PrP^C interacts with tetraspanin-7 through bovine PrP$_{154–182}$ containing alpha-helix 1

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Abstract

The cellular prion protein (PrP^C) is highly conserved in the evolution of mammals, and therefore, thought to have important cellular functions. Despite decades of intensive research, the physiological function of PrP^C remains enigmatic. We carried out a yeast two-hybrid screen on a bovine brain cDNA expression library and identified the transmembrane protein tetraspanin-7 (CD231), as a PrP^C interacting protein. We confirmed the interaction between PrP^C and tetraspanin-7 by yeast two-hybrid assay, immunofluorescent co-localization, and immunocoprecipitation. Our mutational studies further demonstrated that PrP^C specifically binds tetraspanin-7 through the region corresponding to bovine PrP$_{154–182}$ containing alpha-helix 1.
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The prion diseases or transmissible spongiform encephalopathies (TSEs) are invariably fatal neurodegenerative diseases that include bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt–Jacob disease (CJD) in human, scrapie in sheep [1]. Prion diseases have been subjected to much recent attention due to the epidemic of BSE and the subsequent emergence of its human form, variant CJD (vCJD).

PrP^C is a highly conserved GPI-anchored sialoglycoprotein and expressed in neurons, glia, and a variety of non-neuronal tissues [2,3]. It is also distributed widely in the immune system, in haematopoietic stem cells and mature lymphoid and myeloid compartments [4]. However, the physiological function of this widely expressed prion protein is unclear. It has been proposed that PrP^C is part of a multiprotein complex that modulates various cellular functions, depending on both protein compositions in the complex and cell type. Moreover, a variety of molecules have been proposed to be PrP^C binding protein, such as mouse STLI [5,6], Synapsin, Grb-2, and Pint 1 [7], and human 37-kDa/67-kDa LRP [8,9]. It is yet unknown whether PrP^C functions through these binding proteins. Thus, attempts have been made in order to look for new PrP^C binding proteins that may provide new insights into its physiological and pathological functions.

We employed a yeast two-hybrid approach to search for new potential cellular prion protein interacting partners by using a brain expression library. A novel PrP^C interacting protein, tetraspanin-7 (also referred to as CD231, TALLA-1, A15, CCG-B7, or TM4SF2) [10–12], was identified. Our results showed PrP^C interacts with tetraspanin-7 through the peptide region bovine PrP$_{154–182}$ containing alpha-helix 1.

Materials and methods

Yeast two-hybrid screen. *Saccharomyces cerevisiae* strain PJ69-4A cells, and vectors pGBKT7 and pGADT7, were obtained from Clontech. The bovine brain cDNA library (Uni-ZAP XR Library) was purchased from Stratagene and the cDNA fragments were ligated into pGADT7 at the EcoRI–XhoI sites, resulting in pGADT7-cDNA library as the prey. The gene encoding mature bovine PrP^C (25–242) was amplified by polymerase chain reaction (PCR) using bovine genomic DNA as the template and then cloned into pGBDKT7 vector via NdeI–EcoRI restriction sites, yielding $pGBDKT7$-PrP^C as the bait. The yeast two-hybrid Gal4 interaction trap assayed was done as previously described [13].

Plasmid construction. For the construction of fusion proteins with fluorescent tags, commercial pEGFP-N1 and pDsRed-N1 (Clontech) vectors were used. For the pEGFP-N1/tetraspanin-7 construct, the tetraspanin-7 coding region was amplified from bovine brain cDNA library, and cloned upstream of the EGFP gene in the pEGFP-N1 plasmid by using EcoRI/BamHI restriction sites. For the construct $pDsRed/PrP^C$ and $pcDNA3/PrP^C$, PrP^C (25–242) coding region was cloned into the pDsRed-N1 and $pcDNA3/PrP^C$, respectively, by using the EcoRI/BamHI sites.

Cell cultures and transfections. HeLa cells were cultured at 37 °C, 5% CO_2, in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cell transfections were performed with Lipofectamine^{TM} 2000 (Invitrogen) according to the manufacturer's instructions, and the cells were analyzed for 2 days after transfection.

Immunofluorescent co-localization. The fluorescent vectors $pDsRed/PrP^C$ and pEGFP-N1/tetraspanin-7 were transiently co-transfected in HeLa cells. After 48 h, the fluorescent signals were analyzed with the Leica TCS-AOBS-SP2-MP laser confocal microscope.

