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RESEARCH PAPERS

Identification of novel putative-binding proteins for cellular prion protein and a specific interaction with the STIP1 homology and U-Box-containing protein 1

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ABSTRACT. Prion diseases involve the conversion of the endogenous cellular prion protein, PrP^{C}, into a misfolded infectious isoform, PrP^{Sc}. Several functions have been attributed to PrP^{C}, and its role has also been investigated in the olfactory system. PrP^{C} is expressed in both the olfactory bulb (OB) and olfactory epithelium (OE) and the nasal cavity is an important route of transmission of diseases caused by prions. Moreover, $Prnp^{-/-}$ mice showed impaired behavior in olfactory tests. Given the high PrP^{C} expression in OE and its putative role in olfaction, we screened a mouse OE cDNA library to identify novel PrP^{C}-binding partners. Ten different putative PrP^{C} ligands were identified, which were involved in functions such as cellular proliferation and apoptosis, cytoskeleton and vesicle transport, ubiquitination of proteins, stress response, and other physiological processes. In vitro binding assays confirmed the interaction of PrP^{C} with STIP1 homology and U-Box containing protein 1 (Stub1) and are reported here for the first time. Stub1 is a co-chaperone with ubiquitin E3-ligase activity, which is associated with neurodegenerative diseases characterized by protein misfolding and aggregation. Physiological and pathological implications of PrP^{C}-Stub1 interaction are under investigation. The PrP^{C}-binding proteins identified here are not exclusive to the OE,

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suggesting that these interactions may occur in other tissues and play general biological roles. These data corroborate the proposal that PrP^C is part of a multiprotein complex that modulates several cellular functions and provide a platform for further studies on the physiological and pathological roles of prion protein.

KEYWORDS. prion, yeast two-hybrid, olfactory epithelium, Stub1, protein interaction, CHIP

ABBREVIATIONS. PrP, prion protein; CHIP, C-terminus of Hsc70-interacting protein; OB, olfactory bulb; OE, olfactory epithelium; OSN, olfactory sensory neurons; Stub1, STIP1 homology and U-Box containing protein 1; TSE, transmissible spongiform encephalopathies; STI1, stress-inducible protein 1

INTRODUCTION

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy (BSE) in cattle. These diseases involve the conversion of the endogenous cellular prion protein, PrP^C, into a misfolded and infectious isoform named PrP^{Sc}.[1,2]

PrP^C is predominantly an extracellular glicosylphosphatidilinositol (GPI)-anchored glycoprotein that is expressed in most tissues and is enriched in the central nervous system.[3] Despite intensive research and the fact that PrP^C is conserved across different species, a consensus about its physiological function has not yet been reached.[2,4] Several functions have been attributed to PrP^C, including stress and behavior, sleep-wake cycle, memory, neuritogenesis, neuroprotection, and cellular adhesion.[2] Many of these roles were elucidated with the discovery of PrP-binding partners using different biophysical assays such as conventional yeast two-hybrid screening, co-immunoprecipitation, and other methods. These findings suggest that PrP^C may serve as a scaffolding protein for the assembly of signaling modules and therefore it must be related to a variety of cellular processes, rather than having a specific function.[2,5]

The physiological role of PrP^C has also been investigated in the olfactory system. PrP^C is expressed in different areas of the olfactory system including the olfactory epithelium (OE) and the olfactory bulb (OB), and is localized in the axons of both olfactory sensory neurons (OSNs) and mitral cells.[6] Interestingly, $Prnp^{-/-}$ mice showed impaired behavior in olfactory tests and have altered electrophysiological activity in the dendrodendritic synapse in the olfactory bulb. Both the behavioral and electrophysiological deficits observed in $Prnp^{-/-}$ mice were rescued by transgenic neuronal-specific expression of PrP^C.[7] It was reported that the OE from $Prnp^{-/-}$ mice showed no alterations in the odorant-evoked electro-olfactogram, suggesting that the PrP^C plays an olfactory function in the OB, rather than in the OE.[7] However, PrP^C expression in olfactory sensory neurons, both in dendrites and cell bodies (in a lower abundance) and in axons (more abundantly) is evident[6,8] and suggests that this protein should play a physiological role in these sensory cells. Moreover, the OE has been previously demonstrated to be a site of prion infection in humans[9] and animals.[10]

