyr/Sho(25–100), pMyr/Sho(25–112), pMyr/Sho(77–122) and pMyr/Sho(61–122).

For co-immunoprecipitation experiments, pcDNA3.1(-) and pcDNA3.1(-) myc-His plasmids (Invitrogen) were used. The gene encoding mature mouse PrP^C (23–231) was amplified from mouse genomic DNA by PCR and digested with *Eco*RI and *Bam*HI. The *Eco*RI and *Bam*HI fragment was cloned into the pcDNA3.1(-) vector, yielding pcDNA3.1/PrP^C (23–231). Similarly, the gene encoding mature Sho (25–122) was amplified from mouse genomic DNA and digested with *Eco*RI and *Bam*HI. The fragment was then cloned into pcDNA3.1(-) myc-His vector to obtain pcDNA3.1/myc-Sho (25–122).

Yeast two-hybrid assay

To determine whether PrP^C physically interacts with Sho in vivo and further identify the site of interaction between PrP^C and Sho, we utilized the CytoTrap yeast two-hybrid system (Stratagene). The CytoTrap two-hybrid system utilizes a yeast host strain (Cdc25H) harboring a temperature-sensitive (ts) allele of CDC25, which is an essential guanine nucleotide exchange factor of the RAS-signaling pathway. The plasmid pMyr expresses a fusion partner which is anchored at the plasma membrane via myristoylation. The effector component of the CytoTrap system is provided by a second plasmid pSos (expressing the human hSos protein), which activates Ras and the Ras-dependent signal transduction pathway only if it is localized at the plasma membrane. If two components associate at the plasma membrane due to a protein–protein interaction, a signal transduction cascade is activated and monitored by a growth assay. The bait plasmid, pSos-PrP^C or pSos-PrP^C mutants, and the prey plasmid, pMyr vector containing Sho or Sho mutants, were used to co-transform yeast strain cdc25H using the standard lithium acetate transformation procedure, as described in the CytoTrap kit (Stratagene). Transformants were grown on synthetic complete media lacking uracil and leucine (–Ura/–Leu), for 7 days at 25°C, and replica plated onto synthetic complete media (–Ura/–Leu) with galactose to induce expression of the myristoylation sequence-tagged proteins, which are anchored to the cell membrane. Interactions were generally detected as yeast cell growth after incubation for 10 days at 37°C on galactose plates. Self-interaction between pSos/MafB and pMyr/MafB was tested as positive controls. Vector combinations for negative binding interactions were empty pSos versus empty pMyr, pSos/PrP^C(23–231) versus empty pMyr, and pMyr/Sho(25–122) versus empty pSos. Only those clones growing on galactose media at the restrictive temperature of 37°C after 10 days were defined as “true” positives. The experiments were performed three times.

Co-immunoprecipitation

For co-immunoprecipitation, HEK293T cells (5×10^5) were co-transfected with 10 μg of each plasmid DNA (pcDNA3.1/PrP^C(23–231) and pcDNA3.1/myc-Sho (25–122)) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h post-transfection, the cells were washed with PBS, resuspended in an ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM MgCl₂, 5 mM EGTA, 0.01% bovine serum albumin, 1 mM dithiothreitol, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin), and left to lyse on ice for 20 min. They were then centrifuged at 12,000 g for 15 min at 4°C. Supernatants were recovered, and protein concentration was measured by the Bradford assay. One hundred μg total protein was incubated with either 4 μg of anti-myc antibody (Beyotime, Haimen, China) or anti-PrP antibody (SPI-BIO, Massy, France) for 2 h at 4°C and then with protein A-Sepharose beads (GE Healthcare) for 1 h at 4°C. The beads were washed with ice-cold lysis buffer, and bound proteins were analyzed by SDS-PAGE.

