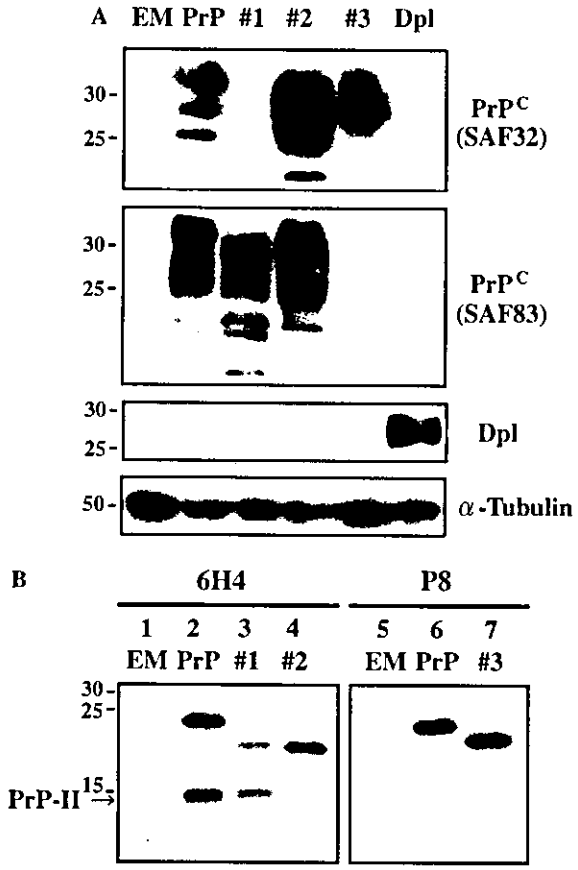

Fig. 2. Expression of PrP and truncated PrP. (A) Western blot analysis with anti-PrP SAF32, anti-PrP SAF83, anti-Dpl DDC39 or anti-α-tubulin B-5-1-1 of equal quantity (60 μg) of protein from HpL3-4 cells expressing wild-type PrP (PrP: HpL3-4-PrP), PrP(Δ53–94, Q52H) (#1: HpL3-4-Δ#1), PrP(Δ95–132) (#2: HpL3-4-Δ#2), and PrP(Δ124–146) (#3: HpL3-4-Δ#3) or the empty vector per se (EM: HpL3-4-EM). It should be noted that SAF32 recognizes residues 79–92 of PrP [32], whereas SAF83 recognizes residues 126–164 of PrP [33]. Therefore, SAF32 recognizes PrP, PrP(Δ95–132), and PrP(Δ124–146) but not PrP(Δ53–94, Q52H). SAF83 recognizes PrP, PrP(Δ53–94, Q52H), and PrP(Δ95–132) but not PrP(Δ124–146). (B) Western blot analysis with anti-PrP 6H4 (lanes 1–4) or P8 (lanes 5 and 6) of equal quantity of deglycosylated protein from HpL3-4-EM (lanes 1 and 5), HpL3-4-PrP (lanes 2 and 6), HpL3-4-Δ#1 (lane 3), HpL3-4-Δ#2 (lane 4) or HpL3-4-Δ#3 (lane 7). It should be noted that 6H4 monoclonal anti-PrP recognizes residues 144–152 of mouse PrP [23], whereas P8 polyclonal anti-PrP recognizes amino acids 92–109 of mouse PrP [16]. Therefore, 6H4 recognizes wild-type PrP, PrP(Δ53–94, Q52H), and PrP(Δ95–132) but not PrP(Δ124–146), while P8 recognizes wild-type PrP and PrP(Δ124–146) but not PrP(Δ53–94, Q52H) and PrP(Δ95–132). PrP-II is proteolytic fragment during normal metabolism [35].

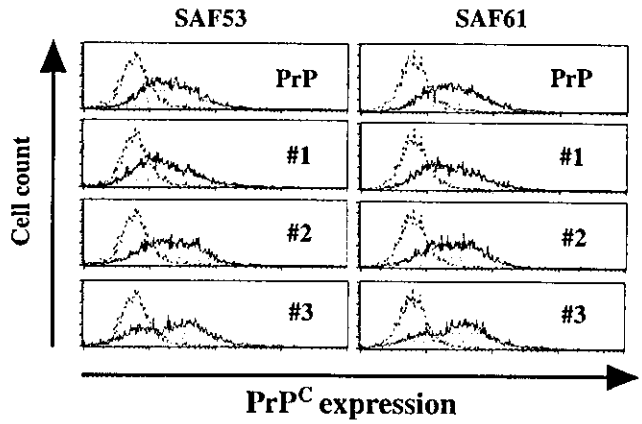

Fig. 3. Cell surface staining of wild-type PrP and the deletion mutants. HpL3-4 cells expressing wild-type PrP (PrP: HpL3-4-PrP), PrP(Δ53–94, Q52H) (#1: HpL3-4-Δ#1), PrP(Δ95–132) (#2: HpL3-4-Δ#2), and PrP(Δ124–146) (#3: HpL3-4-Δ#3) were incubated with anti-PrP SAF53 or SAF61 and a PE-conjugated secondary antibody, and the intensity of the labelled cells was determined by FACScan (filled areas). HpL3-4-EM cells incubated with SAF53 or SAF61 and a PE-conjugated secondary antibody were used as negative control (open areas).

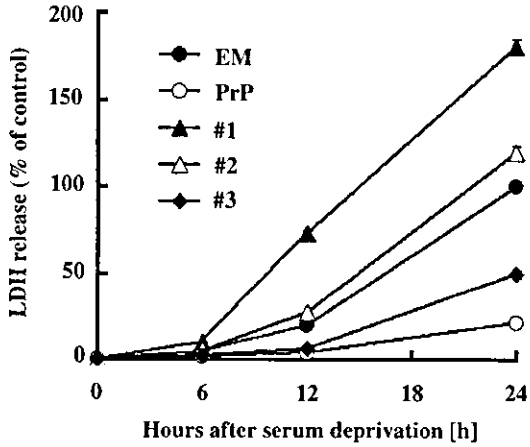

Fig. 4. Effect of PrP mutants on cell death induced by serum deprivation. HpL3-4-EM (EM: closed circles), HpL3-4-PrP (PrP: open circles), HpL3-4-Δ#1 (#1: closed triangles), HpL3-4-Δ#2 (#2: open triangles), and HpL3-4-Δ#3 (#3: closed diamonds) cells were cultured in the absence of serum for the indicated times. Cell death was examined by LDH assay as described in Materials and methods. Cell death is measured by the release of LDH into culture medium. LDH activity was measured as indicator of cell number of dead cells. The dead cell number of HpL3-4-EM cells serum-deprived for 24 h was taken as 100% (control). Values are expressed as means ± SEM ($N = 3$).

to HpL3-4-EM cells. The absence of anti-apoptotic function in PrP(Δ95–132) could not be explained simply by the relative levels of protein expression as assayed by Western blot and flow cytometry. The deletion proteins were expressed at levels equivalent to the full-length PrP (Fig. 2A). Furthermore, flow cytometry with SAF53 and SAF61 showed that the deletion proteins were localized at the cell membrane in the same way as full-length PrP (Fig. 3). Deglycosylation with PNGase F demonstrated that unlike full-length PrP, which is proteolytically cleaved during cellular metabolism, PrP(Δ95–132) did not exhibit any proteolytic fragment possibly due to absence of cleavage site 111/112 of PrP (Fig. 2B).

