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# Prion protein expression in Chinese hamster ovary cells using a glutamine synthetase selection and amplification system

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Syrian hamster prion protein (PrP$^C$) and a truncated Syrian hamster prion protein lacking the glycosylphosphatidylinositol (GPI) anchor C-terminal signal sequence (GPI$^-$) were expressed in Chinese hamster ovary cells using a glutamine synthetase selection and amplification system. The CHO cell clones expressing the GPI$^-$ PrP secreted the majority of the protein into the media, whereas most of the PrP produced by clones expressing the full-length protein with the GPI anchor was located on the cell surface, as demonstrated by its release upon treatment with phosphatidylinositol-specific phospholipase C (PIPLC). A cell clone that expressed the highest levels of full length PrP was subcloned to obtain clone 30C3-1. PrP from clone 30C3-1 was shown to be sensitive to proteolysis by proteinase K and to react with monoclonal and polyclonal antibodies that recognize native PrP$^C$. The recombinant PrP migrated as a diffuse band of 19–40 kDa but removal of the N-linked oligosaccharides with peptide *N*-glycosidase F (PNGase F) revealed three protein species of 19, 17 and 15 kDa. The 19 kDa band corresponding to deglycosylated full-length PrP was quantified and found to be expressed at a level ~14-fold higher than that of PrP$^C$ found in Syrian hamster brain.

*Keywords*: Chinese hamster ovary cells/glutamine synthetase selection and amplification system/prion protein expression

## Introduction

Prions are defined as proteinaceous, infectious particles which contain no nucleic acid and cause neurodegenerative disorders in a range of different species (Prusiner, 1996). The current epidemics of bovine spongiform encephalopathy (BSE), or 'mad cow disease', which has resulted in more than 160 000 cattle deaths in Great Britain since 1986, is thought to be caused by prions initially derived from sheep scrapie (Wilesmith *et al.*, 1991). Human prion diseases identified to date include kuru, Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS) and fatal familial insomnia (Gajdusek, 1977; Masters *et al.*, 1981; Medori *et al.*, 1992). Epidemiological studies suggest that a new variant form of CJD manifest in Great Britain may be caused by BSE prions (Will *et al.*, 1996).

The prion protein (PrP) is a normal cellular protein of unknown function which has been found in both neuronal and nonneuronal tissues (Oesch *et al.*, 1985; Kretzschmar *et al.*, 1986; Bendheim *et al.*, 1992; Manson *et al.*, 1992). It is located on the exterior cell surface and is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor (Stahl *et al.*, 1987, 1990). Fourier transform infrared (FTIR) and circular dichroism (CD) spectroscopic studies show that the normal cellular isoform (PrP$^C$) is predominantly α-helical in structure with little, if any, β-sheet (Pan *et al.*, 1993; Pergami *et al.*, 1996; Riek *et al.*, 1996). In contrast, the secondary structure of the pathogenic form (PrP$^{Sc}$) which is derived from PrP$^C$ has less α-helical structure and substantial amounts of β-sheet (Caughey *et al.*, 1991; Gasset *et al.*, 1993; Pan *et al.*, 1993; Safar *et al.*, 1993). It is believed that this conformational transition is the fundamental event in the conversion of PrP$^C$ to PrP$^{Sc}$. To understand better the formation of PrP$^{Sc}$, it seemed useful to develop a system for producing large quantities of PrP$^C$, particularly because purification of PrP$^C$ from rodent brain has been difficult (Turk *et al.*, 1988; Pan *et al.*, 1992; Pergami *et al.*, 1996).

Although the level of PrP mRNA in brain is higher than in any other tissue in rodents, its abundance is low (Oesch *et al.*, 1985; Kretzschmar *et al.*, 1986). Similarly, the level of PrP$^C$ in brain is higher than in other tissues (Bendheim *et al.*, 1992), but it represents less than 0.1% of the total CNS protein (Turk *et al.*, 1988; Pan *et al.*, 1992, 1993). Since there is no natural source of PrP$^C$, we examined many cultured cell types for PrP synthesis (Scott *et al.*, 1988) and attempted expression of PrP from vectors transfected into cells. Although many investigators have reported expression of recombinant PrP, few of these have produced the PrP$^C$ in high amounts. Milligram quantities of recombinant PrP have been isolated and purified from bacterial and insect cells (Weiss *et al.*, 1995; Hornemann and Glochshuber, 1996; Mehlhorn *et al.*, 1996). In most of the mammalian expression systems where the PrP$^C$ was post-translationally modified by addition of Asn-linked oligosaccharides and a GPI anchor, the quantity of recombinant PrP$^C$ was low and purification was not reported. In the present study, we sought to establish a mammalian cell system capable of generating PrP$^C$ at high levels of expression and we therefore chose a glutamine synthetase gene amplification system (Bebbington *et al.*, 1987).