We performed a yeast two-hybrid screen to address the physiological roles of PrP^C using PrP^C as bait. Given the high PrP^C expression in OE and its putative role in olfaction, we screened a mouse OE cDNA library, in attempt to identify novel PrP^C-binding partners. Ten different putative PrP^C-binding proteins were identified to provide insights into the physiological and pathological functions of PrP in OE and other tissues. The PrP^C interaction with STIP1 homology and U-Box containing protein 1 (Stub1) was also confirmed by in vitro binding assays and are reported here for the first time. Stub1 functions both as a molecular

co-chaperone, and as an ubiquitin E3 ligase protein.[11] Moreover, Stub1 is associated with several neurodegenerative diseases characterized by protein misfolding and aggregation.[12] Hence, the interaction between PrP^C and Stub1, identified in our study, may occur in vivo and modulate PrP^C stability, which is implicated in the PrP^{Sc} conversion.

RESULTS

Identification of PrP^C-Interacting Proteins by Yeast Two-Hybrid Screen

We conducted a yeast two-hybrid screening of an OE cDNA library to identify new interactors for the cellular prion protein using PrP^C as bait. Fifty-three clones were isolated for both *LEU2* and *Lac-z* reporter genes. These clones were sequenced and their identities are listed in **Table 1**. Eighteen out of the total clones had their insert sequence in the correct frame. Most of the putative ligands were found in one single positive clone, except for a few that were found in 2 (*Srpk2, Pcsk5, Stub1*) or 4 (*Dynlt1b*) different clones.

In order to confirm these interactions, we performed cross-mating assays. As shown in **Figure 1**, 10 of the interactions were validated, i.e., the reporter gene (β-galactosidase) was only expressed when diploid yeasts contained both the target and bait vectors.

Additional information about these 10 PrP^C-binding proteins is given in **Table 2**. Several inserts contain most of the coding region sequence, but only the clones corresponding to Dynlt1b have the entire coding sequence.

The identified PrP^C ligands are involved in many functions such as cellular proliferation and apoptosis, cytoskeleton and vesicle transport, ubiquitination of proteins, stress response, and other physiological processes (**Table 2**). Interestingly, these putative PrP^C-binding proteins are not exclusive to the OE, suggesting that these interactions may also occur in other tissues and play general biological functions.

Validation of PrP^C Interaction with Stub1 Using In Vitro Binding Assays

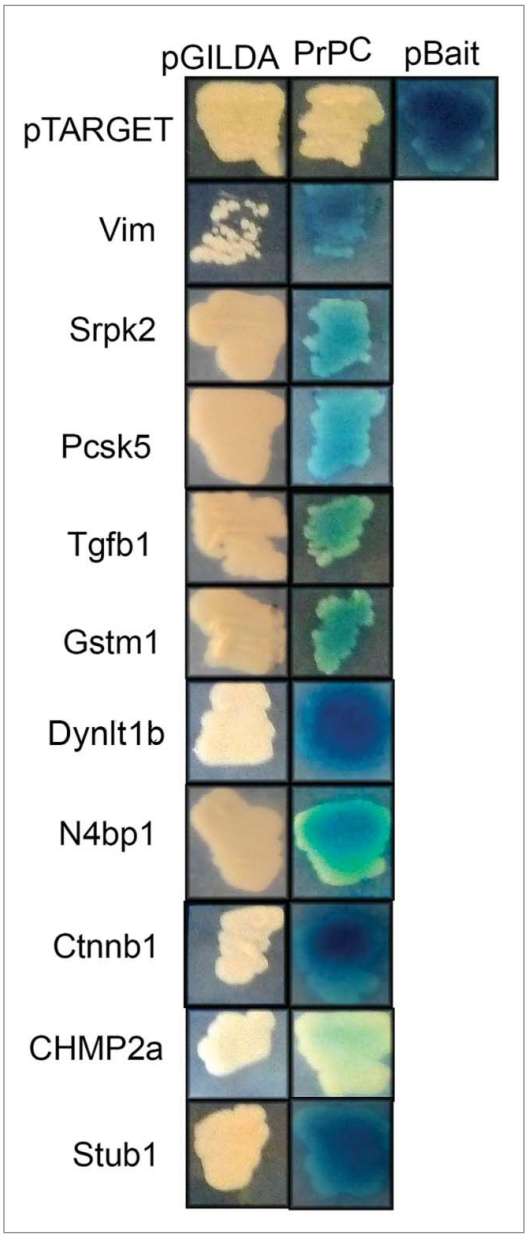
Out of the 10 ligands, the following 4 showed strong interaction (based on the intensity of blue color) with PrP^C: vimentin (Vim),