To confirm the anti-apoptotic function of PrP deletion mutants, apoptosis assays including the DNA ladder assay, measurement of the histone-associated DNA fragments (mono- and oligonucleosomes) in

cytosol fractions of the cells with ELISA was performed (Figs. 5 and 6). When compared with HpL3-4-EM cells, HpL3-4-PrP cells indicated lower levels of fragmented DNA following serum deprivation in the DNA ladder assay and ELISA. Similarly, HpL3-4-Δ#3 cells registered lower levels of fragmented DNA than HpL3-4-EM cells. By contrast, HpL3-4-Δ#1 cells showed higher levels of fragmented DNA when compared with HpL3-4-EM cells. Consistent with the results obtained by the LDH assay, HpL3-4-Δ#2 cells exhibited equivalent levels of fragmented DNA compared with HpL3-4-EM cells by the DNA ladder assay and ELISA. Comparative differences of apoptotic cells in each deletion mutant were also confirmed by DAPI staining (data not shown).

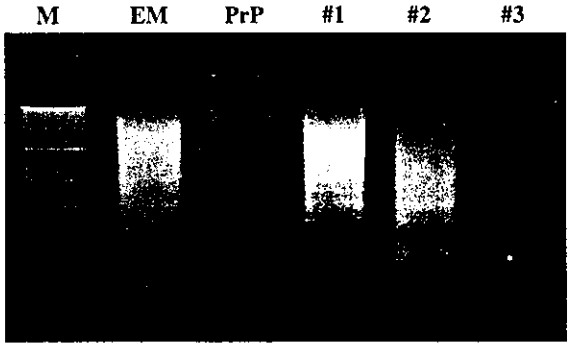

Fig. 5. Effect of PrP mutants on the formation of DNA laddering. HpL3-4 EM (EM), HpL3-4-PrP (PrP), HpL3-4-Δ#1 (#1), HpL3-4-Δ#2 (#2), and HpL3-4-Δ#3 (#3) cells were cultured in the absence of serum. After 24-h incubation, the cells were harvested. The fragmented DNA was analysed on agarose gels as described in Materials and methods. HpL3-4-EM cells exhibited fragmented DNA to a higher extent than HpL3-4-PrP cells after serum deprivation for 24 h. HpL3-4-Δ#1 cells elicited an increased extent of fragmented DNA compared with HpL3-4-PrP cells or HpL3-4-EM cells. HpL3-4-Δ#2 cells demonstrated fragmented DNA to a similar extent as HpL3-4-EM cells. HpL3-4-Δ#3 cells showed fragmented DNA to a lesser extent than HpL3-4-EM cells.

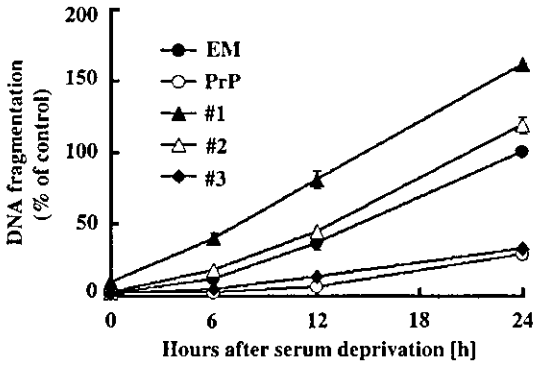

Fig. 6. Effect of PrP mutants on the formation of cytoplasmic histone-associated DNA fragments. HpL3-4-EM (EM: closed circles), HpL3-4-PrP (PrP: open circles), HpL3-4-Δ#1 (#1: closed triangles), HpL3-4-Δ#2 (#2: open triangles), and HpL3-4-Δ#3 (#3: closed diamonds) cells were serum-deprived for the indicated times. Apoptotic cell death was quantitatively measured using a cell death detection ELISA kit. DNA fragmentation was measured by quantitation of cytosolic oligonucleosome-bound DNA as indicator of cell number of apoptotic cells. The apoptotic cell number of HpL3-4-EM cells serum-deprived for 24 h was taken as 100% (control). Values are expressed as means ± SEM ($N = 3$).

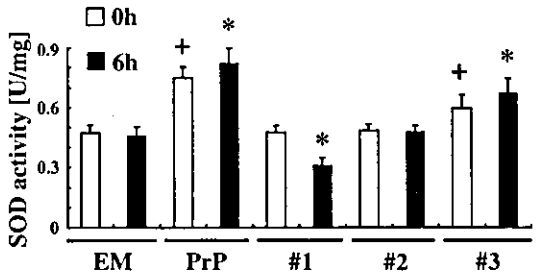

Fig. 7. Effect of PrP mutants on SOD activity. HpL3-4-EM (EM), HpL3-4-PrP (PrP), HpL3-4-Δ#1 (#1), HpL3-4-Δ#2 (#2), and HpL3-4-Δ#3 (#3) cells were serum-deprived for 0 h (white bars) or 6 h (black bars). SOD activity was determined by xanthine-based spectrophotometric assay as described in Materials and methods. Results are expressed as units/mg protein. Differences where $p < 0.01$ (*) were compared with HpL3-4-EM cells serum-deprived for 6 h. Differences where $p < 0.01$ (+) were compared with HpL3-4-EM cells serum-deprived for 0 h.

To examine whether the susceptibility of each deletion mutants to serum deprivation was due to the activity of SOD, SOD activity in the presence and absence of serum was analysed in each deletion mutant (Fig. 7). HpL3-4-Δ#1 cells demonstrated significantly lower activity of SOD than HpL3-4-EM cells whereas HpL3-4-PrP and HpL3-4-Δ#3 cells showed significantly higher activity of SOD than HpL3-4-EM cells under serum deprivation for 6 h. In contrast, HpL3-4-Δ#2 cells showed SOD activity comparable with that of HpL3-4-EM cells.

Discussion

To date, the function of the cellular isoform of prion protein (PrP^C) remains unclear. In a previous analysis of PrP, it has been reported that removal of serum from the cell culture causes apoptosis in a *Prnp*-deficient immortalized hippocampal cell line but not in $Prnp^{-/-}$ cells transfected with *Prnp* [15]. Furthermore, deletion analysis of PrP^C demonstrated that deletion of the N-terminal OR of PrP^C enhances apoptosis under serum-free conditions [16]. The OR has been shown to be involved in copper binding [18], SOD-like activity of PrP^C [17], and neuroprotection for Bax-induced apoptosis [36]. However, the data presented here further suggest that the anti-apoptotic function of PrP^C can be regulated by not only the OR but also the N-terminal half of HR. Proteolytic cleavage is not involved in the anti-apoptotic activity of PrP^C because not only deletion of the N-terminal half of HR but also that of OR lead to inactivation of the anti-apoptotic function of PrP^C. This quantitative difference in the anti-apoptotic function of

PrP deletion mutants correlates with the level of SOD activity in transfectants expressing PrP deletion mutants. The data are consistent with the notion that both OR and N-terminal half of HR are necessary for activation of cellular SOD. The OR and HR of PrP are well conserved among mammals [25,37]. Therefore, the high evolutionary conservation of these regions is consistent with the notion that these regions are important domains for the function of PrP.

The ability of deletion mutants to endow the anti-apoptotic function of PrP accounts for the differences in the ability of the deletion mutants to exhibit cellular SOD activity. Recent paper by Cui et al. [38] has reported that amino acids 51–89 and 112–136 are necessary domains for SOD-like activity of PrP. However, as the SOD activity of PrP itself has been shown to be about 10-fold less than that of Cu/Zn SOD [17], it may not exert a crucial effect on cell death. Therefore, further studies on the mechanisms by which OR and N-terminal half of HR are required for SOD activation are warranted to fully elucidate the anti-apoptotic roles of PrP^C.

