FEBS Letters 580 (2006) 4231–4235

# *In vitro* self-propagation of recombinant $PrP^{Sc}$-like conformation generated in the yeast cytoplasm

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Received 8 June 2006; revised 23 June 2006; accepted 27 June 2006

Available online 5 July 2006

Edited by Jesus Avila

**Abstract Self-propagation is characteristic property for a prion conformation. Previous studies revealed that prion protein expressed in the cytoplasm gained a $PrP^{Sc}$-like conformation. However, it remains unclear whether the $PrP^{Sc}$-like conformation has the self-propagating property. We found that PrP partially purified from yeast cytoplasm formed amyloid fiber like structures, and we found that the $PrP^{Sc}$-like conformation is able to convert normal $PrP^{C}$ in the brain homogenate to a proteinase K-resistant conformation. These results suggest that yeast cytoplasm expressed recombinant $PrP^{Sc}$-like conformation has the characteristic self-propagating property of a prion, which may have implications in the pathogenesis of sporadic and inherited prion diseases.**



*Keywords:* Prion; Transmissible spongiform encephalopathies; Cytoplasm; Protein misfolding

## 1. Introduction

$PrP^{C}$ is α-helical rich, detergent soluble, and sensitive to protease digestion. During prion disease, a portion of PrP molecules converts to a β-sheet rich, aggregated, and protease resistant $PrP^{Sc}$ conformation [1]. The ''protein-only'' hypothesis proposes that $PrP^{Sc}$ is the infectious agent [1,2]. By virtue of its unusual self-propagating characteristic, $PrP^{Sc}$ converts normal host $PrP^{C}$ into the $PrP^{Sc}$ conformation, and subsequently leads to neurodegeneration. Compelling experimental evidence, particularly the recent findings that amyloid fibers made from recombinant PrP89-230 is able to cause prion disease in transgenic mice overexpressing PrP89-230 [3] and *in vitro* amplified infectious mammalian prion caused disease in wild-type mice [4], strongly supports the ''protein-only'' hypothesis.

The self-propagating property of $PrP^{Sc}$ was first demonstrated in the cell free system [5], showing that highly purified $PrP^{Sc}$ is able to convert purified normal PrP into an aggregated and proteinase K (PK) resistant conformation [5]. Recently, a more sensitive and robust assay, the protein-misfolding cyclic amplification (PMCA), was developed [6]. Both the $PrP^{Sc}$ conformation and prion infectivity were successfully propagated *in vitro* using PMCA [4]. The mechanism of PrP conversion process was dissected with a modified PMCA assay [7], revealing that polyanions stimulate PrP conversion [8]. Among all the polyanions tested, RNA molecules (>0.2 kb in length) are the most potent stimulators [9]. These results clearly established that $PrP^{Sc}$ conformation is a prion with the self-propagating characteristic.

In all the *in vitro* conversion assays, $PrP^{Sc}$ from diseased brains were used as seed to study its propagation. Till now, no recombinant $PrP^{Sc}$-like conformation has been reported to successfully seed PrP conversion *in vitro*. Previous studies revealed that a recombinant $PrP^{Sc}$-like conformation could be generated by high-level expression of wild-type PrP in the yeast cytoplasm [10]. A recent study revealed that a N-terminal hydrophobic amino acid stretch (amino acid 112–119) is crucial for PrP to gain the $PrP^{Sc}$-like conformation in the yeast cytoplasm [11]. This study also revealed that the same amino acid stretch is essential for prion propagation in prion-infected mammalian cells, while it remains unclear whether yeast cytoplasm generated $PrP^{Sc}$-like conformation has the self-propagating property.

We analyzed recombinant PrP expressed in the yeast cytoplasm to determine whether it has this unusual property of a prion. Our results suggest that, besides the biochemical similarities to $PrP^{Sc}$, cytoplasm generated recombinant $PrP^{Sc}$-like conformation also has the characteristic self-propagating property as a prion.

## 2. Materials and methods

### 2.1. Plasmid construction and yeast strain

Yeast expression plasmid mPrP (23-230)-p2UGPD was described previously [10]. In all experiments described here, a pep4 knockout yeast strain W303 (MATa *ade2-1 can1-100 his3-12, 16 leu2-3,112 trp1-1 ura3-1*) was used (a kind gift from Susan Lindquist's lab).

