GPI-anchorless human prion protein is secreted and glycosylated but lacks superoxide dismutase activity

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Abstract. Prion protein (PrP) is a glycosylphosphatidylinositol (GPI)-anchored membrane protein that is thought to play a role in anti-oxidative stress. It remains controversial whether PrP elicits superoxide dismutase (SOD) activity itself or indirectly by activating cellular SOD. Our previous studies showed that soluble PrP produced by a baculovirus expression system did not exhibit any SOD activity in a marginally glycosylated form. In the present study, we developed a mammalian expression system for a truncated soluble form of human prion protein with the native signal peptide but without a GPI-anchor site, driven by the peptide chain elongation factor 1α promoter in stably transfected rabbit-kidney epithelial RK13 cells, to investigate the SOD activity of mammalian PrP. This recombinant product, denoted sPrP, is secreted in large quantities in medium and can be isolated in very high purity and yield (more than 1 mg sPrP per 2 litres medium). Characterization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and tunicamycin treatment revealed that a fully glycosylated form of sPrP was secreted from the cells. SOD activity in cell lysate showed a decrease in sPrP-expressing RK13 cells and an increase in wild-type PrP-expressing RK13 cells compared to empty vector-transfected RK13 cells or parent cells. Purified or immunoprecipitated sPrP did not show any SOD activity. In conclusion, the GPI-anchor site, but not glycosylation, appears to be essential for the secretion of PrP. In addition, mammalian PrP itself does not act as a functional SOD enzyme.

Introduction

Cellular prion protein (PrP^C) is a glycosylphosphatidylinositol (GPI)-anchored protein present at the surface of cells (1). PrP^C is widely distributed in a variety of tissues, and although especially abundant in neurons, it is also expressed in non-neuronal cells (1). Experiments with cell culture models have shown that PrP^C has anti-oxidative roles (1). Moreover, there is considerable evidence that PrP^C binds copper (2-4). However, the mechanism behind the anti-oxidative functions of PrP^C has not been elucidated despite several biochemical studies. Brown *et al* reported that copper-bound prion protein (PrP), itself produced by *Escherichia coli* (*E. coli*), exhibits superoxide dismutase (SOD)-like activity (5). Affinity-purified PrP from mouse brain and cultured cells has been reported to display SOD-like activity (6). In contrast, the SOD-like activity of PrP produced by *E. coli* was not reproduced in other studies (7). Furthermore, our previous study showed that PrP^C produced by a baculovirus expression system (bacMuPrP) did not demonstrate SOD-like activity, although the bacMuPrP was in a marginally glycosylated and soluble monomeric form (8). Therefore, it remains unclear whether the anti-oxidative functions of PrP^C observed in cell culture systems are due to an indirect mechanism, the activation of SOD, or are a direct result of the SOD-like activity of PrP^C.

As PrP^C is modified with a GPI-anchor and by *N*-glycosylation, the protein is best expressed in mammalian cells to preserve the correct folding and posttranslational modifications. Therefore, in the present study, we established a mammalian soluble PrP-expression system driven by the peptide chain elongation factor 1α (EF-1α) promoter using rabbit-kidney epithelial RK13 cells. Then, to evaluate this system and characterize the protein's features, the SOD activity of soluble PrP was analyzed in cell lysate, medium, or immunoprecipitates. This expression system facilitates the efficient production and normal secretion of soluble PrP. The secreted protein in culture media could be readily assayed and purified on an Ni-NTA column. The data on SOD activity using RK13 cells expressing PrP, soluble PrP (sPrP), or empty vector *per se* showed that PrP increases SOD activity in cell lysate, while sPrP decreases cellular SOD activity in cell lysate but does not show any SOD activity itself.

Materials and methods

Plasmid construction. A DNA fragment that translates 251 amino acids (1-230 of human PrP and an additional 21 amino acids) for sPrP and 253 amino acids (1-253 of human PrP) for wild-type PrP, was amplified by the polymerase chain

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reaction (PCR) from human placental cDNA, using primer 1 (5'-CTCGAG ATG GCG AAC CTT GGC TG-3') and primer 2 (5'-GGATCC TCT CTG GTA ATA GGC CTG-3') for soluble PrP; and primer 1 and primer 3 (5'-GCGGCCGC TCA TCC CAC TAT CAG GAA GAT G-3') for wild-type PrP. The respective restriction enzyme recognition sites (*Xho*I in primer 1, *BamH*I in primer 2, and *Not*I in primer 3) are underlined. After proofreading with DNA sequencing, the human PrP gene (*Prnp*) fragments were subcloned into the mammalian expression vector pEF-BOS (9) containing six histidine sites in the 5'-3' sense orientation relative to the EF-1α promoter. sPrP additionally has IEGRIVKDYKDD DDKHHHHHH at the C-terminal.