In summary, we have shown that PrP^C has an anti-apoptotic function for cells. The anti-apoptotic function of PrP^C is mediated by activation of cellular SOD. The OR and N-terminal half of HR are indispensable for SOD activation. Indeed, SOD activation is essential for neuroprotection, albeit our data do not exclude the possibility of additional sites of PrP^C action for neuroprotection such as the cAMP/PKA pathway [39] and Bax-regulated mitochondrial apoptosis cascade [36]. When animals are infected with prions, PrP^{Sc} is converted from PrP^C before being accumulated. The conversion of PrP^C to PrP^{Sc} leads to PrP^C deficiency [40], suggesting that not only a gain-of-function of PrP^{Sc} but also a loss-of-function of PrP^C contributes to the etiology of prion diseases. Our present studies suggest that $Prnp^{-/-}$ neuronal cells are susceptible to cell death due to reduced SOD activity induced by the absence of PrP^C signals. In short, alteration in the signals mediated by PrP^C may be due to another mechanism, whereby modification in the function of PrP might have contributed to prion diseases. Therefore, this suggests that pharmacological stimulation of PrP^C signals may be a useful approach in the treatment of prion disease.

Acknowledgments

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References

[1] S.B. Prusiner, Prions, Proc. Natl. Acad. Sci. USA 95 (1998) 13363–13383.
[2] H. Büeler, A. Aguzzi, A. Sailer, R.A. Greiner, P. Autenried, M. Aguet, C. Weissmann, Mice devoid of PrP are resistant to scrapie, Cell 73 (1993) 1339–1347.
[3] I. Tobler, S.E. Gaus, T. Deboer, P. Achermann, M. Fischer, T. Rulicke, M. Moser, B. Oesch, P.A. McBride, J.C. Manson, Altered circadian activity rhythms and sleep in mice devoid of prion protein, Nature 380 (1996) 639–642.
[4] J. Collinge, M.A. Whittington, K.C. Sidle, C.J. Smith, M.S. Palmer, A.R. Clarke, J.G. Jefferys, Prion protein is necessary for normal synaptic function, Nature 370 (1994) 295–297.
[5] R. Walz, O.B. Amaral, I.C. Rockenbach, R. Roesler, I. Izquierdo, E.A. Cavalheiro, V.R. Martins, R.R. Brentani, Increased sensitivity to seizures in mice lacking cellular prion protein, Epilepsia 40 (1999) 1679–1682.
[6] E. Graner, A.F. Mercadante, S.M. Zanata, O.V. Forlenza, A.L. Cabral, S.S. Veiga, M.A. Juliano, R. Roesler, R. Walz, A. Minetti, I. Izquierdo, V.R. Martins, R.R. Brentani, Cellular prion protein binds laminin and mediates neuritogenesis, Brain Res. Mol. Brain Res. 76 (2000) 85–92.
[7] S. Gauczynski, J.M. Peyrin, S. Haik, C. Leucht, C. Hundt, R. Rieger, S. Krasemann, J.P. Deslys, D. Dormont, C.I. Lasmezas, S. Weiss, The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein, EMBO J. 20 (2001) 5863–5875.
[8] R. Rieger, F. Edenhofer, C.I. Lasmezas, S. Weiss, The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells, Nat. Med. 3 (1997) 1383–1388.
[9] B. Oesch, D.B. Teplow, N. Stahl, D. Serban, L.E. Hood, S.B. Prusiner, Identification of cellular proteins binding to the scrapie prion protein, Biochemistry 29 (1990) 5848–5855.
[10] F. Edenhofer, R. Rieger, M. Famulok, W. Wendler, S. Weiss, E.L. Winnacker, Prion protein PrPc interacts with molecular chaperones of the Hsp60 family, J. Virol. 70 (1996) 4724–4728.
[11] G. Schmitt-Ulms, G. Legname, M.A. Baldwin, H.L. Ball, N. Bradon, P.J. Bosque, K.L. Crossin, G.M. Edelman, S.J. DeArmond, F.E. Cohen, S.B. Prusiner, Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein, J. Mol. Biol. 314 (2001) 1209–1225.
[12] C. Kurschner, J.I. Morgan, The cellular prion protein (PrP) selectively binds to Bcl-2 in the yeast two-hybrid system, Brain Res. Mol. Brain Res. 30 (1995) 165–168.
[13] G.I. Keshet, O. Bar-Peled, D. Yaffe, U. Nudel, R. Gabizon, The cellular prion protein colocalizes with the dystroglycan complex in the brain, J. Neurochem. 75 (2000) 1889–1897.
[14] G.I. Keshet, H. Ovadia, A. Taraboulos, R. Gabizon, Scrapie-infected mice and PrP knockout mice share abnormal localization and activity of neuronal nitric oxide synthase, J. Neurochem. 72 (1999) 1224–1231.
[15] C. Kuwahara, A.M. Takeuchi, T. Nishimura, K. Haraguchi, A. Kubosaki, Y. Matsumoto, K. Saeki, T. Yokoyama, S. Itohara, T. Onodera, Prions prevent neuronal cell-line death, Nature 400 (1999) 225–226.
[16] A. Sakudo, D.C. Lee, K. Saeki, Y. Nakamura, K. Inoue, Y. Matsumoto, S. Itohara, T. Onodera, Impairment of superoxide

dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line, Biochem. Biophys. Res. Commun. 308 (2003) 660–667.
[17] D.R. Brown, B.S. Wong, F. Hafiz, C. Clive, S.J. Haswell, I.M. Jones, Normal prion protein has an activity like that of superoxide dismutase, Biochem. J. 344 (Pt. 1) (1999) 1–5.
[18] D.R. Brown, K. Qin, J.W. Herms, A. Madlung, J. Manson, R. Strome, P.E. Fraser, T. Kruck, A. von Bohlen, W. Schulz-Schaeffer, A. Giese, D. Westaway, H. Kretzschmar, The cellular prion protein binds copper in vivo, Nature 390 (1997) 684–687.
[19] P.C. Pauly, D.A. Harris, Copper stimulates endocytosis of the prion protein, J. Biol. Chem. 273 (1998) 33107–33110.
[20] S.M. Zanata, M.H. Lopes, A.F. Mercadante, G.N. Hajj, L.B. Chiarini, R. Nomizo, A.R. Freitas, A.L. Cabral, K.S. Lee, M.A. Juliano, E. de Oliveira, S.G. Jachieri, A. Burlingame, L. Huang, R. Linden, R.R. Brentani, V.R. Martins, Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection, EMBO J. 21 (2002) 3307–3316.
[21] A. Sakudo, D.C. Lee, E. Yoshimura, S. Nagasaka, K. Nitta, K. Saeki, Y. Matsumoto, S. Lehmann, S. Itohara, S. Sakaguchi, T. Onodera, Prion protein suppresses perturbation of cellular copper homeostasis under oxidative conditions, Biochem. Biophys. Res. Commun. 313 (2004) 850–855.
[22] A. Sakudo, D.C. Lee, K. Saeki, Y. Matsumoto, S. Itohara, T. Onodera, Tumor necrosis factor attenuates prion protein-deficient neuronal cell death by increases in anti-apoptotic Bcl-2 family proteins, Biochem. Biophys. Res. Commun. 310 (2003) 725–729.
[23] C. Korth, B. Stierli, P. Streit, M. Moser, O. Schaller, R. Fischer, W. Schulz-Schaeffer, H. Kretzschmar, A. Raeber, U. Braun, F. Ehrensperger, S. Hornemann, R. Glockshuber, R. Riek, M. Billeter, K. Wuthrich, B. Oesch, Prion (PrPSc)-specific epitope defined by a monoclonal antibody, Nature 390 (1997) 74–77.
[24] K. Peoc'h, C. Serres, Y. Frobert, C. Martin, S. Lehmann, S. Chasseigneaux, V. Sazdovitch, J. Grassi, P. Jouannet, J.M. Launay, J.L. Laplanche, The human "prion-like" protein Doppel is expressed in both Sertoli cells and spermatozoa, J. Biol. Chem. 277 (2002) 43071–43078.
[25] H.M. Schatzl, M. Da Costa, L. Taylor, F.E. Cohen, S.B. Prusiner, Prion protein gene variation among primates, J. Mol. Biol. 245 (1995) 362–374.
[26] R.S. Hegde, J.A. Mastrianni, M.R. Scott, K.A. DeFea, P. Tremblay, M. Torchia, S.J. DeArmond, S.B. Prusiner, V.R. Lingappa, A transmembrane form of the prion protein in neurodegenerative disease, Science 279 (1998) 827–834.
[27] S.G. Chen, D.B. Teplow, P. Parchi, J.K. Teller, P. Gambetti, L. Autilio-Gambetti, Truncated forms of the human prion protein in normal brain and in prion diseases, J. Biol. Chem. 270 (1995) 19173–19180.
[28] D.R. Brown, B. Schmidt, H.A. Kretzschmar, Role of microglia and host prion protein in neurotoxicity of a prion protein fragment, Nature 380 (1996) 345–347.
[29] G. Forloni, N. Angeretti, R. Chiesa, E. Monzani, M. Salmona, O. Bugiani, F. Tagliavini, Neurotoxicity of a prion protein fragment, Nature 362 (1993) 543–546.
[30] R.C. Moore, I.Y. Lee, G.L. Silverman, P.M. Harrison, R. Strome, C. Heinrich, A. Karunaratne, S.H. Pasternak, M.A. Chishti, Y. Liang, P. Mastrangelo, K. Wang, A.F. Smit, S. Katamine, G.A. Carlson, F.E. Cohen, S.B. Prusiner, D.W. Melton, P. Tremblay, L.E. Hood, D. Westaway, Ataxia in prion protein (PrP)-deficient mice is associated with upregulation of the novel PrP-like protein Doppel, J. Mol. Biol. 292 (1999) 797–817.
[31] A. Li, S. Sakaguchi, R. Atarashi, B.C. Roy, R. Nakaoke, K. Arima, N. Okimura, J. Kopacek, K. Shigematsu, Identification of a novel gene encoding a PrP-like protein expressed as chimeric transcripts fused to PrP exon 1/2 in ataxic mouse line with a disrupted PrP gene, Cell. Mol. Neurobiol. 20 (2000) 553–567.
[32] B. Vincent, E. Paitel, P. Saftig, Y. Frobert, D. Hartmann, B. De Strooper, J. Grassi, E. Lopez-Perez, F. Checler, The disintegrins ADAM10 and TACE contribute to the constitutive and phorbol ester-regulated normal cleavage of the cellular prion protein, J. Biol. Chem. 276 (2001) 37743–37746.
[33] A. Barret, F. Tagliavini, G. Forloni, C. Bate, M. Salmona, L. Colombo, A. De Luigi, L. Limido, S. Suardi, G. Rossi, F. Auvre, K.T. Adjou, N. Sales, A. Williams, C. Lasmezas, J.P. Deslys, Evaluation of quinacrine treatment for prion diseases, J. Virol. 77 (2003) 8462–8469.
[34] O. Milhavet, S. Lehmann, Oxidative stress and the prion protein in transmissible spongiform encephalopathies, Brain Res. Brain Res. Rev. 38 (2002) 328–339.
[35] W.S. Perera, N.M. Hooper, Proteolytic fragmentation of the murine prion protein: role of Tyr-128 and His-177, FEBS Lett. 463 (1999) 273–276.
[36] Y. Bounhar, Y. Zhang, C.G. Goodyer, A. LeBlanc, Prion protein protects human neurons against Bax-mediated apoptosis, J. Biol. Chem. 276 (2001) 39145–39149.
[37] F. Wopfner, G. Weidenhofer, R. Schneider, A. von Brunn, S. Gilch, T.F. Schwarz, T. Werner, H.M. Schatzl, Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein, J. Mol. Biol. 289 (1999) 1163–1178.
[38] T. Cui, M. Daniels, B.S. Wong, R. Li, M.S. Sy, J. Sassoon, D.R. Brown, Mapping the functional domain of the prion protein, Eur. J. Biochem. 270 (2003) 3368–3376.
[39] L.B. Chiarini, A.R. Freitas, S.M. Zanata, R.R. Brentani, V.R. Martins, R. Linden, Cellular prion protein transduces neuroprotective signals, EMBO J. 21 (2002) 3317–3326.
[40] T. Yokoyama, K.M. Kimura, Y. Ushiki, S. Yamada, A. Morooka, T. Nakashiba, T. Sassa, S. Itohara, In vivo conversion of cellular prion protein to pathogenic isoforms, as monitored by conformation-specific antibodies, J. Biol. Chem. 276 (2001) 11265–11271.
[41] N. Stahl, D.R. Borchelt, K. Hsiao, S.B. Prusiner, Scrapie prion protein contains a phosphatidylinositol glycolipid, Cell 51 (1987) 229–240.

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PrP cooperates with STI1 to regulate SOD activity in PrP-deficient neuronal cell line

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Abstract

Cellular prion protein (PrP^C) plays anti-apoptotic and anti-oxidative roles in apoptosis induced by serum deprivation in an immortalized prion protein gene (*Prnp*)-deficient neuronal cell line. The octapeptide repeat region (OR) and N-terminal half of the hydrophobic region (HR) of PrP^C are indispensable for PrP^C activity, but the mechanisms remain unclear. In the present study, elucidation of the mechanisms by which PrP^C elicits the anti-oxidative activities was facilitated by evidence of stress-inducible protein 1 (STI1) mediating PrP^C-dependent superoxide dismutase (SOD) activation. Immunoprecipitation revealed that PrP^C was associated with STI1. The inhibitory peptides against PrP^C–STI1 binding [STI1 pep.1 and PrP(113–132)] indicated toxic activity in PrP^C-expressing cells by inhibiting SOD activity but not in $Prnp^{-/-}$ cells. Furthermore, OR and N-terminal half of the HR were required for the inhibitory effect of PrP(113–132) but not STI1 pep.1. These data are consistent with results established with a model where OR and N-terminal half of the HR mediate the action of STI1 upon cell survival and upregulation of SOD activity.

Keywords: Prion disease; Prion protein; Apoptosis; PrP-deficient cell line; STI1

Transmissible spongiform encephalopathies (TSE) are fatal neurological disorders that include the Creutzfeldt–Jakob disease and Gerstmann–Sträussler Scheinker syndrome in humans, scrapie in sheep and goats, and bovine spongiform encephalopathy in cattle [1]. The pathological characteristics of the diseases encompass neuronal cell loss, vacuolation, astrocytosis, and amyloid plaques in the brain [1]. After prion infection, cellular prion protein (PrP^C) is converted into an abnormal isoform of prion protein (PrP^{Sc}), which has been proposed to be responsible for the disease [1]. Therefore, PrP^C is hypothetically required to induce the disease and eventually provoke neural damages. The fact that prion protein (PrP) gene (*Prnp*)-knockout mice are resistant to infectivity and toxicity induced by pathogenic inoculates [2] is clear evidence of such a hypothesis.