Chinese hamster ovary (CHO) cells growing in culture require glutamine for their growth and survival. Glutamine can either be obtained from the medium or synthesized by the cells from glutamate and ammonia through catalysis by the enzyme glutamine synthetase (GS). CHO cells grown in a selection medium that is glutamine free and contains methionine sulfoximine (MSX), an inhibitor of GS (Sanders and Wilson, 1984), do not survive. Specific gene expression can be achieved by plating the CHO cells in an MSX selection medium and then transfecting the cells with a vector that codes for the GS gene along with the foreign gene of interest (Cockett *et al.*, 1990). Only those cells that incorporate the vector and express GS at levels sufficiently

high to overcome the MSX block will survive. Expression of the foreign gene can be amplified by growing the cells in successively higher concentrations of MSX. The GS system has been used to produce a wide variety of recombinant proteins including HIV-1 envelope gp120 (Moore *et al.*, 1990), mb-1 (Brown and Barclay, 1994), a T cell receptor V domain (Brown and Barclay, 1994), metalloproteinases (Murphy *et al.*, 1991), metalloproteinase inhibitors (Cockett *et al.*, 1990), IgG4 antibody (Bebbington *et al.*, 1992), type-II membrane protein AMOG/β2 (Gloor *et al.*, 1992), the human thyrotrophin receptor (Harfst *et al.*, 1992), CD2 (Davis *et al.*, 1993), CD4 (Davis *et al.*, 1990), CD5 (Brown and Barclay, 1994), CD18 (Robinson *et al.*, 1992), CD45 (McCall *et al.*, 1992) and CD48 (van der Merwe *et al.*, 1993). The crystal structures of CD2 (Jones *et al.*, 1992) and CD4 (Brady *et al.*, 1993) have been obtained from recombinant proteins produced by the GS–CHO system. Here we report the creation of CHO cell lines expressing Syrian hamster (SHa) PrP through the use of the GS selection and amplification system.

## Materials and methods

### Generation of constructs

The pEE12-27 and pEE14-27 vectors were generated by removing the polylinker region from pEE12 and pEE14 glutamine synthetase vectors (Celltech, Slough, Berkshire, UK) via a *Hin*dIII–*Bcl*I digestion and then ligating an adaptor sequence into the *Hin*dIII–*Bcl*I site. The following DNA strands were synthesized using a Millipore Expedite 8909 (Perseptive Biosystems, Framingham, MA): ADAP1, 5′ AGCTTGCGTGATCAGCGCTCGAGCGCG 3′; and ADAP2, 5′ GATCCGCGCTCGAGCGCTGATCACGCA 3′. The linker was created by annealing the two DNA strands together. The open reading frame coding for full length SHaPrP was released from a p805H4 vector (Scott *et al.*, 1988) via a *Bam*HI–*Xho*I digestion and then subcloned into pEE12–27 and pEE14–27 to generate pEE12–27 SHaPrP and pEE14–27 SHaPrP, respectively. A carboxy-terminal deletion construct of SHaPrP (pMSR547) (M.Rogers, unpublished plasmid) containing only the first 231 nucleotides of the open reading frame and lacking the GPI signal sequence was removed from the pMSR547 vector via a *Bgl*II–*Xho*I digestion and then ligated into pEE12–27 and pEE14–27 to generate pEE12–27–547 and pEE14–27–547, respectively.