Western blot and co-immunoprecipitation. The expression vectors $pcDNA3/PrP^C$, pEGFP-N1/tetraspanin-7, and pEGFP were transiently transfected into HeLa cells either individually or in combination. The transfected cells were washed with PBS, and resuspended in an ice-cold lysis buffer (Beyotime institute of Biotechnology). The cells were then lysed on ice for 2 min. Subsequently they were centrifuged at 12,000g for 15 min at 4 °C. The supernatants from cells transfected with individual plasmid were analyzed by Western blotting with antibodies against PrP or GFP (Beyotime institute of Biotechnology). The co-transfected lysates (2–3×10^7 cells/ml) were cleared by centrifugation and PrP-specific polyclonal anti-PrP antibody was added and incubated at 4 °C for 2–4 h. Protein G-Plus Agarose beads (Beyotime institute of Biotechnology) were added and incubated for 2 h at 4 °C. Proteins isolated from the beads were transferred onto NC membrane and EGFP-tetraspanin-7 fusion protein or EGFP protein was detected with EGFP antiserum and anti-rabbit HRP secondary antibody.

Characterization of the interacting domains of the proteins by using yeast two-hybrid assay. Different PrP deletion constructs were made with the pGBKT7 plasmid. These included pGBKT7/PrP(25–242), pGBKT7/PrP(51–242), pGBKT7/PrP(103–242), pGBKT7/PrP(154–242), pGBKT7/ PrP(185–242), pGBKT7/PrP(212–242), pGBKT7/PrP(25–53), pGBKT7/ PrP(25–103), pGBKT7/PrP(25–136), and pGBKT7/PrP(25–182). All were made by using primer pairs containing NdeI–EcoRI restriction sites (Fig. 4). The different deletion bait plasmids and the prey plasmids were co-transformed into yeast.

Results and discussion

To identify proteins that physically interact with the bovine prion protein, we performed a yeast two-hybrid screen by using a bovine brain library. This revealed that several yeast clones had positive signals in the assay. The bait plasmid ($pGBDKT7$-PrP^C) and the empty vectors (pGBKT7 and pGADT7), when individually tested for self-activation of the reporter cassette, in the absence of interacting partners, produced no activation and thus confirmed the specificity of the interactions in the positive clones. The pGADT7-cDNA plasmids isolated from the positive clones were transformed into *Escherichia coli* for amplification and DNA purification. The purified DNA from the positive clones was then verified by yeast two-hybrid assay. Sequencing of the isolated positive plasmids showed that several of these positive clones encode the bovine tetraspanin-7 protein (NM_001076384, from nucleotides 72–552). Noticeably, tetraspanin-7 protein is well conserved between bovine and *Homo sapiens*, showing 99% of nucleotide sequence identity. Fig. 1D shows that tetraspanin-7 had no auto-activity in the yeast two-hybrid assay and Fig. 1E shows that tetraspanin-7 interacted with PrP^C.

Tetraspanin-7 is a member of the tetraspanin family proteins which are highly expressed in the central nervous system, including the cerebral cortex and hippocampus [14]. Tetraspanin-7 is a membrane protein containing four transmembrane domains [15]. PrP is also a membrane protein with a glycosylphosphatidylinositol membrane anchor. These suggest that tetraspanin-7 and PrP^C may interact with each other on the membrane. To further confirm whether PrP^C and tetraspanin-7 interact with each other, we first carried out co-localization studies on PrP^C and tetraspanin-7 in mammalian cells. We produced vectors
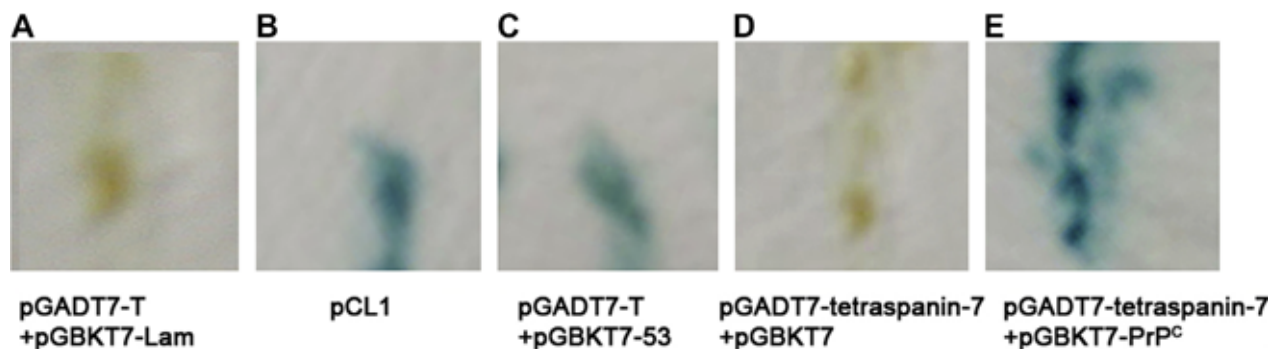