Several reports are devoted to the putative physiological function of PrP^C. Aberrant circadian rhythms [3], electrophysiological abnormalities [4], and high susceptibility to seizure [5] in $Prnp^{-/-}$ mice have been reported. Recently, participation of PrP^C in the inhibition of apoptosis has been demonstrated. Although removal of serum from cell cultures causes apoptosis in *Prnp*-deficient immortalized hippocampal neuronal cells, transfection

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of *Prnp* suppresses the apoptosis of *Prnp*$^{-/-}$ cells under serum-free conditions [6]. We have further demonstrated that reintroduction of *Prnp* upregulates superoxide dismutase (SOD) activity and inhibits superoxide generation, suggesting that PrP^{C} suppresses apoptosis by upregulation of SOD activity [7]. However, PrP^{C} lacking an octapeptide repeat region (OR) or an N-terminal half of the hydrophobic region (HR) loses its anti-apoptotic and anti-oxidative functions [7,8]. Several potential mediators of PrP^{C} signals have been recently reported. Copper specifically binds the OR of PrP^{C} [9] and enhances endocytosis of PrP^{C} [10]. A PrP^{C}-binding molecule, stress-inducible protein 1 (STI1), binds with amino acid residues 113–128 located in the N-terminal half of HR of PrP^{C} [11]. These studies prompted us to perform additional studies in order to determine how the STI1 might contribute to PrP^{C}-dependent anti-oxidative signaling.

To investigate whether the STI1 is important for the biological activities displayed by PrP^{C}, the effect of the inhibitory peptides against PrP^{C}–STI1 binding on *Prnp*$^{-/-}$ cells was compared to that on PrP^{C}-expressing *Prnp*$^{-/-}$ cells under serum-free conditions. The inhibitory peptides are toxic to PrP^{C}-expressing cells by inhibiting the SOD activity, although such is not the case for *Prnp*$^{-/-}$ cells. Furthermore, immunoprecipitation indicated that STI1 interacted with PrP^{C} in PrP^{C}-expressing cells. Therefore, we propose that STI1 involves in PrP^{C}-dependent SOD activation that can inhibit apoptosis.

Materials and methods

Cell cultures and animals. Murine *Prnp*-deficient neuronal cells HpL3-4 [6] and transfectants including HpL3-4-EM [8], HpL3-4-PrP [8], HpL3-4-Δ#1 [8], HpL3-4-Δ#2 [8], and HpL3-4-Δ#3 [8] cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified 5% CO_2 incubator. Unless otherwise specified, serum deprivation was performed as previously described [7]. Occasionally, peptides [PrP(113–132) peptide: GAAAAGAVVGGLGGYMLGSA corresponding to p10 peptide [11] and residues 113–132 of mouse PrP; STI1 pep.1: ELGNDAYKKKDFDKAL corresponding to residues 230–245 of mouse STI1 [11]] synthesized and purified by American Peptide Company (Sunnyvale, CA) were added to the media for inhibition of PrP^{C}–STI1 binding.

Preparation of cellular fractions. To fractionate cell samples, the cell homogenate was prepared in phosphate-buffered saline (PBS) by sonication. After centrifugation at 600*g* for 15 min at 4 °C, supernatants were further ultracentrifuged at 200,000*g* for 1 h at 4 °C. The pellets and supernatants were solubilized in a radio-immunoprecipitation assay (RIPA) buffer to yield membrane and soluble fractions. The RIPA buffer was composed of 10 mM Tris-HCl (pH 7.4) containing 1% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 0.15 M sodium chloride supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF).

SOD activity assay. Cells were sonicated in ice-cold RIPA buffer supplemented with PMSF and centrifuged at 15,000*g* for 5 min at 4 °C. Protein concentrations of the supernatants were measured by the DC protein assay (Bio-Rad, Hercules, CA). Each protein extract (20 μg) was assayed by the SOD assay kit-WST (Dojindo, Kumamoto, Japan). The SOD activity was compared with 1 U of bovine erythrocyte Cu/Zn-SOD (Sigma S2515) activity and estimated using the standard curve of SOD activity versus absorbance at 450 nm. The SOD activity was expressed as U/mg protein.

Immunoprecipitation. HpL3-4-EM and HpL3-4-PrP cells were resuspended in RIPA buffer, sonicated on ice, and centrifuged at 15,000*g* for 5 min at 4 °C. The supernatants were precleared using protein G–Sepharose beads suspension (Amersham–Pharmacia Biotech, Piscataway, NJ) for 1 h at 4 °C, and then incubated with 2.5 μg SAF32 for 1 h at 4 °C. Next, 20 μl of protein G–Sepharose beads suspension was added to each of the immunocomplexes and the mixtures were rotated for 1 h at 4 °C. After washing four times with the RIPA buffer, the immunoprecipitated proteins were subjected to SDS–PAGE and Western blotting.

Western blot assay. The Western blot assay was performed as described previously [12]. Briefly, cell lysates were prepared in RIPA buffer. The protein concentration was measured using the Bio-Rad DC assay, and SDS/polyacrylamide gel electrophoresis was conducted before electrical transfer onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham–Pharmacia Biotech). PrP, STI1 or Cu/Zn-SOD was detected as described previously [12] with anti-PrP 6H4 (Prionics, Zürich, Switzerland) [13], anti-PrP SAF83 (SPI Bio, Montigny le Bretonneux, France), anti-recombinant mSTI1 [11] or anti-Cu/Zn-SOD (Stressgen, Victoria, BC) antibody and horseradish peroxidase-conjugated secondary antibody. The probed proteins were detected using an enhanced chemiluminescence detection kit (Amersham–Pharmacia Biotech).

Cell survival assay. In cell survival assays, cells were seeded on 96-well plates at 5000 cells/well. Two days later, cells were washed twice with serum-free DMEM followed by incubation in serum-free DMEM or 10% FCS–DMEM. Incubation of PrP(113–132) or STI1 pep.1 peptides was carried out in serum-free DMEM plus peptides. Viable cell counts were estimated by the Tetra Color One cell proliferation assay system (Seikagaku Kogyo, Tokyo, Japan). In this assay system, the sodium salt of 4-[3-(iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate, which is a water-soluble tetrazolium (WST), was utilized to count the viable cells. It has been reported that the cell viability assay using WST produces formazan dye that correlates linearly with the number of viable cells over the range of 1000–50,000 cells/well [14]. As such, cells were treated with WST (10 μl/well) before further incubation for 4 h. Absorbance at 450 nm was measured to quantify the level of formazan by a microplate reader (Bio-Rad).

Results and discussion

To examine the mechanism by which PrP^{C} prevents apoptosis and upregulates SOD activity, we investigated whether STI1 (reported to bind PrP^{C} in the HR [11]) could mediate PrP-dependent SOD activation. We first examined the localization of PrP^{C}, STI1, and Cu/Zn-SOD in HpL3-4 cells in the absence or presence of PrP^{C}, followed by analysis of the distributions of PrP^{C}, STI1, and Cu/Zn-SOD by cellular fractionation. The resulting precipitate after ultracentrifugation (200,000*g* for 1 h at 4 °C) of the cell homogenate (considered to be the membrane fraction) and the supernatant (the soluble fraction) were subjected to Western blot analysis with anti-PrP 6H4, anti-recombinant STI1, and anti-Cu/Zn-SOD antibody (Fig. 1). PrP^{C} was detected only in the membrane fraction of HpL3-4-PrP cells, whereas Cu/

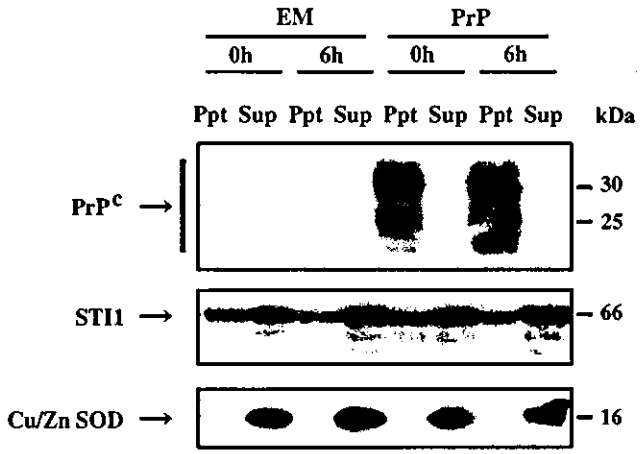


Fig. 1. Localization of STI1 is not altered by PrP expression. STI1 (66 kDa) was detected in fractionated membrane fraction (Ppt) and soluble fraction (Sup) in HpL3-4-EM and HpL3-4-PrP cells previously subjected to serum deprivation for 0 or 6 h (see Materials and methods). The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and detected with anti-PrP 6H4, anti-mSTI1 or anti-Cu/Zn SOD antibody.