### Cloning, selection and amplification

Chinese hamster ovary (CHO) K1 cells (American Type Culture Collection) grown in GMEM-S medium supplemented with 10% heat-treated, dialyzed fetal calf serum (Gibco-BRL) were seeded at $10^6$ cells per 10 cm dish (Bebbington *et al.*, 1987). After 24 h, each plate of cells was transfected with 10 µg of either pEE12–27 SHaPrP, pEE14–27 SHaPrP, pEE12–27–547 or pEE14–27–547 vector, via calcium phosphate (Gorman, 1985). The cells were then grown in the presence of 25 µM MSX (Sigma) for 14 days. Sixty clones were selected and then grown in the presence of 100, 200 or 400 µM MSX for 14 days. The surviving clones were then analyzed by Western blot analysis to examine PrP expression (Towbin *et al.*, 1979). Clone 30 was subcloned via limiting dilution to obtain clone 30C3-1.

### Western blot analysis

Cells were grown to confluency in 10 cm dishes. Prior to processing for Western blot analysis, the medium was removed

from the cells, which were then rinsed three times with phosphate-buffered saline and incubated with UltraCHO medium (Biowhittaker) while on a rocker at 37°C. After 4 h, the medium was removed from the cells, passed through a 0.45 µM filter and then precipitated with 10 volumes of methanol at −20°C for 1 h. The medium was then centrifuged for 30 min at 4000 *g* and the pellet resuspended in Laemmli sample buffer at one tenth of the original volume of medium. The cells were rinsed three times with phosphate-buffered saline and then lysed with Laemmli sample buffer (Laemmli, 1970) which contained no β-mercaptoethanol or bromophenol blue. The lysates were boiled for 5 min and then passed through a 26 gauge syringe needle five times. Golden Syrian hamster (LVG:Lak) brains (Charles River Laboratories), Chinese hamster (*Cricetulus griseus*) brains (George Yerganian, Cytogen) and Swiss CD-1 mouse brains (Charles River Laboratories) were homogenized in Laemmli sample buffer containing no β-mercaptoethanol or bromophenol blue at a 1:9 mass to volume ratio. The homogenate was passed five times each through 14, 16, 18, 20 and 22 gauge syringe needles. The protein concentration of the cell lysates and brain homogenates was measured by BCA Assay (Pierce). Prior to loading the samples on to 12% polyacrylamide gels, β-mercaptoethanol and bromophenol blue were added to the lysates and brain homogenates and then all samples were boiled for 5 min. Amounts of 30 and 300 µg of cell lysate protein were loaded on to an SDS–polyacrylamide gel. After electrophoresis, the samples were electrotransferred to Immobilon P (Millipore) and then incubated with one of the following anti-PrP antibodies: RO73 (Bendheim *et al.*, 1984), 3F4 (Kascsak *et al.*, 1987), 13A5, P1, P2, P3 (Barry *et al.*, 1985; Barry and Prusiner, 1986) or N-10 (H.Serban, unpublished work). All secondary antibodies were conjugated to horseradish peroxidase and their binding was visualized by enhanced chemiluminescence (Amersham). The molecular weight standards used for all blots were myosin (H-chain) (199.1 kDa), phosphorylase B (106.3 kDa), bovine serum albumin (69.1 kDa), ovalbumin (43.6 kDa), carbonic anhydrase (28.4 kDa), β-lactoglobulin (18.9 kDa) and lysozyme (15.4 kDa); these were obtained from Gibco-BRL. The molecular weight standards used for Coomassie-stained polyacrylamide gels were phosphorylase B (101 kDa), bovine serum albumin (83 kDa), ovalbumin (50.6 kDa), carbonic anhydrase (35.5 kDa), soybean trypsin inhibitor (29.1 kDa) and lysozyme (20.9 kDa); these were obtained from Bio-Rad.

### Enzymatic treatments

Cell samples treated with proteinase K were first lysed for 5 min at room temperature in 10 mM Tris (pH 7.4), 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 0.5% deoxycholate at a volume of 1.0 ml per 10 cm dish. The lysates were centrifuged at 12 000 *g* for 45 s. The supernatant was recovered and its protein concentration determined via BCA assay (Pierce). Proteinase K (Boehringer Mannheim) was added to the samples at a ratio of 1 µg of enzyme per 100 µg of protein. The samples were incubated at 37°C for 45 min. The incubation was stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 2 mM and then incubating for an additional 5 min.

Cell lysates to be treated with PNGase F were processed as described for the Western blot analysis. A 30 µg amount of protein was incubated in 40 µl of 20 mM sodium phosphate (pH 7.0), 25 mM EDTA, 0.6% NP-40, 1% β-mercaptoethanol,

0.1% SDS in the presence of 1200 mU PNGase F (Boehringer Mannheim). Incubations were carried out for 24 h at 37°C and then the samples were frozen.