Cell cultures. Rabbit-kidney epithelial RK13 cells and the derived transfectants were maintained in minimum essential medium (MEM) (Nakalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS) at 37˚C in a humidified 5% CO_2 incubator. The cells were transfected with the pEF-BOS constructs and pcDNA3.1(-) (Invitrogen Corp., Carlsbad, CA) by the Lipofectamine 2000-mediated method (Invitrogen) following the manufacturer's directions. The cells were further cultured in the presence of neomycin for selection. Stable and highly expressed cell clones expressing wild-type PrP (RK13-PrP) and soluble PrP (RK13-sPrP) were used for the study. RK13 cell clone-expressing empty vector *per se* (RK13-EM) was also used as a control.

Western blotting. Cells collected with a scraper or medium were used for Western blotting. The Western blot assay was performed as described previously (10). Briefly, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was conducted before electrical transfer onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Pharmacia Biotech, Piscataway, NJ). PrP or sPrP was detected with anti-PrP antibodies, SAF32 (SPI-Bio, Montigny le Bretonneux, France), SAF83 (SPI-Bio), or 7A1 (kindly provided by Professor N. Kitamoto, University of Hyogo), and horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA). The probed proteins were detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). The electrophoreogram was recorded on X-ray film (Amersham Pharmacia Biotech).

Indirect immunofluorescence assay (IFA). After being washed with phosphate-buffered saline (PBS), RK13-EM, RK13-PrP, and RK13-sPrP cells were fixed with 4% paraformaldehyde at 4˚C for 30 min and further incubated with SAF32 in the presence or absence of 0.1% Triton X-100 for 1 h. The cells were washed three times with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Jackson Immunoresearch) in darkness at room temperature for 1 h. Cells were then washed three times and observed under a fluorescence microscope.

Purification of sPrP. Purification of sPrP was performed according to the manufacturer's directions for the purification of 6X His-tagged proteins under native conditions (Qiagen Inc., Valencia, CA, USA) with minor modifications. After the addition of phenylmethylsulfonyl fluoride (PMSF), aliquots of the supernatants of RK13-sPrP cell cultures were precipitated by the addition of ammonium sulphide, solubilized in phosphate buffer, and mixed with Ni-NTA magnetic agarose beads (Qiagen). After incubation at 4˚C, the mixtures were loaded into an Ni-NTA agarose column, washed twice with wash buffer composed of 50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole (pH 8.0), and eluted four times with 100 μl of elution buffer composed of 50 mM NaH_2PO_4, 300 mM NaCl, and 150 mM imidazole (pH 8.0). After the elution, imidazole was removed by dialysis with phosphate buffer. The purity of sPrP was assessed by Western blotting and Coomassie brilliant blue (CBB) staining. The concentration of sPrP was measured by Bio-Rad DC protein assay (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as a standard.

SOD activity assay. A SOD assay kit-WST (Dojindo Laboratories, Kumamoto, Japan) was used for the quantification of SOD activity. Culture medium or immunoprecipitate of anti-PrP antibody, SAF32 from the culture medium, cell lysate in ice-cold RIPA buffer [10 mM Tris-HCl (pH 7.4), 1% deoxycholate, 1% Nonidet P-40, 0.1% SDS, and 150 mM NaCl], or purified sPrP was assayed and compared with 1 unit of bovine erythrocyte Cu/Zn-SOD activity (S2515; Sigma-Aldrich, St. Louis, MO). In the case of cell lysate, each protein extract (20 μg) was assayed. In the case of medium, purified sPrP, and immunoprecipitate, a 20-μl sample was used for the assay. The SOD activity was estimated using a standard curve of SOD activity versus absorbance at 450 nm. The SOD activity was expressed as units/mg protein.

Immunoprecipitation. The culture media (10 ml) of RK13, RK13-EM, RK13-PrP, and RK13-sPrP cells in 10-cm dishes were precleared using 40 μl of protein G sepharose bead suspension (Amersham Pharmacia Biotech) for 1 h at 4˚C, and then incubated with 4 μg of SAF32 for 1 h at 4˚C. Next, 100 μl of protein G sepharose bead suspension was added to each of the immunocomplexes and the mixtures were rotated for 1 h at 4˚C. After 4 washes with the RIPA buffer, the immunoprecipitated proteins were solubilized in 200 μl of RIPA buffer and subjected to an SOD activity assay or Western blotting.