Surface plasmon resonance assay

Interaction assays between PrP^C and Sho were performed on a BIAcore 3000 (BIAcore AB, Uppsala, Sweden) instrument using HBS-EP (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3 mM EDTA and 0.005% (v/v) surfactant P20) as the running buffer. All experiments were performed at room temperature. PrP^C and Sho were respectively expressed with an N-terminal His-tag and purified by nickel-affinity chromatography (Ni-NTA-agarose, Qiagen), following the manufacturer's instructions. PrP^C was covalently immobilised using amine coupling on a BIAcore CM5 carboxymethyl dextran sensor chip by the amine coupling method as specified by the manufacturer (BIAcore). Interactions were detected as changes in the SPR response, which is proportional to a change in mass at the sensor chip surface and therefore represents binding of analyte. Surfaces were regenerated using a 2 min pulse of 50 mM glycine pH 2.7 to remove bound protein before each analysis.

Results

PrP^C and Sho interaction in a yeast two-hybrid system

To investigate the interaction between PrP^C and Sho, binding between PrP^C(23–231) and Sho(25–122) was tested by the CytoTrap two-hybrid system analysis. As shown in Fig. 1, PrP^C interacted with Sho.

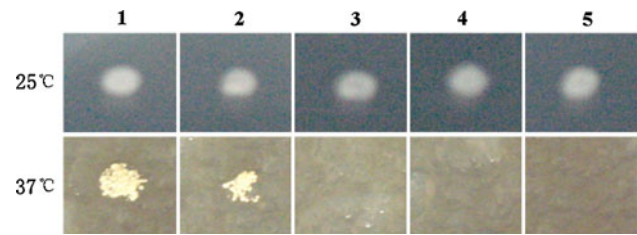


Fig. 1 PrP^C and Sho association in vivo using the CytoTrap two-hybrid system. Various bait (pSos) and prey (pMyr) plasmids were used for co-transformation of cdc25H yeast cells, representing a positive control (lane 1 (pSos/MafB + pMyr/MafB)) and negative controls (lane 3 (pSos/PrP^C(23–231) + pMyr), lane 4 (pSos + pMyr/Sho(25–122)), lane 5 (pSos + pMyr)). cdc25H transformants were spotted on glucose medium at 25°C (top panel) and on galactose medium at 37°C (bottom panel). Yeast transformants expressing PrP^C(23–231) and Sho(25–122) grew efficiently on glucose medium at 25°C (top panel, lane 2) and on galactose medium at 37°C (bottom panel, lane 2)

Verification of PrP^C–Sho interaction using co-immunoprecipitation and surface plasmon resonance

To investigate the interaction of PrP^C with Sho in a cellular environment, we performed immunoprecipitation experiments in HEK293T cells transfected with pcDNA3.1/PrP^C(23–231) and pcDNA3.1/myc-Sho (25–122). PrP^C and Sho-myc in cell extracts were incubated with an anti-myc mAb, and immune complexes were captured on protein A-agarose. The antibody–protein complexes were then resolved by 10% SDS–PAGE and subjected to Western blotting using anti-PrP antibodies. PrP^C was co-immunoprecipitated with Sho-myc by the anti-myc antibodies (Fig. 2, lane 1). In contrast, PrP^C was not precipitated by the anti-myc antibody when Sho-myc was absent from the reaction mix (Fig. 2, lane 2). Similarly, Sho-myc could be co-immunoprecipitated with PrP^C by the anti-PrP antibody (Fig. 2, lane 4) and it was not immunoprecipitated by the anti-PrP antibody when PrP^C was absent from the reaction mix (Fig. 2, lane 5). These results indicate that PrP^C interacted with Sho in vitro.

To further verify the interaction between PrP^C and Sho, real-time biomolecular interaction analysis using SPR on a BIAcore apparatus (BIAcore, Uppsala, Sweden) was performed. Partially purified recombinant PrP^C expressed in *E. coli* was immobilized on a sensor surface. Immunoaffinity-purified Sho preparations were passed over the immobilised proteins and interactions were detected as changes in the SPR response. Figure 3 shows that Sho specifically binds to PrP^C in a concentration dependent manner.