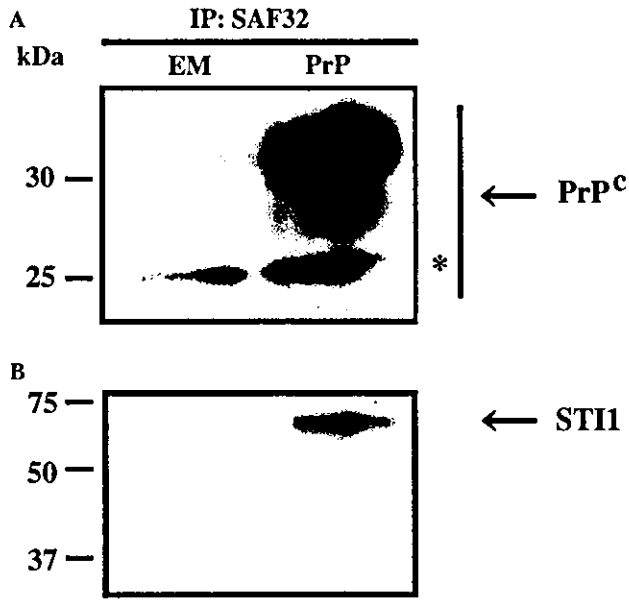


Fig. 2. PrP^C interacts with STI1. HpL3-4-EM and HpL3-4-PrP cells were immunoprecipitated with anti-PrP SAF32. The immunoprecipitates were then immunoblotted with anti-PrP SAF83 (A) or anti-recombinant STI1 (B) antibody. The asterisk represents the immunoglobulin light chain.

Zn-SOD was detected in the soluble fraction of HpL3-4-EM and HpL3-4-PrP cells. STI1 was detected in both the membrane and soluble fractions in HpL3-4-EM and HpL3-4-PrP cells in the absence and presence of serum. The expression level and ratio of STI1 protein in the soluble fraction were compared with those in the membrane fraction in HpL3-4-EM and HpL3-4-PrP cells. STI1 was detected as doublet or triplet with lower molecular weight bands, which mobilized from the membrane to the soluble fraction upon serum deprivation in HpL3-4-EM and HpL3-4-PrP cells, suggesting the dephosphorylated form of STI1 protein [15–17]. We next examined if PrP^C would bind to STI1 in HpL3-4-PrP cells. Interestingly, coimmunoprecipitation with SAF32 (Fig. 2) indicated that PrP^C bound to STI1 in HpL3-4-PrP cells but not HpL3-4-EM cells.

The PrP(113–132) peptide (corresponding to p10 peptide [11] that contains the STI1-binding domain, i.e., amino acid residues 113–128 of mouse PrP [11]) and STI1 pep.1 peptide (corresponding to amino acid residues 230–245 of STI1 [11] or the PrP^C-binding sites) were used for the third analysis. The PrP(113–132) and STI1 pep.1 peptides have already been reported to inhibit PrP^C–STI1 interaction, and STI1 pep.1 reduces anisomycin-induced cell death in retinal explants from neonatal mice and rats [11]. STI1 pep.1 and PrP(113–132) peptides significantly inhibited SOD activity of HpL3-4-PrP cells but not HpL3-4-EM cells (Fig. 3). Furthermore, STI1 pep.1 and PrP(113–132) peptides inhibited the cell survival in HpL3-4-PrP cells without influencing the cell survival of HpL3-4-EM cells (Fig. 4). The effect of peptides on cell survival of HpL3-4 cells expressing deletion mutants was also investigated (Fig. 4). STI1 pep.1 peptide inhibited cell survival of HpL3-

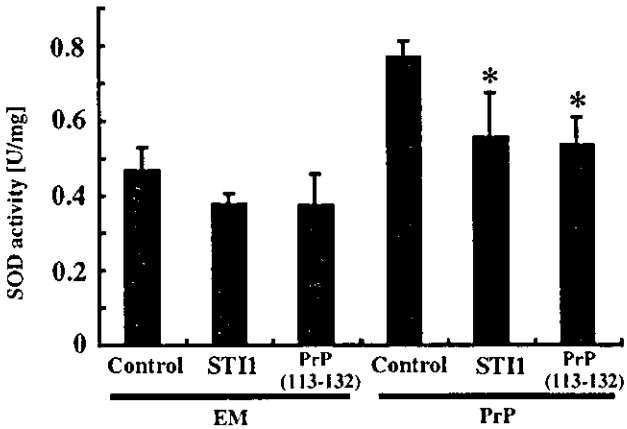
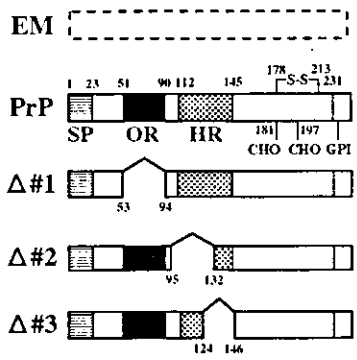


Fig. 3. Inhibitory effect of STI1 and PrP^C-binding peptide on SOD activity. The SOD activities of HpL3-4-EM and HpL3-4-PrP cells previously serum-deprived for 6 h in the absence (Control) or presence of STI1 pep.1 (5 μM) or PrP(113–132) peptide (4 μM) were measured as described in Materials and methods. Values are expressed as means ± SEM ($N = 4$). Differences where $p < 0.01$ (*) versus Control were considered significant when verified by the non-repeated measures ANOVA followed by the Bonferroni correction.

4 cells expressing PrP with removal of the OR (HpL3-4-Δ#1), the N-terminal half of HR (HpL3-4-Δ#2) or the C-terminal half of HR (HpL3-4-Δ#3). In contrast, PrP(113–132) peptide inhibited cell survival of HpL3-4-Δ#3 cells, while the peptide did not inhibit those of HpL3-4-Δ#1 and HpL3-4-Δ#2 cells.