Fig. 1. PrP^C and tetraspanin-7 interact with each other in the yeast two-hybrid assay as detected by the β-galactosidase activities. (A) Negative control, human lamin C did not interact with SV40 large T-antigen. (B) Positive control, pCL1 encoding full-length, wild-type GAL4 protein. (C) Positive control, murine P53 interacted with SV40 large T-antigen. (D) No auto-activity was found in pGADT7-tetraspanin-7. (E) PrP^C interacted with tetraspanin-7.

for fluorescent fusion proteins of the two interacting proteins (pDsRed-N1/PrP^C and pEGFP-N1/tetraspanin-7). The expression vectors were co-transfected transiently into HeLa cells. Confocal microscopic analysis revealed that PrP^C and tetraspanin-7 proteins were co-localized in mammalian cells (Fig. 2).

Next, co-immunoprecipitation assays were carried out. As shown in Fig 3, GFP antiserum recognized EGFP (lane 1) and tetraspanin-7 fusion EGFP (lane 2); PrP antibody recognized PrP protein (lane 5) in Western blot using HeLa cell lysates. When the lysates were immunoprecipitated with the PrP antiserum followed by Western blot with the anti-EGFP antibody, which recognizes the EGFP-tetraspanin-7 fusion protein. The results showed that tetraspanin-7 was co-immunoprecipitated (the upper band in lane 3), indicating that tetraspanin-7-PrP^C proteins interact with each other in vivo (Fig. 3).

To map the tetraspanin-7 interaction domain, deletion mutants of PrP^C were made and analyzed by using the yeast two-hybrid assay. The results indicate that tetra-
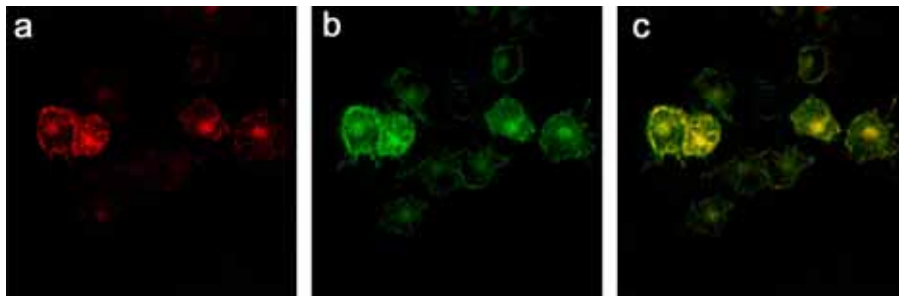

Fig. 2. Co-localization of PrP^C and tetraspanin-7 fusion proteins. PrP^C and tetraspanin-7 fusion proteins had red (a) and green (b) colors, respectively, and were co-localized as demonstrated by yellow color in the overlaid image (c). (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)
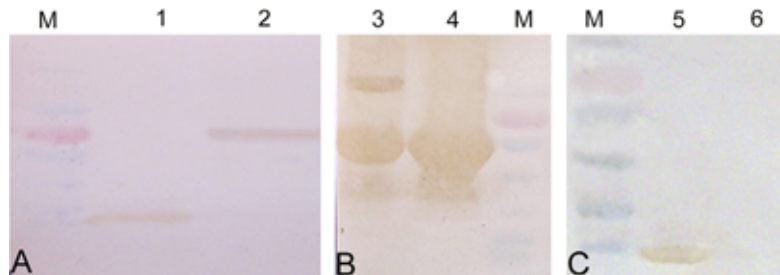