TABLE 1. Gene identities obtained from yeast two-hybrid screening

Gene description	Symbol	Accession#	Gene ID	# of clones obtained
Dynein light chain Tctex type 1 D	Dynlt1b	NM_009342.2	42476289	4
NEDD4 binding protein1	N4bp1	NM_030563.2	242117940	1
Catenin (cadherin associated protein) beta 1	Ctnnb1	NM_001165902.1	260166641	1
Collagen type3 alpha 1	Col3a1	NM_009930.2	226423932	1
Collagen type1 alpha 2	Col1a2	NM_007743.2	111120328	1
Ribosomal protein AS	Rpsa	NM_011029.4	224994259	1
Charged multivesicular body protein 2A	Chmp2a	NM_026885.3	254826731	1
Vimentin	Vim	NM_011701.4	227430362	1
Heterogeneous nuclear ribonucleoprotein K	Hnrnpk	NM_025279.2	142350515	1
Serine/Arginine-rich protein specific kinase 2	Srpk2	NM_009274.2	47059479	2
DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 3 x-linked	Ddx3x	NM_010028.3	164607180	1
Proprotein convertase subtilisin/kexin type 5	Pcsk5	NM_001163144.1	253314508	2
Transforming growth factor beta regulated gene 1	Tgfb1	NM_025289.3	224967131	1
Glutathione S transferase	Gstm1	NM_010358.5	239937552	1
Pyruvate kinase	Pkm2	NM_001253883.1	359807366	1
Basic transcription factor 3	Btf3	NM_001170540.1	281485610	1
STIP1 homology and U-Box containing protein 1	Stub1	NM_019719.3	118130581	2
S100 calcium binding protein A5	S100a5	NM_011312.2	113930759	1
Not in frame*	—	—	—	35

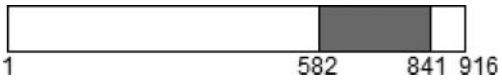
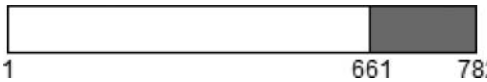
*Inserts sequences were not in frame with the B42 transcription activation domain in the library vector.

FIGURE 1. PrP^C interacts with 10 different ligands in cross-mating assay. Bait strain expressing PrP^C and the *Lac-Z* reporter gene were mated with target strains expressing the putative PrP^C interactors. X-gal was used to score positive interactions (blue color development). pBait and pTarget were used as a positive control for interaction, since they express known interaction partners. The empty bait vector (pGilda), which expresses only Lex A domain, was used as negative control.



dynein light chain Tctex-type 1B (Dynlt1b), catenin beta 1 (Ctnnb1), and STIP1 homology and U-Box containing protein 1 (Stub1) (**Fig. 1**). Since Stub1 is homologous to stress-inducible protein 1 (STI1), a well-known PrP^C ligand,[13,14] we decided to further investigate the putative interaction between Stub1 and PrP^C. Western blotting assays were performed using protein extracts prepared from these mouse tissues to examine protein expression of