Although numerous attempts to elucidate the prion-related pathogenic mechanism(s), limited data concerning the putative physiological function of PrP^C have



	Inhibition [%]	
	STI1	PrP(113-132)
EM	4.6	0
PrP	100	100
Δ#1	84.1	26.5
Δ#2	69.9	0
Δ#3	141.4	167.5

Fig. 4. Inhibitory effect of STI1 and PrP^C-binding peptide on cell death. Schematic presentations of PrP deletion mutants of mouse PrP [PrP: wild-type PrP; Δ#1: PrP(Δ53–94, Q52H); Δ#2: PrP(Δ95–132); and Δ#3: PrP(Δ124–146)] are shown on the left. Schematic locations of the deletions as compared with the wild-type protein are shown by a space within the bar next to the indicated protein. Numbers refer to the amino acid residues in the mouse PrP sequence. The disulfides (S–S), two Asn-linked glycosylation sites (CHO), signal peptide sequence (SP), octapeptide repeat region (OR), hydrophobic region (HR), and glycosylphosphatidylinositol anchor (GPI) are shown. HpL3-4 cells expressing wild-type PrP (PrP: HpL3-4-PrP), PrP(Δ53–94, Q52H) (Δ#1: HpL3-4-Δ#1), PrP(Δ95–132) (Δ#2: HpL3-4-Δ#2), PrP(Δ124–146) (Δ#3: HpL3-4-Δ#3) or the empty vector per se (EM: HpL3-4-EM) cells were serum-deprived at 6.25 μM STI1 pep.1 or PrP(113–132) peptides. The inhibitory effect of the peptides on cell survival was examined 48 h later by the Tetra Color One cell proliferation assay. The rate of inhibition of HpL3-4-PrP cells by STI1 pep.1 or PrP(113–132) peptide was taken as 100%, respectively.

been documented. In a previous analysis of PrP, we have reported that serum deprivation of cell cultures induces apoptosis in a *Prnp*-deficient immortalized hippocampal cell line but not in *Prnp*-transfected $Prnp^{-/-}$ cells [6]. Furthermore, deletion analysis of PrP^C has demonstrated that deletion of the OR or N-terminal half of the HR induces apoptosis under serum-free conditions [7,8]. As the OR and HR of PrP are well conserved among mammals [18,19], the high evolutionary conservation of these regions is consistent with the notion that these regions are the important domains for PrP functions.

In this study, we investigated the possibility of PrP^C regulating the cellular SOD activity by interaction with STI1, which binds HR of PrP [11]. In addition, PrP has recently been reported to mediate neuroprotective signals [11]. While Lassle et al. [15] have localized murine STI1 in cytoplasm of NIH3T3 cells, Zanata et al. [11] have located STI1, at least in part, in the cell membrane of HEK293T cells. In the present study, STI1 was detected in the soluble and membrane fractions of HpL3-4-EM and HpL3-4-PrP cells. Furthermore, STI1 pep.1 and PrP(113–132) peptide, which inhibit PrP^C–STI1 binding [11], prevented the SOD activity and promoted cell death in HpL3-4-PrP but not in HpL3-4-EM cells. Moreover, immunoprecipitation demonstrated that PrP^C was associated with STI1 in HpL3-4-PrP cells. These results suggest two possibilities: (i) direct interaction between PrP and STI1 involves PrP-dependent SOD activation, leading to the inhibition of cell death, and (ii) copper may transduce PrP-dependent SOD activation mediated by STI1. In (i), as a transmembrane domain or a signal-peptide domain was not found in STI1, the mechanism(s) of STI1 interaction with PrP^C remains unclear. Both STI1 and PrP^C could be phosphorylated, and phosphorylation may therefore be related to the signal transduction [15,20]. As PrR peptide, which has a high resemblance to the hydropathic profile of the STI1-binding site for PrP, transduces the cAMP/protein kinase A (PKA) pathway [21], STI1 probably mediates the cAMP/PKA pathway by phosphorylation. As for (ii), it is supported by the important roles played by copper in PrP^C and Cu/Zn-SOD activity [22,23]. Close coordination with the latter recent data from our laboratories has suggested that PrP^C stabilizes cellular copper concentration under oxidative conditions [24]. Furthermore, as not only STI1-binding site but also OR is needed for STI1-mediated anti-apoptotic signal, suggesting that copper binding to PrP plays essential role for the signal. However, the binding of copper to recombinant PrP does not influence its ability to interact with STI1 [11]. Therefore, further studies on the role(s) of STI1–PrP^C interaction in relation with copper are warranted to fully elucidate the anti-apoptotic functions of PrP^C. Interestingly, PrP(113–132) peptide did not inhibit the cell survival of HpL3-4-Δ#1 and HpL3-4-Δ#2 cells. These results suggest that not only N-terminal half of HR, which includes STI1-binding site, but also OR are required for STI1-mediated anti-apoptotic signals. Moreover, as STI1 pep.1 but not PrP(113–132) peptide inhibited cell survival of HpL3-4 cells expressing all of the PrP deletion mutants, PrP^C suggests to mediate the action of STI1 upon cell survival but not vice versa. Finally, it should be noted that the concentration of pep.1 and PrP (113-132) peptide needed in interaction as an interfering peptide to induce a neuroprotective effect on nervous tissues is lower than those required to induce pro-degenerative effects in our in vitro study [11]. According to the results, it is possible that the reaction against the interfering peptides may depend on the type of cells or neurons.

In summary, PrP^C indicated an anti-apoptotic function in cells [6–8]. The anti-apoptotic function of PrP^C is mediated by upregulation of cellular SOD, while STI1 is involved in the PrP^C-dependent SOD activation. This activation is abrogated by STI1 pep.1 and PrP(113–132) peptide inhibiting the PrP^C–STI1 binding, as a result of direct interaction between PrP^C and STI1 (because PrP^C associates with STI1 in this cell system). Furthermore, the OR and N-terminal half of HR, which harbor the STI1-binding site, are indispensable for

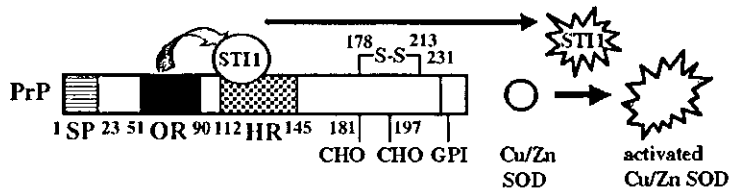


Fig. 5. Model of PrP^C-dependent SOD activation. After STI1 binds to N-terminal half of hydrophobic region (HR) on PrP^C, STI1 may be activated by octapeptide repeat region (OR), because both the OR and HR are indispensable for PrP(113–132) peptide inhibition (Fig. 4) and SOD activation [8]. PrP^C is localized to membrane, whereas STI1 to membrane and cytosol. Therefore, STI1 may be activated by PrP^C in membrane. The activated STI1 may shift to cytosol and activate Cu/Zn-SOD. The mechanisms of activation of STI1 by PrP^C or those of Cu/Zn-SOD by STI1 remain unknown. Copper might play an important role in the mechanisms of PrP^C-dependent activation of STI1 and Cu/Zn-SOD because: (1) activity of Cu/Zn-SOD is regulated by copper incorporation [23], and (2) copper enhances endocytosis of PrP^C [10]. PrP^C prevents apoptotic cell death at least in part by upregulating Cu/Zn-SOD activity [7].

PrP(113–132) peptide inhibition and anti-apoptotic function, suggesting that not only PrP^C–STI1 binding but also the presence of the OR of PrP^C is essential for STI1-mediated anti-apoptotic signals. The data are consistent with the hypothesis that after STI1 has bound to HR, STI1 is then activated by the OR to eventually mediate PrP-dependent SOD activation (Fig. 5). When animals are infected with prions, PrP^{Sc} is converted from PrP^C before being accumulated. The conversion of PrP^C to PrP^{Sc} leads to PrP^C deficiency [25], suggesting that not only a gain-of-function of PrP^{Sc} but also a loss-of-function of PrP^C contributes etiologically to induction of prion diseases. Our present studies suggest that susceptibility of $Prnp^{-/-}$ neuronal cells to cell death is probably due to reduced SOD activity induced by the absence of PrP^C–STI1 signals. In short, alteration of the signals mediated by PrP^C–STI1 binding may be due to another mechanism, whereby functional modification of PrP might have contributed to induction of prion diseases. Therefore, pharmacological stimulation of PrP^C–STI1 signals may serve as a useful approach in the treatment of prion disease.