### 2.2. Solubility and PK digestion of yeast expressed PrP

Yeast spheroplast was obtained with Zymolyase 100 T (MP Biochemicals) for 2 or 3 h at 30 °C, and lysed in two volumes PBS plus protease inhibitors (aprotinin, pepstatin and leupeptin) with or without 10% (w/v) Sarkosyl (Sigma). Yeast lysates were pre-cleared with a 5 min $2000 \times g$ spin. For separation of supernatant and pellet fractions, the yeast lysate was sedimented at $16000 \times g$ for 30 min at 4 °C. For PK digestion, pre-cleared yeast lysates were digested with PK (Merck) at 37 °C for 30 min at indicated PK concentrations, and 5 mM phenyl-methyl sulphonyl fluoride (PMSF) was added to terminate PK digestions.

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*Abbreviations:* PrP, prion protein; PMCA, protein misfolding cyclic amplification; PK, proteinase K; ER, endoplasmic reticulum; EM, electron microscopy

0014-5793/$32.00 
doi:10.1016/j.febslet.2006.06.074

### 2.3. Purification of yeast cytoplasm expressed PrP for electron microscopy analysis

Transformed yeast cells were collected by centrifugation, and cell pellet was resuspended in ice-cold PBS and disrupted by FRENCH Press FA-080-E1 (Thermo Spectronic) according to user's manual. Lysates were pre-cleared, and the supernatant fraction was collected and centrifuged again at $16000 \times g$ for 30 min at 4 °C. The pellet was resuspended in PBS containing 10% (w/v) Sarkosyl. The centrifugation and resuspension procedures were repeated twice, and the final pellet was resuspended in PBS.

### 2.4. Electron microscopy (EM)

Samples of partially purified PrP were absorbed to glow discharged carbon-coated copper grids. After washed twice with deionized water, samples were negatively stained with 2% uranyl acetate for 30 s and air-dried. Grids were analyzed using a Hitachi H600A electron microscope.

### 2.5. Preparation of normal brain homogenates for conversion assay

Whole brain was harvested from normal, pathogen free BALB/C mice or Sprague-Dawley rats. Normal brain was homogenized in PBS plus protease inhibitors. Crude homogenates were pre-cleared with a 30 s centrifugation at $1000 \times g$ and aliquots of the supernatant were stored at −70 °C.

### 2.6. In vitro conversion assay

We employed a modified PMCA assay as previously described [7]. Briefly, 50 μl of 5% or 20% normal brain homogenate was mixed with 50 μl of total yeast lysates. The mixture was incubated for 16 h at 37 °C with continuous shaking at 200 rpm. 20 μl of each conversion mixture were digested at 37 °C with PK with indicated concentrations. The PK digestion was carried out for 30 (for experiments described in Fig. 5) or 60 min (for experiments described in Figs. 3 and 4). PMSF (5 mM) was added to terminate PK digestions.

### 2.7. Immunoblot analysis

Following electrophoresis, proteins were transferred to PVDF membrane. The anti-PrP 8H4 monoclonal antibody (a gift kindly provided by Dr. Man-sun Sy) was used at 1:1000 and a horseradish peroxidase-labeled anti-mouse IgG secondary antibody (GE Healthcare) was used as secondary antibody with a dilution at 1:5000. The blot was developed with the ECL-plus reagent (GE Healthcare).

### 2.8. Preparation of RNA

RNA was extracted with Trizol reagent (Invitrogen) and precipitated with isopropanol, according to manufacturer's instructions.

## 3. Results

### 3.1. Expression and characterization of PrP expressed in yeast cytoplasm

We expressed PrP in the cytoplasm of yeast in which a major vacuolar protease PEP4 was deleted [12]. Immunoblot analysis revealed that PrP exhibited a molecular mass of approximately 25 kDa (Fig. 1A), as expected for unglycosylated PrP lacking the glycosylphosphatidylinositol (GPI)-anchor.

The majority of PrP was insoluble in 10% Sarkosyl and appeared in the pellet fraction (Fig. 1B). Yeast cytoplasm expressed PrP resists PK digestion with PK concentrations ranging from 2 to 20 μg/ml. The apparent molecular weights of PK resistant PrP fragments were around 20 kDa and 15 kDa (Fig. 1C). As a control, the $PrP^C$ present in the brain homogenates was completely removed with PK concentration as low as 5 μg/ml (Fig. 1D).