TABLE 2. Ten putative PrP^C ligands

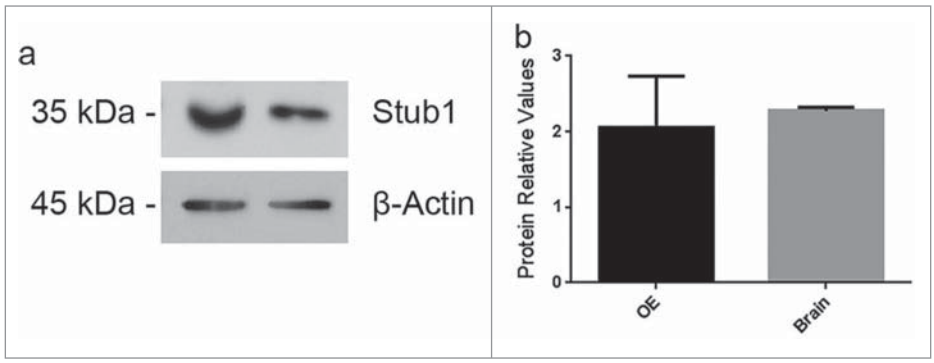
Name/Symbol	Protein region contained in the target clone[a]	Function
Vimentin (Vim)		Class-III intermediate filaments found in various non-epithelial cells
Serine/arginine-rich protein specific kinase 2 (Srpk2)		Protein involved in the phosphorylation of SR-splicing factors and the regulation of splicing. Promotes neuronal apoptosis by upregulating cyclin-D1 (CCND1) expression
Proprotein convertase subtilisin/kexin type 5 (Pcsk5)		Likely to represent a widespread endoprotease activity within the constitutive and regulated secretory pathway
Transforming growth factor beta-1 (Tgfb1)		Multifunctional protein that controls proliferation, differentiation, and other functions in many cell types. Many cells synthesize TGFB1 and have specific receptors for it
Glutathione S-Transferase Mu 1 (Gstm1)		Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles
Dynein light chain Tctex type 1 D 1 (Dynlt1b)		Acts as one of several noncatalytic accessory components of the cytoplasmic dynein 1 complex that are thought to be involved in linking dynein to cargos and to adapter proteins that regulate dynein function. Cytoplasmic dynein 1 acts as a motor for the intracellular retrograde motility of vesicles and organelles along microtubules
NEDD4 binding protein 1 (N4bp1)		Inhibitor of the E3 ubiquitin-protein ligase ITCH
Catenin (cadherin-associated protein) beta 1 (Ctnnb1)		Downstream component of the canonical Wnt signaling pathway. Component of an E-cadherin/catenin adhesion complex located to adherens junctions
Charged multivesicular body protein 2a (Chmp2a)		Core component of the endosomal sorting required for transport complex III (ESCRT-III) which is involved in multivesicular bodies (MVBs) formation and sorting of endosomal cargo proteins into MVBs
STIP1 homology and U-Box containing protein 1 (Stub1)		E3 ubiquitin-protein ligase which targets misfolded chaperone substrates toward proteasomal degradation

[a]Gray color indicates the region found in the target clone.

Stub1 in the OE and the brain. As shown in **Figure 2**, Stub1 is expressed both in the OE and the brain with similar expression levels.