Fig. 3. Co-immunoprecipitation of PrP^C and tetraspanin-7 from transfected HeLa cells. (A) Western blot analyses were performed with anti-GFP. EGFP (lane 1) and EGFP-tetraspanin-7 (lane 2) were specifically detected. (B) HeLa cells were co-transfected with pcDNA3/PrP^C and pEGFP-N1/tetraspanin-7 or pcDNA3/PrP^C and pEGFP. The extract was immunoprecipitated with anti-PrP antibody followed by Western blot with anti-EGFP antibody. Note that EGFP-tetraspanin-7 was co-immunoprecipitated (the upper band in lane 3, the lower band is PrP antibody heavy chain) while EGFP was not (lane 4, the lower band matches the lower band in lane 3). (C) Western blot analyses were performed with anti-PrP antibody, showing the specific recognition of PrP^C (lane 5). The negative control is shown in lane 6.
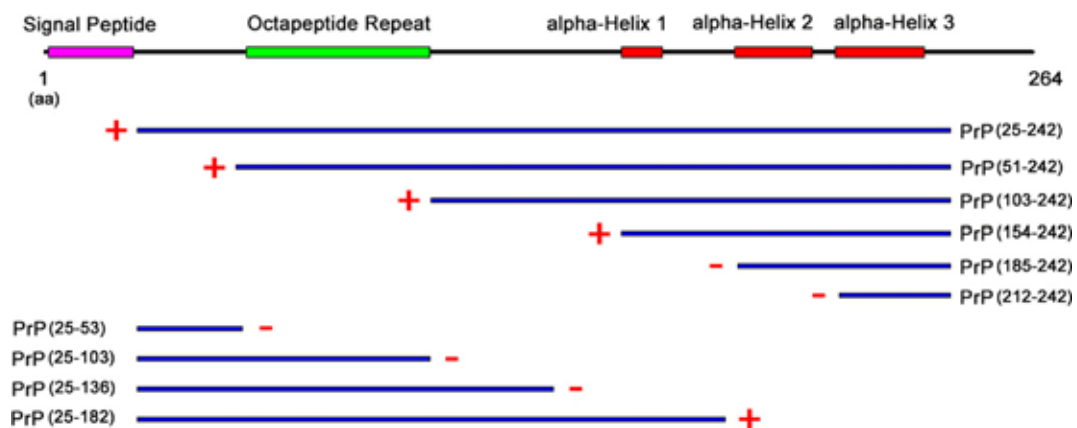

Fig. 4. Schematic representation of the PrP^C deletion mutants and their interaction with tetraspanin-7 as detected by using yeast two-hybrid assay. +, positive interaction; −, no interaction. The result shows that PrP^C binds tetraspanin-7 through the peptide region PrP$_{154-182}$ containing the alpha-helix 1.

spanin-7 binds the peptide region PrP$_{154-182}$ in bovine PrP^C that contains the alpha-helix 1 domain (Fig. 4). The alpha-helix 1 is known to play a important role in prion aggregation [16,17]. Thus, it will be of interest in the future to determine whether the interaction between tetraspanin-7 and PrP^C affects prion aggregation process.

Although the prion protein is expressed widely in the immune system, including haematopoietic stem cells, mature lymphoid and myeloid compartments [4], PrP$^{-/-}$ mice have been reported to have only minor abnormality in immune function. For this reason, the field of TSEs has not attracted much attention [18]. On the other hand, recently, it has been suggested that the lack of a clear immune response in prion disease is likely be due to tolerance to PrP^{Sc}. Furthermore, the immune system contributes to pathogenesis by amplifying prion in lymphoid compartments, thereby facilitating efficient neuroinvasion [4,19]. In addition, incubation of T cells with anti-PrP monoclonal antibodies for >10 min results in internalization of a large proportion of surface PrP into Limp-2 positive endosomes[20]. Our discovery here that PrP binds to the immune molecule tetraspanin-7 may help to understand how immune cells respond to PrP. The tetraspanin-family proteins are evolutionally conserved. They regulate a variety of normal and pathological processes such as immunological responses, protein trafficking, cell adhesion, virus invasion, and cancer metastasis through formation of a network of multi-molecule complexes via interactions among tetraspanins and between tetraspanins and other proteins [21,22]. Of interest, tetraspanin-7(CD231) is associated with X-linked mental retardation and neuropsychiatric diseases such as Huntington's chorea [14]. The finding that PrP interacts with tetraspanin raises the possibility that the interaction may have a role in TSEs immune response or efficient transportation of prion protein by tetraspanin. Whether the association between PrP^C and tetraspanin can affect immunological responses to influence neuroinvasion awaits further study.

Acknowledgments

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