### 3.2. Amyloid fibrils spontaneously formed by PrP expressed in the yeast cytoplasm

Amyloid fibers are ordered aggregates that are generally associated with self-propagating property [13]. EM analysis was performed to determine the physical properties of PrP aggregates. We used a purification scheme based on Sarkosyl extraction and differential centrifugation to obtain partially purified PrP. A variety of amyloid fiber like and/or bead-like structures were observed (Fig. 2A–G). The diameter of the fibrils varied, generally ranging from 10 nm to around 40 nm. The length of the fibers was also diverse, with some as long as 800 nm. The predominant protein aggregates were in a bead-like structure with a diameter around 20 nm (Fig. 2A–G). Besides these two structures, some irregular shaped protein aggregates were also observed. Control yeast lysates were prepared from yeast without PrP expression using exactly the same purification regime. As shown in Fig. 2, none of the structures described above were observed in control samples.

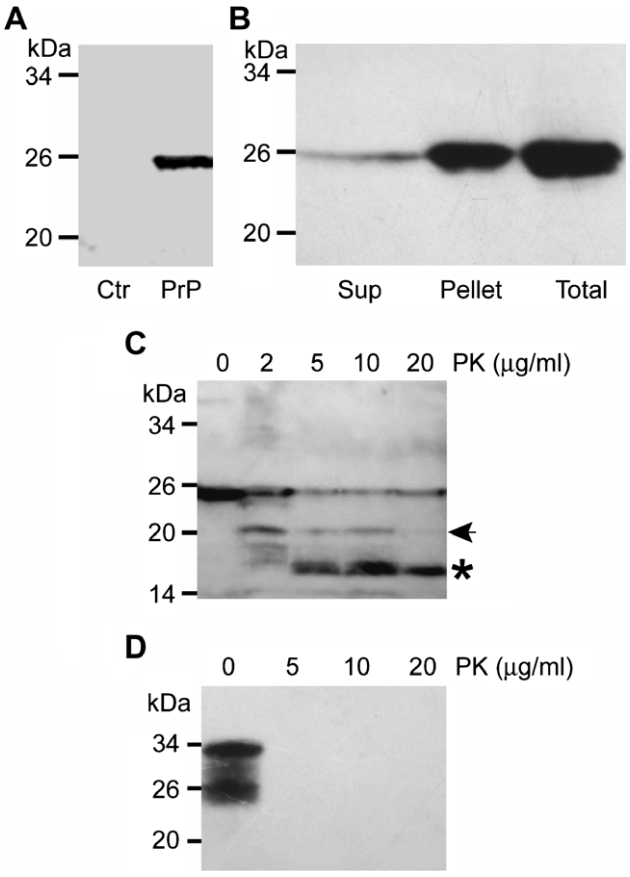


Fig. 1. Analysis of PrP protein generated in the yeast cytosol. (A) Total proteins from W303 yeast lysates (Ctl) or transformed yeast lysates (PrP). (B) Transformed yeast lysates (Total) were extracted with 10% Sarkosyl and subjected to sedimented, and PrP in the supernatant (Sup) and pellet fractions (Pellet) were detected. (C) Proteins from transformed yeast lysates were digested with PK at the indicated concentrations at 37 °C for 30 min. The arrow and the asterisk indicate specific proteinase-resistant bands. (D) Proteins from mouse brain homogenate were digested with PK at the indicated concentrations at 37 °C for 30 min.