Tunicamycin treatment. For tunicamycin treatment, the medium was exchanged with 10% FCS-MEM containing 5 μg/ml of tunicamycin and the cells were incubated for 48 h. Protein (20 μg) from the cell lysate was used for Western blotting. Methanol precipitation at -80˚C for more than 24 h was also used for the precipitation of proteins in media.

Statistical analysis. The statistical significance of differences between groups was tested with the Kruskal-Wallis H test followed by the Dunn's multiple comparison test. P values <0.05 were considered to be statistically significant.

Results

PrP is attached to the cell membrane by a GPI-anchor. The GPI-anchor signal is composed of a stretch of hydrophobic amino acid residues in the C-terminal region of the GPI-

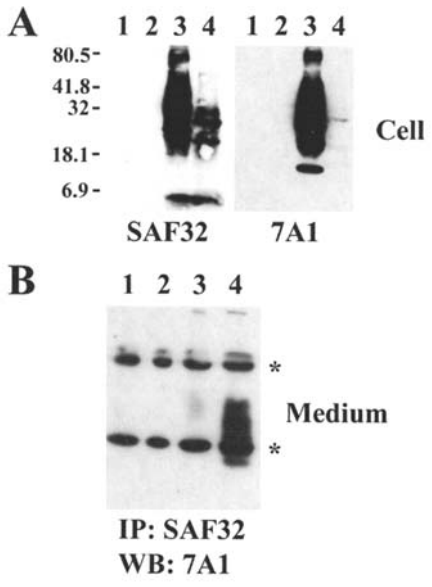

Figure 1. Secretion of sPrP into medium by RK13 cells expressing glycosylphosphatidylinositol (GPI)-anchorless PrP (RK13-sPrP). PrP in cell lysate (A) and immunoprecipitates of medium obtained with SAF32 (B) from RK13 (lane 1), RK13-EM (lane 2), RK13-PrP (lane 3), and RK13-sPrP (lane 4) cells were subjected to SDS-PAGE and then immunoblotted with anti-PrP antibody, 7A1, and HRP-conjugated anti-mouse IgG. The asterisk represents the immunoglobulin light or heavy chain. PrP could be immunoprecipitated with SAF32 from a medium of RK13-sPrP cells and slightly immunoprecipitated in a medium of RK13-PrP cells. PrP was mainly detected in cell lysate, whereas sPrP was mainly detected in the culture medium.

anchored protein. In the endoplasmic reticulum, after PrP is synthesized, the GPI-anchor of PrP is cleaved and replaced by GPI. In our previous study, deletion of the GPI-anchor signal of PrP prevented attachment to the cell membrane by GPI and consequently led to secretion into the medium as a soluble form of PrP in a baculovirus expression system (8). The soluble form of PrP was in an abnormally glycosylated and soluble monomeric form and did not exhibit SOD activity (8). It is conceivable that the lack of SOD activity in the soluble PrP produced by the baculovirus expression system is due to abnormal glycosylation produced by other than mammalian cells. This study prompted us to investigate whether the soluble PrP produced by a mammalian expression system has SOD activity.

We were interested in SOD activity in cells expressing PrP and soluble PrP as well as whether soluble PrP itself could elicit SOD activity. First, we examined the distribution of PrP and sPrP in RK13 cells by Western blotting of the culture medium and cell lysate. Lysate and medium immunoprecipitated by anti-PrP Ab SAF32 were subjected to a Western blot analysis with anti-PrP SAF32 or 7A1 (Fig. 1). SAF32 recognizes residues 79-92 of PrP, while the epitope of 7A1 was estimated to be a C-terminal region based on a competitive enzyme-linked immunosorbent assay (ELISA) with SAF32 (epitope: human PrP 79-92) (11), SAF61 (epitope: human PrP 142-160) (SPI-Bio), SAF53 (epitope: human PrP 142-160) (SPI-Bio), or PRI308 (epitope: human PrP 106-126) (SPI-Bio) (Tsuji S, Sakudo A, and Ikuta K, unpublished data). PrP was mainly detected in the lysate and slightly detected in immunoprecipitates from the culture medium of RK13-PrP cells (Fig. 1A and B). This suggests that most of the PrP was attached to the cell membranes and was not released from cells. On the other hand, sPrP was highly detected in immunoprecipitates from the culture of RK13-sPrP cells, but only slightly detected in lysate of RK13-sPrP cells (Fig. 1A and B). Therefore, sPrP was detected by SAF32 and slightly detected by 7A1 in the Western blotting of cell lysate of RK13-sPrP. PrP and sPrP in RK13-PrP and RK13-sPrP cells, respectively, were observed at a molecular weight <6.9 kDa with SAF32 (Fig. 1A, left panel). These bands were not detected by 7A1, which recognizes the C-terminal of PrP. Therefore, these bands appeared to be an N-terminal fragment of PrP. As recent studies have shown that reactive oxygen species cleave around position 90 (ß-cleavage) at the end of the octapeptide repeat region (12,13), the N-terminal fragment may be produced by ß-cleavage. The expression of the N-terminal fragment of PrP in lysate of RK13-sPrP cells was comparable