Mapping the interacting domains of PrP^C and Sho

To examine the regions of PrP^C and Sho responsible for their binding to each other, a series of deletion mutants

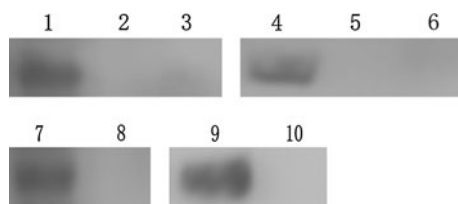


Fig. 2 Co-immunoprecipitation of PrP^C and Sho. Lysates were immunoprecipitated with either anti-myc antibody (lane 1, 2 and 3) or anti-PrP antibody (lane 4, 5 and 6). The antibody-protein complexes were resolved in 10% SDS-PAGE and subjected to immunoblot analysis by either anti-PrP antibody (lane 1, 2 and 3) or anti-myc antibody (lane 4, 5 and 6). PrP^C was co-immunoprecipitated with Sho-myc by the anti-myc antibody (lane 1) and it was not immunoprecipitated by the anti-myc antibody when Sho-myc was absent from the reaction mix (lane 2). Similarly, Sho-myc was co-immunoprecipitated with PrP^C by the anti-PrP antibody (lane 4) and it was not immunoprecipitated by the anti-PrP antibody when PrP^C was absent from the reaction mix (lane 5). Lane 3 and 6 are negative control. 10% of the input lysates was also immunoblotted and probed with antibodies to show the expression level of those proteins (lane 7, PrP^C; lane 9, Sho; lane 8 and 10, negative control)

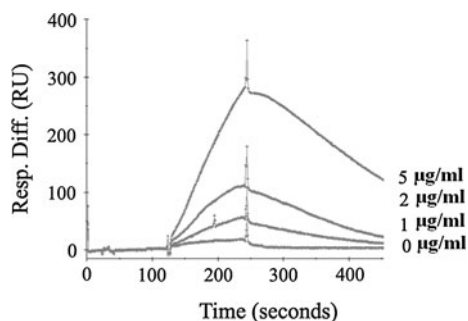


Fig. 3 Interaction between PrP^C and Sho detected by BIAcore 3000. Representative sensorgrams showing SPR responses from immobilized PrP^C following the introduction of Sho. Values plotted represent the difference between the separate measurements from the PrP^C sensor surfaces, over which the Sho protein solutions flowed. An increase in SPR response indicates binding of analyte to the PrP^C

were constructed (Fig. 4) and the interactions were assayed using the yeast two-hybrid system. Whereas all mutants except PrP^C23–108, PrP^C23–90, and PrP^C126–231 interacted with Sho; i.e., deletion of Sho from aa 61 to aa 77 abolished the PrP^C interaction. Thus, the interaction site of PrP^C is 108–126 and of Sho, 61–77.

Discussion

In this work, we describe an interaction between PrP^C and Sho using a CytoTrap yeast two-hybrid system. The CytoTrap system enables researchers to study protein interactions that cannot be assayed by using conventional two-hybrid or interaction trap systems. These include proteins that are transcriptional activators or repressors,

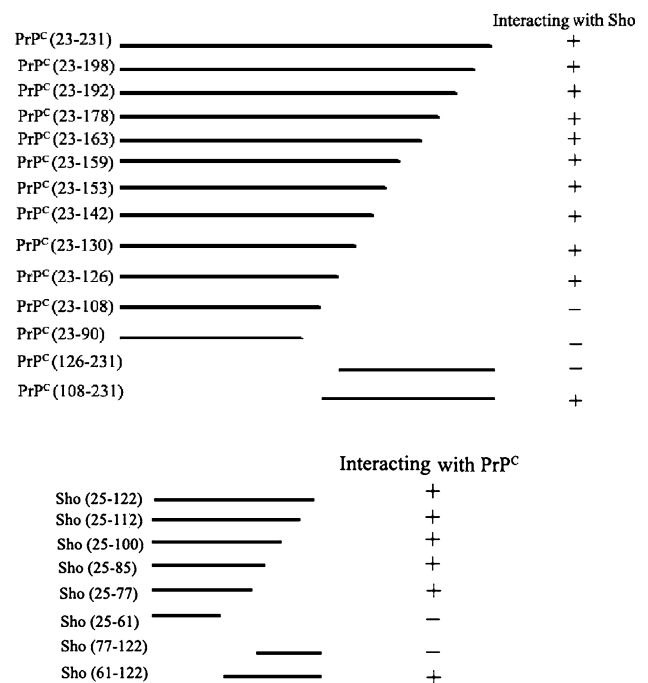


Fig. 4 Mapping of the domains for PrP^C–Sho interaction. Schematic representations of PrP^C, Sho, and their deletion mutants are illustrated. Yeast two-hybrid results are shown at the right