Acknowledgments

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References

[1] S.B. Prusiner, Prions, Proc. Natl. Acad. Sci. USA 95 (1998) 13363–13383.
[2] H. Bueler, A. Aguzzi, A. Sailer, R.A. Greiner, P. Autenried, M. Aguet, C. Weissmann, Mice devoid of PrP are resistant to scrapie, Cell 73 (1993) 1339–1347.
[3] I. Tobler, S.E. Gaus, T. Deboer, P. Achermann, M. Fischer, T. Rulicke, M. Moser, B. Oesch, P.A. McBride, J.C. Manson, Altered circadian activity rhythms and sleep in mice devoid of prion protein, Nature 380 (1996) 639–642.
[4] J. Collinge, M.A. Whittington, K.C. Sidle, C.J. Smith, M.S. Palmer, A.R. Clarke, J.G. Jefferys, Prion protein is necessary for normal synaptic function, Nature 370 (1994) 295–297.
[5] R. Walz, O.B. Amaral, I.C. Rockenbach, R. Roesler, I. Izquierdo, E.A. Cavalheiro, V.R. Martins, R.R. Brentani, Increased sensitivity to seizures in mice lacking cellular prion protein, Epilepsia 40 (1999) 1679–1682.
[6] C. Kuwahara, A.M. Takeuchi, T. Nishimura, K. Haraguchi, A. Kubosaki, Y. Matsumoto, K. Saeki, T. Yokoyama, S. Itohara, T. Onodera, Prions prevent neuronal cell-line death, Nature 400 (1999) 225–226.
[7] A. Sakudo, D.C. Lee, K. Saeki, Y. Nakamura, K. Inoue, Y. Matsumoto, S. Itohara, T. Onodera, Impairment of superoxide dismutase activation by N-terminally truncated prion protein (PrP) in PrP-deficient neuronal cell line, Biochem. Biophys. Res. Commun. 308 (2003) 660–667.
[8] A. Sakudo, D.C. Lee, T. Nishimura, S. Li, S. Tsuji, T. Nakamura, Y. Matsumoto, K. Saeki, S. Itohara, K. Ikuta, T. Onodera, Octapeptide repeat region and N-terminal half of hydrophobic region of prion protein (PrP) mediates PrP-dependent activation of superoxide dismutase, Biochem. Biophys. Res. Commun. 326 (2005) 600–606.
[9] D.R. Brown, K. Qin, J.W. Herms, A. Madlung, J. Manson, R. Strome, P.E. Fraser, T. Kruck, A. von Bohlen, W. Schulz-Schaeffer, A. Giese, D. Westaway, H. Kretzschmar, The cellular prion protein binds copper in vivo, Nature 390 (1997) 684–687.
[10] P.C. Pauly, D.A. Harris, Copper stimulates endocytosis of the prion protein, J. Biol. Chem. 273 (1998) 33107–33110.
[11] S.M. Zanata, M.H. Lopes, A.F. Mercadante, G.N. Hajj, L.B. Chiarini, R. Nomizo, A.R. Freitas, A.L. Cabral, K.S. Lee, M.A. Juliano, E. de Oliveira, S.G. Jachieri, A. Burlingame, L. Huang, R. Linden, R.R. Brentani, V.R. Martins, Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection, EMBO J. 21 (2002) 3307–3316.
[12] A. Sakudo, D.C. Lee, K. Saeki, Y. Matsumoto, S. Itohara, T. Onodera, Tumor necrosis factor attenuates prion protein-deficient neuronal cell death by increases in anti-apoptotic Bcl-2 family proteins, Biochem. Biophys. Res. Commun. 310 (2003) 725–729.
[13] C. Korth, B. Stierli, P. Streit, M. Moser, O. Schaller, R. Fischer, W. Schulz-Schaeffer, H. Kretzschmar, A. Raeber, U. Braun, F. Ehrensperger, S. Hornemann, R. Glockshuber, R. Riek, M. Billeter, K. Wuthrich, B. Oesch, Prion (PrPSc)-specific epitope defined by a monoclonal antibody, Nature 390 (1997) 74–77.

[14] M. Ishiyama, H. Tominaga, M. Shiga, K. Sasamoto, Y. Ohkura, K. Ueno, A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet, Biol. Pharm. Bull. 19 (1996) 1518–1520.
[15] M. Lassle, G.L. Blatch, V. Kundra, T. Takatori, B.R. Zetter, Stress-inducible, murine protein mSTI1. Characterization of binding domains for heat shock proteins and in vitro phosphorylation by different kinases, J. Biol. Chem. 272 (1997) 1876–1884.
[16] G.L. Blatch, M. Lassle, B.R. Zetter, V. Kundra, Isolation of a mouse cDNA encoding mSTI1, a stress-inducible protein containing the TPR motif, Gene 194 (1997) 277–282.
[17] V.M. Longshaw, H.W. Dirr, G.L. Blatch, M. Lassle, The in vitro phosphorylation of the co-chaperone mSTI1 by cell cycle kinases substantiates a predicted casein kinase II-p34cdc2-NLS (CcN) motif, Biol. Chem. 381 (2000) 1133–1138.
[18] F. Wopfner, G. Weidenhofer, R. Schneider, A. von Brunn, S. Gilch, T.F. Schwarz, T. Werner, H.M. Schatzl, Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein, J. Mol. Biol. 289 (1999) 1163–1178.
[19] H.M. Schatzl, M. Da Costa, L. Taylor, F.E. Cohen, S.B. Prusiner, Prion protein gene variation among primates, J. Mol. Biol. 245 (1995) 362–374.
[20] A. Negro, F. Meggio, A. Bertoli, R. Battistutta, M.C. Sorgato, L.A. Pinna, Susceptibility of the prion protein to enzymic phosphorylation, Biochem. Biophys. Res. Commun. 271 (2000) 337–341.
[21] L.B. Chiarini, A.R. Freitas, S.M. Zanata, R.R. Brentani, V.R. Martins, R. Linden, Cellular prion protein transduces neuroprotective signals, EMBO J. 21 (2002) 3317–3326.
[22] D.R. Brown, W.J. Schulz-Schaeffer, B. Schmidt, H.A. Kretzschmar, Prion protein-deficient cells show altered response to oxidative stress due to decreased SOD-1 activity, Exp. Neurol. 146 (1997) 104–112.
[23] P.C. Wong, D. Waggoner, J.R. Subramaniam, L. Tessarollo, T.B. Bartnikas, V.C. Culotta, D.L. Price, J. Rothstein, J.D. Gitlin, Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase, Proc. Natl. Acad. Sci. USA 97 (2000) 2886–2891.
[24] A. Sakudo, D.C. Lee, E. Yoshimura, S. Nagasaka, K. Nitta, K. Saeki, Y. Matsumoto, S. Lehmann, S. Itohara, S. Sakaguchi, T. Onodera, Prion protein suppresses perturbation of cellular copper homeostasis under oxidative conditions, Biochem. Biophys. Res. Commun. 313 (2004) 850–855.
[25] T. Yokoyama, K.M. Kimura, Y. Ushiki, S. Yamada, A. Morooka, T. Nakashiba, T. Sassa, S. Itohara, In vivo conversion of cellular prion protein to pathogenic isoforms, as monitored by conformation-specific antibodies, J. Biol. Chem. 276 (2001) 11265–11271.