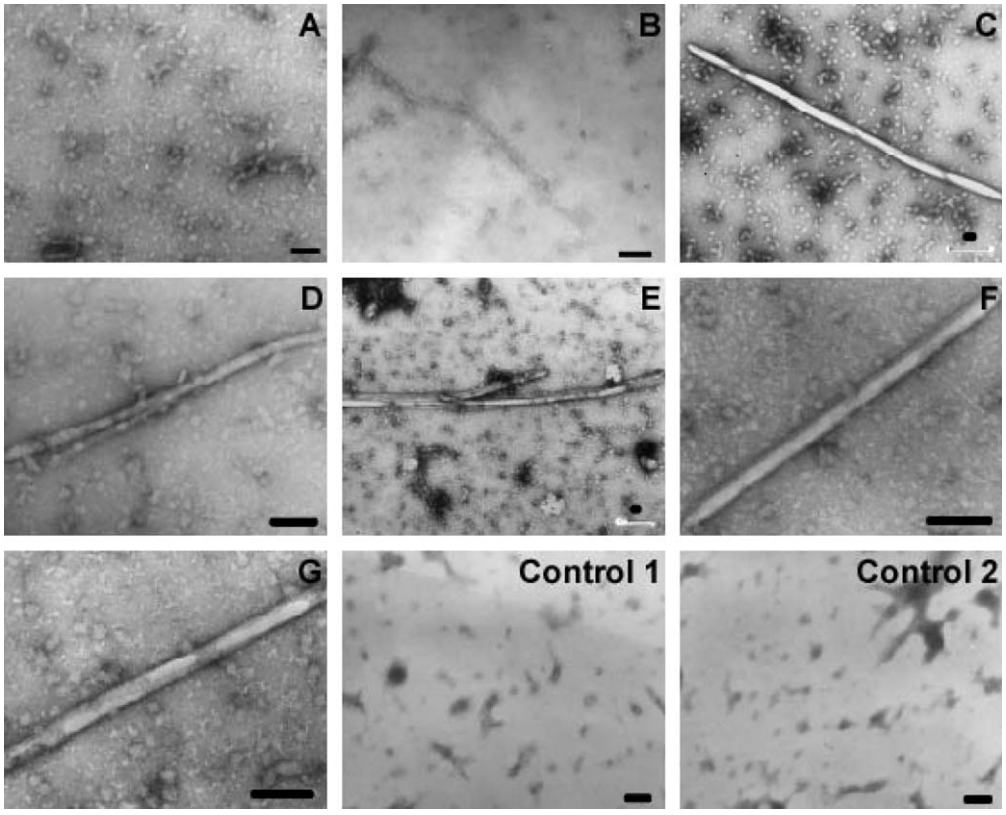


Fig. 2. Electron micrographs show fibrils spontaneously formed by PrP generated in yeast cell system. Purified PrP (A–G) and analogous fractions from control yeast lysates (Controls 1 and 2) were negatively stained with uranyl acetate. Bars represent 100 nm.

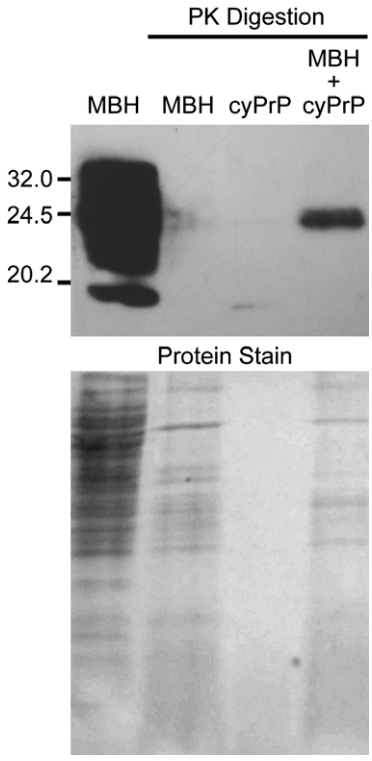


Fig. 3. *In vitro* amplification of PrPres. The mixture of 5% (w/v) normal mouse brain homogenate (MBH) and transformed yeast lysates (CyPrP) was incubated for 16 h at 37 °C. The mixture or MBH or CyPrP were digested with 5 μg/ml PK at 37 °C for 60 min. Equal loading was verified by total stain of the blot.

### 3.3. The yeast cytoplasm expressed PrP seeds the conversion of PrP in brain homogenates to a PK-resistant conformation

The ability of yeast cytoplasm expressed PrP to form ordered aggregates implies that it is able to self-propagate its PK-resistant conformation. To directly test this possibility, we followed a modified PMCA protocol [7]. A significant amount of PK resistant PrP was detected with a molecular weight around 25 kDa (Fig. 3, lane 4). As controls, same amounts of mouse brain homogenate or yeast spheroplast lysates were also subjected to the PK digestion respectively.