proteins that require post-translational modification in the cytoplasm, and proteins that are toxic to yeast in conventional two-hybrid systems. At the cellular membrane, PrP^C and Sho, together with other GPI-anchored proteins, are localized in specialized domains known as lipid rafts, which are rich in cholesterol and sphingomyelin [19].

The results of the two-hybrid assay showing PrP^C–Sho interaction were confirmed by co-immunoprecipitation and SPR. One item of paramount importance in immunoprecipitation experiments is the choice of detergent conditions to allow for weak and transient protein–protein interactions while avoiding artificial or nonspecific interactions. SPR-based sensing has been widely used for studies on biomolecular interactions [20] and has been applied to the detection of pathogens, toxins, proteins and nucleotides. SPR-based biosensor detection offers the advantages of monitoring biomolecular interaction in rapid and real-time without the need for labels, and it is an automated system that improves accuracy, high sensitivity and reliability [21, 22].

Sho is a *bona fide* neuronal glycoprotein and is encoded within a single exon of gene *SPRN* that is widely conserved in nature, being present in the genomes of lower organisms such as zebrafish all the way up to rodents and primates. Sho resembles the N-terminal domain of PrP^C but lacks the C-terminal α -helices and β -sheet regions of the latter. PrP^C has an octarepeat region, while Sho has a series of N-terminal charged tetrarepeats rich in glycine, serine, alanine and arginine. One feature of the octarepeat region

of PrP^C is the binding of copper [23], which might be triggering a copper-mediated protein–protein interaction. Copper-induced aggregation has also been observed in Alzheimer's disease [24]. However, whether or not the tetrapeptide region within the Sho protein is involved in metal-induced dimerization is unknown.

In contrast to PrP^C [25], Sho failed to interact with itself in the yeast two-hybrid system, suggesting that both proteins have a different self-interacting behaviour probably due to significant structural differences.

Murine Sho has been predicted to be a 98 residue protein with N- and C-terminal signal sequences mirroring those in PrP^C [12]. Sho contains a GPI anchor, N-glycosylation at one or two sites and a cleavage event likely positions the N-terminal to the hydrophobic tract. The bulk of the homology between Sho and PrP^C is found within the hydrophobic tract. Recent work has demonstrated that Sho has a number of biochemical and cell biological properties also exhibited by PrP^C. In Prnp^{0/0} cerebellar granular neurons (CGNs), Sho transgenes were PrP^C-like in their ability to counteract neurotoxic effects of either Doppel or ΔPrP. Watts et al. [12] infer that the center of both PrP^C and Sho comprises a functionally conserved and ancient activity domain contributing to neuroprotection. In PrP^C-deficient areas of the brain, Sho may be particularly important in providing a PrP-like activity. Since the lack of PrP^C in Prnp^{0/0} animals does not lead to obvious physiological defects that could provide clues as to its role, Sho might possibly shed some light on the former's possible functions. Beck et al. [26] justified the functional genetic characterisation of *SPRN* and supported the involvement of Sho in prion pathobiology. From our observations, we can also propose that PrP^C directly binds to Sho, an association not involving additional factors. Our results also indicate that the association of PrP^C and Sho is through a specific binding site. No binding partner for Sho has yet been reported, although there is a potential interaction with stress-inducible protein 1 since its binding site in PrP^C is well-conserved in Sho [27].

In conclusion, we have described what appears to be a specific interaction between PrP^C and Sho. Whether this association can prevent the PrP^C from converting to fibril and neurotoxicity is unknown. Our future studies will focus on this, however, since it may be a key step for both an understanding of prion disease pathogenesis and the development of therapeutics.

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