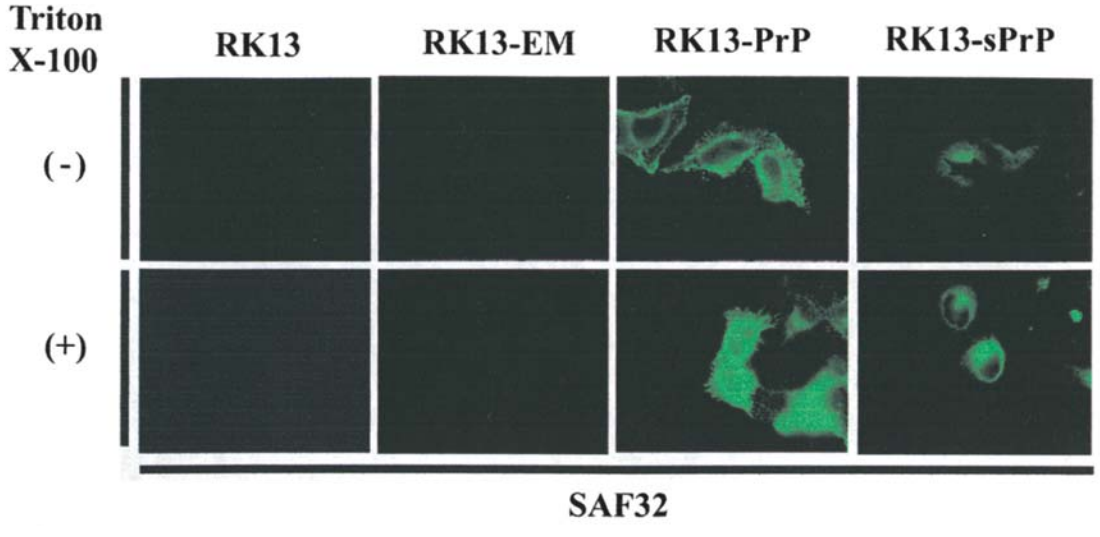

Figure 2. Localization of PrP and sPrP in RK13 transfectants. RK13 cells transfected with pEF-BOS-PrP (RK13-PrP) or pEF-BOS-sPrP (RK13-sPrP) or empty vector pEF-BOS (RK13-EM), as well as the untransfected control (RK13) were fixed with paraformaldehyde, and the localization of PrP was examined using SAF32 and FITC-conjugated anti-mouse IgG in the presence (+) and absence (-) of 0.1% Triton X-100.

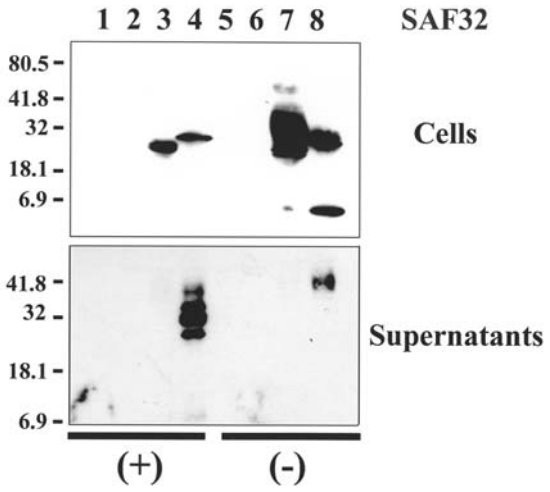

Figure 3. Glycosylation of sPrP in RK13 cells. RK13 cells transfected with empty vector (lanes 2 and 6), PrP-expressing vector (lanes 3 and 7), or soluble PrP-expressing vector (lanes 4 and 8) as well as the parental RK13 cells (lanes 1 and 5) were cultured for 48 h in the presence (+) and absence (-) of tunicamycin. The cells and the methanol precipitate of supernatants were analyzed by Western blotting using SAF32 to detect PrP.