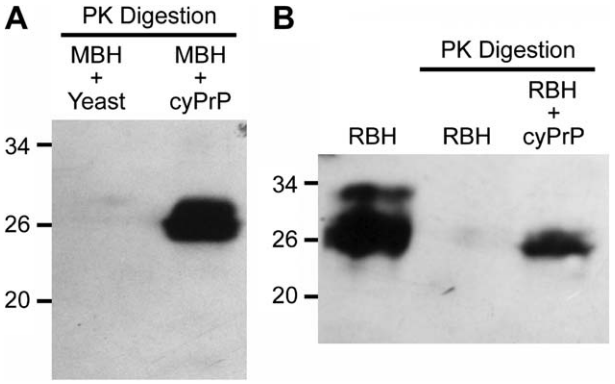


Fig. 4. Specificity of *in vitro* amplification of PrPres. (A) W303 yeast lysates (Yeast) or transformed yeast lysates (CyPrP) was mixed with 5% (w/v) normal mouse brain homogenate (MBH), followed by incubation for 16 h at 37 °C. (B) The mixture of 5% (w/v) normal rat brain homogenate (RBH) and transformed yeast lysates (CyPrP) was incubated for 16 h at 37 °C. The mixture or RBH were digested with 5 μg/ml PK at 37 °C for 1 h.

Notably, PrP in the mouse brain homogenate was almost completely removed by PK digestion (Fig. 3, lane 2). Some PK resistant PrP bands were detected in yeast spheroplast lysates (Fig. 3, lane 3), which may represent the $PrP^{Sc}$-like conformation generated by cytoplasm PrP. Importantly, the amount of PK resistant PrP in the yeast lyates was significantly lower than that in the conversion reaction (compare lane 3 and 4 of Fig. 3). Protein stain of the PVDF blot verified a similar level of PK digestion, ruling out the possibility that a weaker PK digestion was responsible for the PrP detection (Fig. 3, protein stain). The increase of PK resistant PrP fragment after the incubation (Fig. 3, lanes 2–4) suggests that yeast cytoplasm expressed PrP can act as a seed to convert normal PrP in the brain homogenate to a PK-resistant conformation.

To rule out the possibility that the conversion observed here was due to other components in the yeast lysates, we performed the conversion assay with yeast lysates prepared from yeast with or without cytoplasm PrP expression. Notably, PrP conversion was only observed in the presence of lysates prepared from PrP expressing yeast, while no conversion was observed in the reaction with control yeast lysates (Fig. 4A). To rule out the possibility that only mouse PrP in the brain homogenate is susceptible to cytoplasm PrP induced conversion, we performed the same experiment with 5% rat brain homogenates. A similar conversion of PrP to the PK-resistant conformation was observed (Fig. 4B).

Generally, the $PrP^{Sc}$ from diseased brain is highly resistant to PK digestion. However, the newly converted PK resistant PrP could only resist 5 μg/ml of PK digestion (Figs. 3 and 4A and B). One of the possible reasons for this lower level of PK resistance could due to difference in the concentrations of brain homogenate used here. We used 20% mouse brain homogenate for the conversion reaction. Notably, the newly converted PK resistant PrP showed a higher resistance to PK digestion, with final PK concentrations at 50 μg/ml and 100 μg/ml (Fig. 5A).

### 3.4. The conversion seeded by yeast cytoplasm expressed PrP depends on the presence of RNA molecules

Recent reports revealed that RNA plays an important role in the *in vitro* PrP conversion [9]. We included different concentrations of RNase A (Sigma) in the conversion. Notably, the amount of PK resistant PrP decreased with increasing concentrations of RNase A (Fig. 5B). We performed a control experiment to monitor the amount of RNA subject to RNase A treatment. Incubation did cause some RNA degradation (compare lanes 1 and 4 in Fig. 5C). However, a significant decrease of the total amount of RNA was only observed when RNase A was included (lanes 2 and 3 in Fig. 5C).