We conducted pull-down assays to further analyze the interactions of PrP^C with Stub1. Recombinant His_6-PrP^C was incubated with protein extract prepared from mouse brain, as a source of endogenous Stub1. Protein complexes were then pulled down using Ni-NTA-agarose beads. Western blotting analysis

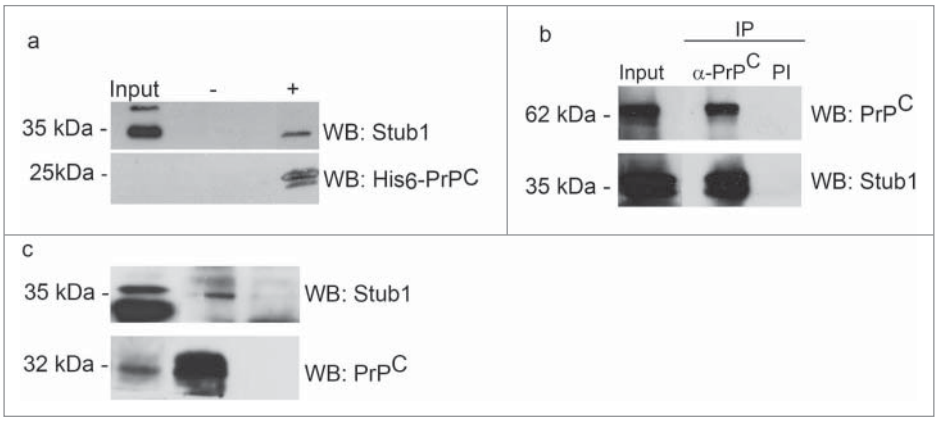
FIGURE 2. Stub1 is expressed in both olfactory epithelium (OE) and brain. (**a**) Protein extracts prepared from mouse brain and OE were analyzed by western blotting with anti-Stub1 (upper panel) antibodies. Anti-β-actin was used as loading control (lower panel). (**b**) Protein relative values were quantified using ImageJ Software® and normalized to β-actin. The results are representative of 4 independent experiments. Statistical analysis was conducted with GraphPad Prism (values were expressed as mean $\pm$SE. Student's t test was used for statistical analysis and $P<0.05$ was considered significant).



showed that recombinant PrP^C was recovered from the resin, attested by the detection of a 25 kDa band when probed with anti-His tag (**Fig. 3a**). Stub1 were pulled down together with recombinant PrP^C. No reaction was observed when the extract was incubated with Ni-NTA-agarose beads alone.

Co-immunoprecipitation assays were also performed to confirm the pull-down results. Protein extracts of HEK293T cells were co-

FIGURE 3. PrP^C interacts with Stub1. (**a**) A pull-down assay was performed using mouse brain extract incubated with His_6-PrP^C bound to Ni-NTA-agarose beads (+) or Ni-NTA-agarose alone (−). Pulled-down proteins were analyzed by western blotting (WB) employing anti-Stub1 antibody (upper panel). The same membranes subjected to pull-down assay were re-probed with anti-his-tag antibody to attest that recombinant PrP^C was recovered from the beads (lower panel). HEK293T cells protein extracts overexpressing GFP-PrP^C and Myc-Stub1 (**b**) or olfactory epithelium (OE) extracts (**c**) were immunoprecipitated with anti-PrP^C or pre-immune serum (PI). Co-precipitated proteins were analyzed using an anti-Stub1 antibody (upper panels). The same membranes were re-probed with anti-PrP^C (lower panels) to confirm that PrP^C was precipitated during IP-reaction.



transfected with pEGFP-PrP^C and pcDNA3-Myc-Stub1 expression vectors were incubated with anti-PrP^C antibody, followed by immunoprecipitation (IP). The blotting reaction of the immunoprecipitated material with anti-Stub1 antibody revealed 2 bands of 35–40 kDa, corresponding to the endogenous Stub1 (lower band) and the myc-tagged Stub1 (higher band) (**Fig. 3b**). No bands were observed when preimmune serum was employed in the immunoprecipitation reaction (**Fig. 3b**, PI). The same membrane was re-probed with anti-PrP^C antibody to confirm that GFP-PrP^C was precipitated during the IP-reaction.