with that in RK13-PrP cells (Fig. 1A, left panel). In RK13-PrP cells, 7A1 reacted with a band at around 15 kDa, which was not detected by SAF32 (Fig. 1A, right panel). The band was thought to be an N-terminal-truncated form of PrP, which may be produced by proteolytic cleavage at around 110 (α-cleavage). As PrP was not detected by SAF32 and 7A1 in the cell lysate and immunoprecipitates from the culture medium of RK13 and RK13-EM cells (Fig. 1A and B), these antibodies specifically reacted with these bands.

To further examine the localization of PrP and sPrP, IFA using SAF32 was performed (Fig. 2). PrP was detected in the cellular membrane of RK13-PrP cells in the absence of Triton X-100, whereas sPrP showed paracrine secretion-like distribution in RK13-sPrP cells in the absence of Triton X-100. In the presence of Triton X-100, immunofluorescence was predominantly localized around the nucleus, particularly in the perinuclear endoplasmic reticulum, in both RK13-PrP and RK13-sPrP cells. No immunofluorescence was detected in RK13 cells or those transfected with empty vector, RK13-EM cells, in the absence or presence of Triton X-100.

Several posttranslational modifications may give rise to large isoforms, particularly through glycosylation. This is also the case for PrP. We used tunicamycin to inhibit *N*-glycosylation and analyzed the change in molecular weight of PrP and sPrP by Western blotting of SAF32 (Fig. 3). Methanol precipitation was used to precipitate protein from the medium. Inhibition of the glycosylation of sPrP by tunicamycin resulted in four lower molecular weight bands from 41.8 to 22 kDa in the medium of RK13-sPrP (Fig. 3, lower panel). The 41.8-kDa band may be a fully glycosylated form. The 41.8-kDa form is the same size of sPrP in the medium with and without tunicamycin. The other three bands may be a form that is *N*-glycosylated at one site, an unglycosylated form of the full-length sPrP, or a C-terminally truncated form. Tunicamycin treatment also decreased the molecular weight of PrP and sPrP in the lysate of RK13-PrP and RK13-sPrP cells, respectively. These results suggest that the PrP and sPrP of the cellular fraction were glycosylated, while the sPrP in medium was also glycosylated. Moreover, these findings suggest that glycosylation is not necessary for the secretion of sPrP, because tunicamycin treatment did not inhibit the secretion of sPrP into medium.

RK13-sPrP cells showed an sPrP of 20-32 kDa and <6.9 kDa (N-terminal fragment of PrP) in the absence of tunicamycin. Interestingly, the N-terminal PrP fragment, which was detected in both RK13-PrP and RK13-sPrP cells in the absence of tunicamycin, was dramatically decreased after tunicamycin treatment. This suggests that production of the N-terminal PrP fragment requires *N*-glycosylation.

Although PrP and sPrP were detected in the lysate of RK13-PrP and RK13-sPrP cells, respectively, SOD activity was increased in the lysate of RK13-PrP cells and decreased in that of RK13-sPrP cells compared to RK13-EM cells (Fig. 4A). On the other hand, no increase in SOD activity was observed in the medium of RK13-PrP and RK13-sPrP compared to RK13-EM cells (Fig. 4B). To confirm the results, immunoprecipitates of SAF32 from culture media were used for SOD activity assays. The immunoprecipitate of the supernatant of RK13-PrP and RK13-sPrP was not significantly different in SOD activity from that of RK13-EM cells (Fig. 4C), whereas sPrP was successfully immunoprecipitated using SAF32 from the supernatant of RK13-sPrP cells, and PrP was slightly immunoprecipitated in that of RK13-PrP