## 4. Discussion

One of the most important characteristics of a prion is its ability to self-propagate its conformation [1]. Our results from the modified PMCA conversion suggest that cytoplasm ex-

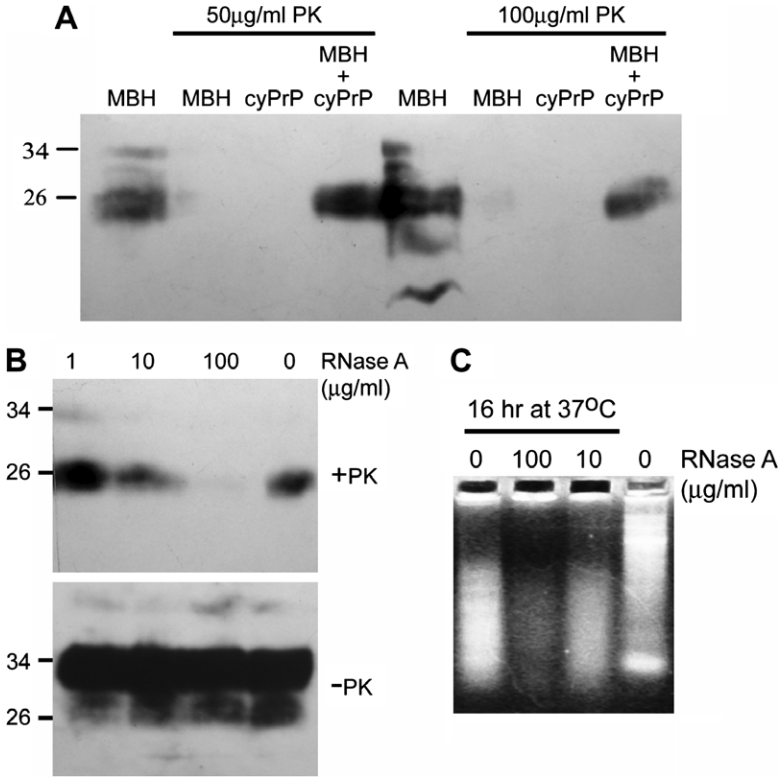


Fig. 5. Characteristics of *in vitro* amplification of PrPres. (A) The mixture of 20% (w/v) normal mouse brain homogenate (MBH) and transformed yeast lysates(CyPrP) was incubated for 16 h at 37 °C. The mixture or MBH or CyPrP were digested with 50 μg/ml or 100 μg/ml PK at 37 °C for 30 min. (B) Effects of RNase A on *in vitro* amplification of PrPres. The mixture of 20% (w/v) normal mouse brain homogenate and transformed yeast lysates was incubated with Rnase A in indicated concentrations. (C) RNase A did degrade RNA molecules in the brain homogenates. Agarose gel electrophoresis of total RNA prepared from the mixture of 20% (w/v) normal mouse brain homogenate and transformed yeast lysates incubated with Rnase A in indicated concentrations.

pressed recombinant PrP can convert normal PrP in the brain homogenate to a PK-resistant conformation. The observation that cytoplasm expressed PrP is able to form amyloid fiber like structures supports that it has the self-propagating ability as a prion.

Previous studies and results from this study showed that PrP could achieve a variety of conformations in the yeast cytoplasm [10], which were illustrated by the size of PK resistant PrP ranging from full-length to 20 kDa or 15 kDa. Since the cytoplasmic environment, such as redox potential and chaperones, differs quite dramatically from that in the lumenal side, it is not surprising that PrP in the cytoplasm can achieve a variety of conformations. However, it is not clear whether all the PK-resistant cytoplasm PrP conformations have the self-propagating property, or just one of them has this property. Further studies are required to develop purification schemes to separate different PrP conformations, which will allow us to find an answer to this question.

Another question needs to be further studied is whether yeast cytoplasm expressed PrP is infectious to animals. Although this PrP could propagate its conformation, it might be no infectious to animals. It is possible that it is not the infectious PrP form in prion diseases, or that an infection-facilitating factor is missing in the yeast lysates. As mentioned above, the existence of such a factor can explain the observations that the recombinant PrP amyloid fiber has a low infectivity [3] while the prion infectivity generated in the presence of brain homogenate is high[4]. In addition, a recent report showed that scrapie brain microsome has a much greater efficiency to infect cultured cells than purified PK resistant PrP [14] also supports the existence of an infection-facilitating factor. Bioassay with PK resistant PrP generated from yeast cytoplasm and from the modified PMCA reaction described here could potentially provide insight of prion infection process.