To determine whether endogenous PrP^C could bind endogenous Stub1, similar co-immunoprecipitation experiments were carried out using mouse OE extract. When PrP^C was immunoprecipitated from OE lysates and subjected to western blot analysis, a specific band corresponding to Stub1 was detected (**Fig. 3c**). This band is slightly higher than in the input and this finding was verified in other co-immunoprecipitation assays, with different source of tissue samples. A weak signal of Stub1 was also obtained when beads coupled with preimmune serum were used (negative control), suggesting residual nonspecific binding. Co-immunoprecipitation assays with anti-Stub1 were also done and produced similar results as shown in **Figure 3c**, confirming the specific interaction between endogenous PrP^C and Stub1 (data not shown).

DISCUSSION

This study aimed to identify novel PrP^C binding proteins using two-hybrid screening of an OE cDNA library. A group of 10 proteins that potentially interact with PrP^C was identified, which provided clues about the enigmatic function of PrP^C. As PrP^C is a GPI-anchored protein, localized predominantly on the extracellular side of the plasma membrane, the relevance of yeast two-hybrid system to analyze interactions of PrP^C could be questioned. However, it is known that a subset of PrP^C is present in the cytosol, designated as cytosolic PrP^C ($cytPrP^C$).[15] There are also 2 PrP^C transmembrane forms that are partially exposed to the cytosol: ^{Ctm}PrP with the N-terminus in the cytosol and ^{Ntm}PrP with the C-terminus in the cytosol.[16] Therefore, interaction of PrP^C with cytosolic proteins is possible and the identification of these ligands should help in understanding its physiological role(s).

Indeed, most of the putative PrP^C ligands identified in the present study are cytoplasmic proteins, which belong to different protein classes and are associated to distinct cellular mechanisms. These potential interactions are consistent with the proposal that PrP^C is part of a multiprotein complex that modulates several cellular functions.[2] A number of other proteins have also been identified that could interact with PrP in the cytosol. Some of them mediate neuroprotection, including the anti-apoptotic protein Bcl-2[17] and the neurotrophin receptor-interacting MAGE homologue, NRAGE.[18] Restelli and colleagues found that PrP molecules that escaped translocation into the ER were associated with an increase in the resistance of cortical and hippocampal cells to apoptosis, suggesting that this form of cytPrP has neuroprotective function.[19,20] The putative interactions obtained that could modulate the neuroprotective role of cytPrP should be investigated.

Several studies indicated that cytPrP can acquire neurotoxic potential. Cytotoxic effects of cytPrP were observed in several mammalian cell lines and animal models.[16] Thus, the interactions found in the present study may have some physiological role in the cytosol or they may participate in the induction of PrP^{Sc} and are potential targets for the treatment of TSEs.

Previous studies also used the yeast two-hybrid methodology to screen for PrP^C ligands; however the interactions found in these studies do not completely overlap with our findings.[17,22-24] A number of possible explanations could account for the different results. First, most of the screens were not exhaustive and may not have reached saturation. Second, it is well known that a significant number of false-positive interactions can be obtained in the yeast two-hybrid method. Moreover, variations in the details of the protocol, such as the vectors used, the nature of the reconstituted

transcription factor, and the nature of the libraries screened, have a great impact on the interactions that can be retrieved.[25] This is the first screening for PrP^C-binding proteins that used a cDNA library from OE in the yeast two-hybrid assay. This approach provided the identification of new PrP^C interactions, although they were not limited to the OE.

In addition to yeast two-hybrid experiments, the interaction between PrP^C and Stub1 was also confirmed by pull-down and co-immunoprecipitation assays.

Stub1, also known as CHIP (C-terminus of Hsc70-interacting protein), is a 35 kDa cytoplasmatic protein that functions both as a molecular co-chaperone and as an ubiquitin E3 ligase.[11] The N-terminal region of Stub1/CHIP has 3 tetratricopeptide repeat (TPR) domains responsible for protein-protein interactions with Hsps 70/90 and other molecular chaperones. The C-terminus contains a U-box, which is the site of its ubiquitin E3 ligase activity.[26] In cooperation with heat-shock chaperone proteins, including Hsc70, Hsp70, and Hsp90, Stub1/CHIP plays a crucial role in recognizing and modulating the degradation of numerous chaperone-client proteins.[27]

Stub1/CHIP is associated with several neurodegenerative disorders characterized by protein misfolding and aggregation, such as Alzheimer and Parkinson diseases.[12] Since prion diseases are also characterized by protein misfolding and aggregations, it would be logical to expect that PrP^C is a substrate for Stub1/CHIP. It is possible that the interaction identified between PrP^C and Stub1/CHIP can occur in vivo and modulate PrP^C stability, which is implicated in the PrP^{Sc} conversion.