Recombinant phosphatidylinositol-specific phospholipase C (PIPLC) was isolated from *Bacillus cereus* (Koke *et al.*, 1991). 30C1-1 cells were used for all the PIPLC studies. PIPLC treatment was performed on cultured cells by first removing the media from a 6 cm plate of cells and then rinsing three times with phosphate-buffered saline; PIPLC was added at 0.5 units/ml in UltraCHO media. The cells were then incubated on a rocker for 4 h at 37°C. A duplicate plate containing the same number of cells was also processed identically except that it received no PIPLC. The media and cell lysates were prepared as described above for Western blot analysis, with the exception that the medium was not passed through a 0.45 μm filter. Aliquots of 2.0% of each sample were loaded on to a 12% polyacrylamide gel. Following transfer on to an Immobilon P membrane, the blots were incubated with RO73 antibody and developed as described above in Western blot analysis.

PIPLC treatment was performed on cell lysates by first removing the medium from cultured 30C1-1 or N2a cells and rinsing three times with UltraCHO media. The cells were then scraped off the plates in UltraCHO media containing 0.16% Triton X-100 (Sigma) and a protease inhibitor cocktail (Boehringer Mannheim) at a concentration of one inhibitor tablet per 30 ml of medium, then Dounce homogenized. Each cell lysate was divided into three equal samples: Laemmli sample buffer was added to one which was then passed five times through a 26 gauge needle then boiled for 5 min; the second was incubated at 37°C for 1 h; PIPLC was added to the third at a concentration of 0.5 units/ml, and the sample was incubated at 37°C for 1 h. Following the incubation, the second and third samples received Laemmli sample buffer and were processed as described for the first sample. Aliquots of 5.0% of each were loaded on to 12% polyacrylamide gels and then detected as described above for the PIPLC treatment on cultured cells. This PIPLC procedure was also used for the time point assays but with some modifications. Lysates from two duplicate plates of cells were prepared in medium as described above. One sample received PIPLC whereas the other did not; neither sample received protease inhibitors. Aliquots of 5% of each were removed from both samples at 0, 2, 5, 10, 30 and 60 min. The samples were treated as described above and then examined via Western blot analysis using RO73 antiserum. Samples visualized via Coomassie Brilliant Blue staining were processed as described above for the 30C1-1 or N2a cells except that the incubation was performed for 4 h.

### PrP$^C$ quantification

Western blots of serial dilutions of PNGase F-treated 30C3-1 cell lysate and PNGase F-treated brain homogenate were incubated with 3F4 antibody, which is specific for SHaPrP. Densitometric scanning of the 19 kDa band from the brain homogenate and cells was performed using Bioquant OS/2 Image Analysis Software Version 2.50 (R&M Biometrics, Nashville, TN) on a Grafika computer (DTK). The relative amounts of PrP$^C$ (densitometric units) were plotted versus the amount of total protein (μg) for both Syrian hamster brain and 30C3-1 cell lysate. For each plot the ratio of the slopes of the best linear fits to the data was calculated to determine the relative expression level of PrP in 30C3-1 cells as compared with Syrian hamster brain.

## Results

### Generation of cell clones

To achieve the highest possible expression of PrP, four different GS vectors expressing PrP were constructed (Figure 1). The pEE12–27 and pEE14–27 vectors differ in the promoter regulating the GS gene. SHaPrP and 547 differ in that SHaPrP contains the entire open reading frame, whereas 547 contains a carboxy-terminal deletion resulting in the loss of the GPI addition signal. Thus, PrP encoded by the SHaPrP cDNA should be attached to the exterior plasma membrane surface via a GPI achor, whereas PrP expressed from the 547 construct should be secreted into the medium.

Following calcium phosphate transfection of the four constructs into CHO-K1 cells, 60 clones were selected in the presence of 25 μM MSX and then amplified in 100, 200 or 400 μM MSX. Seven clones survived amplification at 100 μM MSX and one survived at 200 μM (clone 19–200) (Figure 2). The carboxy-terminal deletion clones received the following constructs: clones 17, 19 and 19–200, pEE14–27–547; clones 37, 50 and 56, pEE12–27–547. Both clones 29 and 30 received the full-length construct pEE 12–27-SHa. No clones were obtained by transfection with the pEE14–27 SHa vector. The majority of immunoreactive protein appeared to be in the cell lysates as opposed to the medium for all clones. However, a larger percentage of PrP was found in the medium of the deletion clones as compared with the full-length clones. This would be expected since the PrP from the deletion clones should not receive a GPI anchor and thus would be secreted into the media. It is interesting that there was some PrP in media taken from clones 29 and 30, which may indicate autocleavage of PrP from the cell surface.