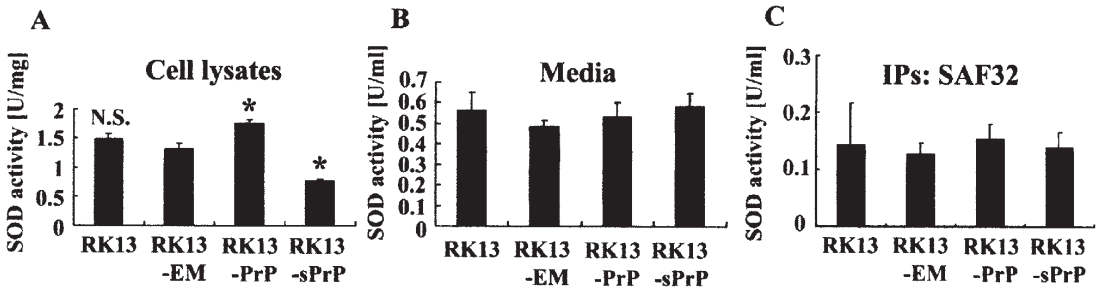

Figure 4. SOD activity of sPrP is absent in medium, while PrP increases and sPrP decreases total cellular SOD activity in cells. The cell lysates (A), media (B), and immunoprecipitates (IPs) obtained with SAF32 (C) from RK13 cells transfected with pEF-BOS-PrP (RK13-PrP) or pEF-BOS-sPrP (RK13-sPrP) or empty vector pEF-BOS (RK13-EM) as well as the untransfected control (RK13), were used for SOD assay. There was no significant difference in SOD activity in the medium among RK13, RK13-EM, RK13-PrP, and RK13-sPrP cells. Immunoprecipitated PrP and sPrP did not exhibit significant SOD activity, whereas the lysate of RK13-PrP cells showed a significant increase in SOD activity compared to that of RK13-EM cells. In contrast, the lysate of RK13-sPrP cells demonstrated significantly less SOD activity than that of RK13-EM cells. Values are mean ± SD of quadruplicate determinations. $^{*}p<0.01$ compared with RK13-EM cells.

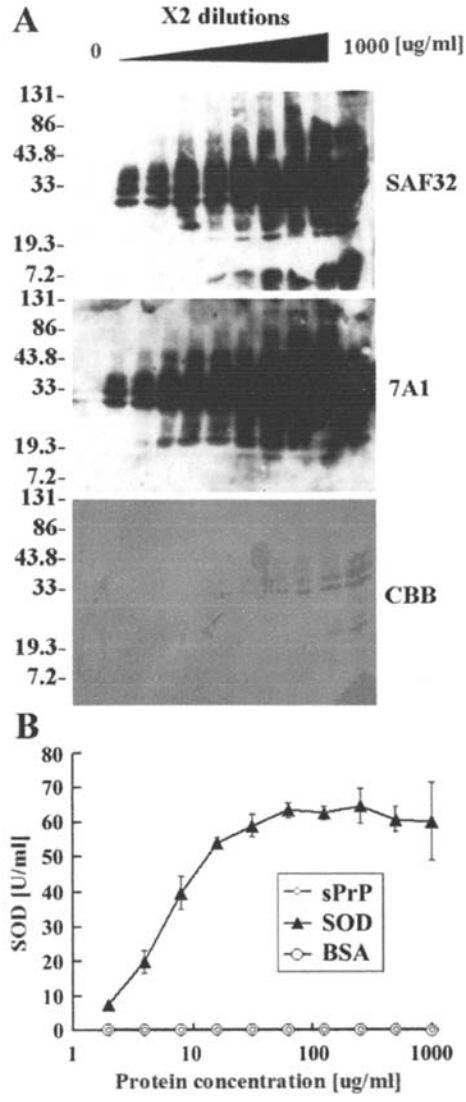

Figure 5. Absence of SOD activity of purified sPrP. (A) The expressed protein in the supernatant of RK13 cells transfected with pEF-BOS-sPrP (RK13-sPrP) was purified with Ni-NTA agarose affinity chromatography. The sPrP samples prepared by a 2-fold serial dilution of 1,000 μg/ml were separated by SDS-PAGE and analyzed by Coomassie brilliant blue staining and Western blotting with anti-PrP antibody, SAF32 or 7A1. (B) The SOD activity of the samples was examined using a SOD assay kit-WST as described in Materials and methods. The bovine erythrocyte Cu/Zn-SOD exhibited SOD activity in a dose-dependent manner, whereas sPrP and bovine serum albumin (BSA) did not show any SOD activity. Values are mean ± SD of quadruplicate determinations.

cells, respectively (Fig. 1B). These results confirmed that sPrP and PrP did not show any SOD activity.