Interestingly, Stub1/CHIP is homologous to stress-inducible protein 1 (STI1) or Hsp70/Hsp90-organizing protein (Hop), a well-known PrP^C ligand.[13,14] Like Stub1/CHIP, STI1/Hop is also a co-chaperone and contains TPR domains that interact with the C-terminal sequence of Hsp70 and Hsp90. Its interaction with PrP^C at the cell surface is implicated in neuritogenesis and neuroprotection.[2,13,14] Some works have demonstrated that both STI1/Hop and Stub1/CHIP are involved together, modulating the balance between folding and degradation for Hsp70/90 client proteins.[28,29] It is possible that a similar mechanism is involved in PrP^C quality control, since PrP^C binds both STI1/Hop and Stub1/CHIP. This regulation of PrP^C by STI1/Hop and Stub1/CHIP is currently under investigation in our group.

To date, there is no evidence in the literature showing that PrP^C would be a substrate for Stub1/CHIP. This possibility was suggested in a study by Zhang and colleagues[30], who reported a specific interaction between Hsp70 and cytosolic PrP and that mutant PrPs are the targets of Hsp70 in the cytosol. They also found that overexpression or activation of Hsp70 in cultured cells selectively mediated the degradation of cytosolic PrPs, contributing to the protective effect against cytotoxicity. They also suggested that the conversion of Hsp70's function in protein folding to its function as a degradation factor could be mediated by the co-chaperone Stub1/CHIP.[30]

Since Stub1 is a cytoplasmic protein and PrP^C is located in the extracellular side of cell membrane, the sub-localization of the identified interactions awaits further investigation. The presence of PrP^C in the cytosol might be a normal feature of PrP^C metabolism, either by retro-translocation or by direct transfer from the ribosomes without entering the endoplasmic reticulum (ER).[31] As mentioned above, some studies indicated that the cytoplasmic PrP^C has neurotoxic effects, but other findings suggested a neuroprotective role for these PrP molecules. Hence, interaction of PrP^C with Stub1 may occur in the cytosol and may be involved in neuroprotective or neurotoxic effects.

The characterization of novel PrP^C ligands should contribute to the understanding of both the physiological and pathological roles played by PrP^C. The functional implications of PrP^C interaction with Stub1 in olfaction and other biological processes should be further investigated.

MATERIALS AND METHODS

Antibodies and Plasmids

The following antibodies were used in this study: rabbit monoclonal anti-Stub1 (ABCAM,

#134064) and mouse monoclonal anti- β-actin (Sigma Aldrich, #A5441). Polyclonal anti-PrP^C antibodies were raised in PrP^C-null mice (a kind gift from Dr. Marilene H. Lopes, University of Sao Paulo); the specificity of this antiserum has already been described.[13,32]

Plasmids used for the cells transfections were as follows: pCDNA 3.1-Myc-Stub1, which harbored a full-length mouse Stub1 cDNA insert; pEGFP-PrP^C, which has been previously described[33], was kindly provided by Dr. Vilma Regina Martins (International Research Center, A.C. Camargo Hospital, São Paulo, Brazil). The yeast two-hybrid vectors are described below.

Production of the Yeast Bait Strain

The sequence encoding the mature mouse PrP^C (residues 23–231) was amplified by PCR using the recombinant plasmid pEGFP-PrP^C as template and subcloned into the *Eco*RI/*Bam*HI restrictions sites of the yeast bait expression vector pGILDA (Clontech, USA). The construct was then transformed into the yeast strain RFY206 together with pSH18-34, a Lac-Z reporter vector.