Our results suggest that, at least, some of the cytoplasm generated PK resistant PrP conformation has the characteristic prion property, the ability to self-propagate its PK-resistant conformation. Although wild-type PrP is cell surface localized, a portion of PrP enters the cytoplasm through either retrotranslocation from the ER [15] or impaired import into the ER [16]. Normally, PrP in the cytoplasm is efficiently removed by the proteasome [17]. However, if proteasome activity is compromised, some PrP molecules might escape proteasome degradation and gain the $PrP^{Sc}$ conformation. Further studies of cytoplasmic PrP will help us to elucidate whether it plays any role in the pathogenesis of prion disease.

*Acknowledgements:* We thank Jiyan Ma for his guidance in experimental design and for preparation of the manuscript. This work was supported by grants from National Frontier Research Program of China (2005CB2301) and Natural Sciences Foundation of China (30270307).

## References

[1] Prusiner, S.B. (1998) Prions. Proc. Natl. Acad. Sci. USA 95, 13363–13383.
[2] Prusiner, S.B. (1982) Novel proteinaceous infectious particles cause scrapie. Science 216, 136–144.
[3] Legname, G., Baskakov, I.V., Nguyen, H.O., Riesner, D., Cohen, F.E., DeArmond, S.J. and Prusiner, S.B. (2004) Synthetic mammalian prions. Science 305, 673–676.
[4] Castilla, J., Saa, P., Hetz, C. and Soto, C. (2005) *In vitro* generation of infectious scrapie prions. Cell 121, 195–206.
[5] Kocisko, D.A., Come, J.H., Priola, S.A., Chesebro, B., Raymond, G.J., Lansbury, P.T. and Caughey, B. (1994) Cell-free formation of protease-resistant prion protein. Nature 370, 471–474.
[6] Saborio, G.P., Permanne, B. and Soto, C. (2001) Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature 411, 810–813.
[7] Lucassen, R., Nishina, K. and Supattapone, S. (2003) *In vitro* amplification of protease-resistant prion protein requires free sulfhydryl groups. Biochemistry 42, 4127–4135.
[8] Deleault, N.R., Geoghegan, J.C., Nishina, K., Kascsak, R., Williamson, R.A. and Supattapone, S. (2005) Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions. J. Biol. Chem. 280, 26873–26879.
[9] Deleault, N.R., Lucassen, R.W. and Supattapone, S. (2003) RNA molecules stimulate prion protein conversion. Nature 425, 717–720.
[10] Ma, J. and Lindquist, S. (1999) *De novo* generation of a $PrP^{Sc}$-like conformation in living cells. Nat. Cell. Biol. 1, 358–361.
[11] Norstrom, E.M. and Mastrianni, J.A. (2005) The AGAAAAGA palindrome in PrP is required to generate a productive $PrP^{Sc}$–$PrP^{C}$ complex that leads to prion propagation. J. Biol. Chem. 280, 27236–27243.
[12] Preston, R.A., Reinagel, P.S. and Jones, E.W. (1992) Genes required for vacuolar acidity in *Saccharomyces cerevisiae*. Genetics 131, 551–558.
[13] Ross, E.D., Baxa, U. and Wickner, R.B. (2004) Scrambled prion domains form prions and amyloid. Mol. Cell. Biol. 24, 7206–7213.
[14] Baron, G.S., Magalhaes, A.C., Prado, M.A. and Caughey, B. (2006) Mouse-adapted scrapie infection of SN56 cells: greater efficiency with microsome-associated versus purified PrP-res. J. Virol. 80, 2106–2117.
[15] Ma, J. and Lindquist, S. (2001) Wild-type PrP and a mutant associated with prion disease are subject to retrograde transport and proteasome degradation. Proc. Natl. Acad. Sci. USA 98, 14955–14960.
[16] Ma, J. and Lindquist, S. (2002) Conversion of PrP to a self-perpetuating $PrP^{Sc}$-like conformation in the cytosol. Science 298, 1785–1788.
[17] Wang, X., Wang, F., Sy, M.S. and Ma, J. (2005) Calpain and other cytosolic proteases can contribute to the degradation of retro-translocated prion protein in the cytosol. J. Biol. Chem. 280, 317–325.