The major PrP band found in the media from the deletion clones was observed at a molecular weight of 19 kDa. Migration of this product to a position lower than native PrP$^C$ from SHa brain (33–35 kDa) could indicate degradation or lack of glycosylation. PrP found in the cell lysates of clones 29 and 30 appeared as a diffuse band of larger molecular size ranging from 19 to 40 kDa. The lower range of the band could represent degradation or processing products. Since equivalent amounts of total protein were loaded on to the gel, clone 30 appeared to be the highest expressor and was therefore chosen for further characterization. It was successively subcloned to obtain clones 30C1-1, 30C2-8 and 30C3-1 (Figure 3). A comparison of the relative expression levels of the four clones is shown in Figure 3D.

### PrP expression

The expression level of PrP in 30C3-1 cells was compared with those found in other cell lines that are commonly used to study PrP (Figure 4). The primary antibody used for the Western blot analysis was the anti-PrP RO73 polyclonal antiserum, which recognizes PrP in all species tested at undefined epitopes (Serban *et al.*, 1990). Controls using Syrian hamster, Chinese hamster and mouse brain homogenates showed that RO73 antiserum recognized PrP with equal affinity in all three species. The non-transfected CHO cells appeared to have a very small amount of immunoreactive protein, none of which was found at the molecular weight of PrP, 33–35 kDa. A mouse neuroblastoma cell line (N2a) and a Syrian hamster cell line (HaB) both produced PrP, but in much lower quantities than found in 30C3-1 cells.
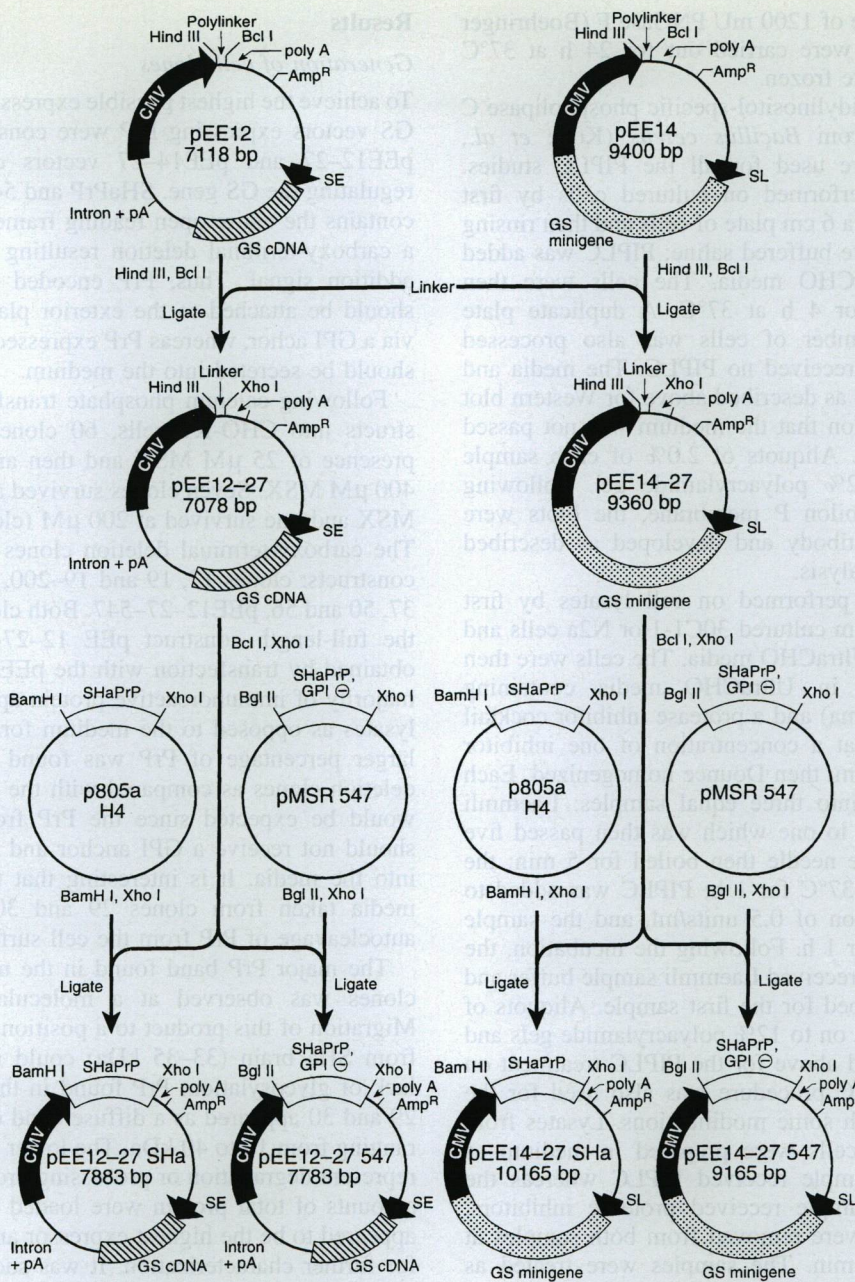
**Fig. 1.** SHaPrP glutamine synthetase constructs transfected into CHO cells. SHaPrP is the Syrian hamster PrP open reading frame (ORF). SHaPrP, GPI⁻ is SHaPrP ORF with the C-terminal GPI anchor addition sequence deleted. CMV represents the human cytomegalovirus promoter. SE refers to the SV40 early promoter. SL denotes the SV40 late promoter. GS refers to glutamine synthetase. Amp^R marks the site of the ampicillin resistance gene. The sequence of the linker is given in the Methods section.