Yeast Two-Hybrid Screening

The pre-transformed OE cDNA library has already been described by Von Dannecker and colleagues and Kerr and colleagues in their studies.[34,35] Yeast two-hybrid screening was carried out using a DupLex-A Yeast Two-Hybrid System (OriGene Technologies, Rockville, MD) and was performed as previously described using a mating assay.[34] Cross-mating test was conducted as described by Von Dannecker et al.[34]

Detection of Stub1 in Mouse Brain and OE Protein Homogenates

Mouse brain and OE tissues were removed and lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.2% sodium deoxycholate, 1 mM PMSF and 1 mM NEM). The extracts were centrifuged at 19,000 x *g* for 30 min at 4°C and the supernatants were saved. All animal procedures undertaken in this study were approved by the Animal Care and Use Committee (Federal University of Paraná) and are in accordance to the ethical guidelines established by CONCEA (National Council of Animal Experiment Control, Brazil).

Protein homogenates (100 μg) were separated in SDS-PAGE, and analyzed by western blotting using antibodies anti-Stub1 (1:2,000) and anti-β actin (1:3,000). The reaction was developed using a chemiluminescent substrate (WestPico, Pierce Co.).

Quantification of band intensity was performed using the Image J Software (NIH, Bethesda, MD, USA). The signal intensity of each protein was normalized with the corresponding β-actin signal. Experiments were conducted at least 3 times and values were expressed as mean $\pm$ standard error. Student's t test was used for statistical analysis and $P < 0.05$ was considered significant.

Pull-Down Assays

Mouse brain homogenate diluted in buffer A (10 mM Tris, pH 7.4; 100 mM NaH_2PO_4; 25 mM imidazole; 1% NP-40) was precleared twice in 30 μl of Ni-NTA-agarose. The resulting supernatant (input; 250 μg) was incubated with 45 μg of mouse recombinant His_6-PrP^C (kindly provided by Dr. Vilma Regina Martins, International Research Center, A.C. Camargo Hospital, São Paulo, Brazil) for 2 h, at 4°C. The same amount of input (without recombinant protein) was used as a negative control. After that, 30 μl of Ni-NTA-agarose was added to each sample and incubated for 1 h, at room temperature. The resins were washed 7 times in buffer A (with 50 mM imidazole) and the last wash was in buffer A with 0.01% SDS. Pulled-down material was eluted with Laemmli sample buffer, resolved by a 12% SDS-PAGE, and analyzed by western blotting using the antibody anti-Stub1, as described above, and with monoclonal anti-penta His antibodies (Invitrogen, Carlsbad, CA).

Immunoprecipitation Assays

HEK 293T cells were co-transfected as previously described[32] with pEGFP-PrP and pcDNA3.1-Myc-Stub1. After 48 h, cells were lysed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM de $MgCl_2$, 1% NP40, and 0.2% sodium deoxycholate plus complete protease inhibitor cocktail and centrifuged at 20,000 × *g*, for 15 min at 4°C. Protein extracts (800 μg of protein, 0.4 μg/μL) were pre-cleared using protein A/G-Sepharose (Sigma-Aldrich), for 3 h, at 4°C. The pre-cleared extract was aliquoted into 2 samples containing 400 μg of protein each. Both samples were incubated for 4 h at 4°C under gentle agitation; one of them was added with 30 μl of protein A/G-Sepharose cross-linked to anti-PrP^C polyclonal antibodies, the other one (negative control) was added with an equal volume of protein A/G-Sepharose cross-linked to pre-immune mice serum. Beads were washed 5 times in lysis buffer, and bound proteins were eluted with 1x Laemmli buffer at 100°C. Recovered proteins were separated using 10% SDS-PAGE followed by western blot analysis using anti-PrP^C (1:1,000) or anti-Stub1 (1:2,000).

The same assay described above was performed using protein lysates from mouse OE (500 μg, 0.5 μg/μL).

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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