Finally, we purified sPrP from culture medium using Ni-NTA agarose affinity chromatography. CBB staining showed that specific bands observed at around 40-25 kDa and 20 kDa were selectively purified by Ni-NTA agarose affinity chromatography in the supernatant of RK13-sPrP cells (Fig. 5A, lower panel). The bands were detected by Western blotting of SAF32 and 7A1. In Western blotting using SAF32 but not 7A1, a band of <6.9 kDa (N-terminal fragment) was also detected. Quantitative protein assay compared to BSA standard indicated that the yield of purified sPrP is in total >1 mg per 2 litres of culture medium. We examined the SOD activity of sPrP produced by this mammalian expression system by conducting a xanthine oxidase-based SOD activity assay. Purified sPrP was subjected to the SOD assay at a 2-fold serial dilution of 1,000 μg/ml. Although a dose-dependent increase of SOD activity was observed in bovine erythrocyte Cu/Zn-SOD, this was not the case for sPrP, which did not show any SOD activity at similar levels to BSA (Fig. 5B).

Discussion

The function of PrP^C has not been fully elucidated, partly due to the difficulty of obtaining an adequate quantity of mammalian PrP^C for biochemical and structural characterization. Here we demonstrated that a soluble His-tagged prion protein secreted from mammalian cells into the medium was easily obtained in a large quantity from the culture medium. The C-terminal His-tag enabled us to purify the protein. Although a previous report indicated hundreds of normal hamster brains yielded only a few micrograms of protein (14), the yield of sPrP after purification in our system was in the order of 1 mg per 2 litres of culture medium. The yield is better than our previous result for soluble PrP using a baculovirus expression system (1 μg per 10 ml of culture medium) (8). Furthermore, biochemical assays such as IFA and Western blotting demonstrated that sPrP was distributed not in the cell membrane but rather in the perinuclear endoplasmic reticulum of the cells. Most of the sPrP was released into the medium as an *N*-glycosylated form, while the sPrP in the cells was only slightly *N*-glycosylated. Tunicamycin treatment did not inhibit the secretion of sPrP into the medium, suggesting that glycosylation was not necessary for the secretion. However, as the secreted sPrP was highly glycosylated, the *N*-glycosylated form is more readily secreted. Although affinity-purified or immunoprecipitated PrP from mouse brain has been reported to display SOD activity (6), sPrP does not have such activity. As refolding in the presence of copper is required to endow SOD activity on recombinant PrP produced by *E. coli* (5), some factors or treatment such as refolding in the presence of copper might be necessary to endow SOD activity on sPrP. However, the results after such treatment might not reflect the natural state of this protein and may be obtained only under artificial conditions. Therefore, the present results are important to clearly show that PrP itself does not have any SOD activity under more native conditions than in the past study. In addition, although glycosylation of PrP expressed in a baculovirus expression system (bacMuPrP) is apparently distinct from mammalian PrP^C, bacMuPrP did not show any SOD activity (8). This is consistent with the present result that sPrP did not elicit any SOD activity. Western blotting demonstrated that non-glycosylated PrP and sPrP in the cells were 26 and 30 kDa in size, respectively (Fig. 3), which is consistent with values calculated from the deduced amino acid sequence of mature PrP and sPrP: PrP is composed of 208 amino acids (from the 23rd to the 230th amino acid of human PrP) and sPrP, 251 amino acids (from the 23rd to the 230th amino acids of human PrP and an additional 20 amino acids including six histidines), suggesting that the N-terminal signal peptide was removed in the cells. Furthermore, secreted sPrP showed a higher molecular weight than sPrP in

cells in the absence and presence of tunicamycin, suggesting that the more glycosylated form is secreted more readily. However, inhibition of *N*-glycosylation by tunicamycin did not inhibit the secretion, so *N*-glycosylation is not always required for secretion. These results were important and suggested significant differences in glycosylation and SOD activity, as well as meaningful similarities in signal peptide proteolysis between PrP from different species.

Western blotting demonstrated that sPrP was detected not only in the cellular fraction but also in the medium. Furthermore, sPrP is more efficiently secreted into the medium than PrP (Fig. 3). In addition, the inhibition of *N*-linked glycosylation by tunicamycin did not result in impaired sPrP secretion. These results were supported by the IFA results that showed staining in the peripheral region around nuclei, including the endoplasmic reticulum and Golgi apparatus with Triton X-100 treatment where *N*-glycosylation occurred, as well as secretion into the medium, possibly due to deletion of the C-terminal hydrophobic region which is essential for attachment of PrP to the cell membrane via the GPI-anchor.

There have been several reports regarding the association between PrP and SOD. However, it remains unclear whether PrP regulates SOD activity directly or indirectly (1). The present study demonstrated that GPI-anchored PrP up-regulates cellular SOD activity but GPI-anchorless PrP does not. Furthermore, there is no SOD activity in soluble PrP produced by a mammalian expression system. These findings suggest that PrP indirectly regulates cellular SOD activity via GPI-anchorage, and PrP itself does not have any SOD activity.

Soluble His-tagged sPrP expressed in mammalian cells was secreted into the culture medium, from which we were able to obtain large amounts of the soluble form of sPrP by purification using an Ni-NTA column. Considering the results of biochemical assays, it is rational to conclude that sPrP is a fully glycosylated soluble form composed of the amino acid sequence of mature sPrP and does not exhibit SOD activity. Soluble sPrP could also be useful for structural, functional, and biochemical analyses. Furthermore, by comparing the effect of media from RK13, RK13-EM, RK13-PrP and RK13-sPrP, studies on the effects of PrP^{C} on cells can be facilitated. This convenient system should be used to investigate the influence of sPrP on *Prnp*-transfected or non-transfected *Prnp*$^{-/-}$ neuronal cells (15).

Finally, we emphasize that soluble PrP is useful for revealing the mechanism behind, not only the normal functions of PrP^{C}, but also the accumulation of PrP^{Sc}. Further establishment of expression systems using RK13 cells expressing PrP from various species would facilitate detailed analysis of the species barrier.

Acknowledgements

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References

1. Sakudo A, Onodera T, Suganuma Y, Kobayashi T, Saeki K and Ikuta K: Recent advances in clarifying prion protein functions using knockout mice and derived cell lines. Mini Rev Med Chem 6: 589-601, 2006.
2. Brown DR, Qin K, Herms JW, *et al*: The cellular prion protein binds copper *in vivo*. Nature 390: 684-687, 1997.
3. Thompsett AR, Abdelraheim SR, Daniels M and Brown DR: High affinity binding between copper and full-length prion protein identified by two different techniques. J Biol Chem 280: 42750-42758, 2005.
4. Leach SP, Salman MD and Hamar D: Trace elements and prion diseases: a review of the interactions of copper, manganese and zinc with the prion protein. Anim Health Res Rev 7: 97-105, 2006.
5. Brown DR, Wong BS, Hafiz F, Clive C, Haswell SJ and Jones IM: Normal prion protein has an activity like that of superoxide dismutase. Biochem J 344: 1-5, 1999.
6. Brown DR, Clive C and Haswell SJ: Antioxidant activity related to copper binding of native prion protein. J Neurochem 76: 69-76, 2001.
7. Jones S, Batchelor M, Bhelt D, Clarke AR, Collinge J and Jackson GS: Recombinant prion protein does not possess SOD-1 activity. Biochem J 392: 309-312, 2005.
8. Sakudo A, Hamaishi M, Hosokawa-Kanai T, *et al*: Absence of superoxide dismutase activity in a soluble cellular isoform of prion protein produced by baculovirus expression system. Biochem Biophys Res Commun 307: 678-683, 2003.
9. Mizushima S and Nagata S: pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res 18: 5322, 1990.
10. Sakudo A, Lee DC, Saeki K, Matsumoto Y, Itohara S and Onodera T: Tumor necrosis factor attenuates prion protein-deficient neuronal cell death by increases in anti-apoptotic Bcl-2 family proteins. Biochem Biophys Res Commun 310: 725-729, 2003.
11. Vincent B, Paitel E, Saftig P, *et al*: The disintegrins ADAM10 and TACE contribute to the constitutive and phorbol ester-regulated normal cleavage of the cellular prion protein. J Biol Chem 276: 37743-37746, 2001.
12. Mange A, Beranger F, Peoc'h K, Onodera T, Frobert Y and Lehmann S: Alpha- and beta-cleavages of the amino-terminus of the cellular prion protein. Biol Cell 96: 125-132, 2004.
13. Watt NT, Taylor DR, Gillott A, Thomas DA, Perera WS and Hooper NM: Reactive oxygen species-mediated beta-cleavage of the prion protein in the cellular response to oxidative stress. J Biol Chem 280: 35914-35921, 2005.
14. Pan KM, Baldwin M, Nguyen J, *et al*: Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. Proc Natl Acad Sci USA 90: 10962-10966, 1993.
15. Kuwahara C, Takeuchi AM, Nishimura T, *et al*: Prions prevent neuronal cell-line death. Nature 400: 225-226, 1999.