*Biochemical and immunological characterization of recombinant PrP*

The immunoreactivity of the recombinant PrP against a series of antibodies that recognize the native PrP^C found in brain was examined. A comparison of the immunoreactivities of SHa brain homogenate, non-transfected CHO cell lysate and 30C3-1 cell lysate by Western blot analysis was made using the following antibodies: N-10 (which recognizes PrP^C in all species tested), 13A5 (which recognizes Syrian hamster PrP^C), P1 (specific for the N-terminus of PrP 27–30), P2 (specific for the N-terminus of PrP^C) and P3 (specific for the C-terminus of PrP^C) (Figure 5). The diffuse band corresponding to the

recombinant PrP from the 30C3-1 cells was seen with all five antibodies, and no PrP was seen in the non-transfected CHO cells.

Proteinase K treatment showed that PrP from 30C3-1 cell lysate was sensitive to proteolytic digestion (Figure 6A), as was also the case with PrP^C from SHa brain homogenate. PNGase F treatment of SHa brain homogenate resulted in a shifting of the three PrP bands from 33, 27 and 19 kDa to a single band at 19 kDa (Figure 6B). By comparison, PNGase F treatment of 30C3-1 cell lysate produced three major bands, the most intense of which was at the same position as the deglycosylated PrP^C from brain (19 kDa). The other two 30C3
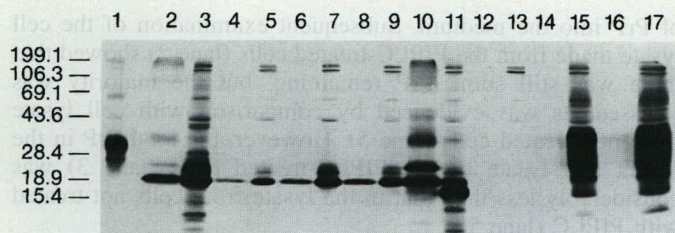
**Fig. 2.** Western blot of media and cell lysates from clones expressing SHaPrP. Lane 1, 30 µg SHa brain homogenate; lanes 2 and 3, clone 17; lanes 4 and 5, clone 19; lanes 6 and 7, clone 19–200; lanes 8 and 9, clone 37; lanes 10 and 11, clone 50; lanes 12 and 13, clone 56; lanes 14 and 15, clone 29; lanes 16 and 17, clone 30. The odd-numbered lanes contain 30 µg of cell lysate protein. The even-numbered lanes contain media corresponding to 300 µg of cell lysate protein. The blot was incubated with RO73 antiserum.
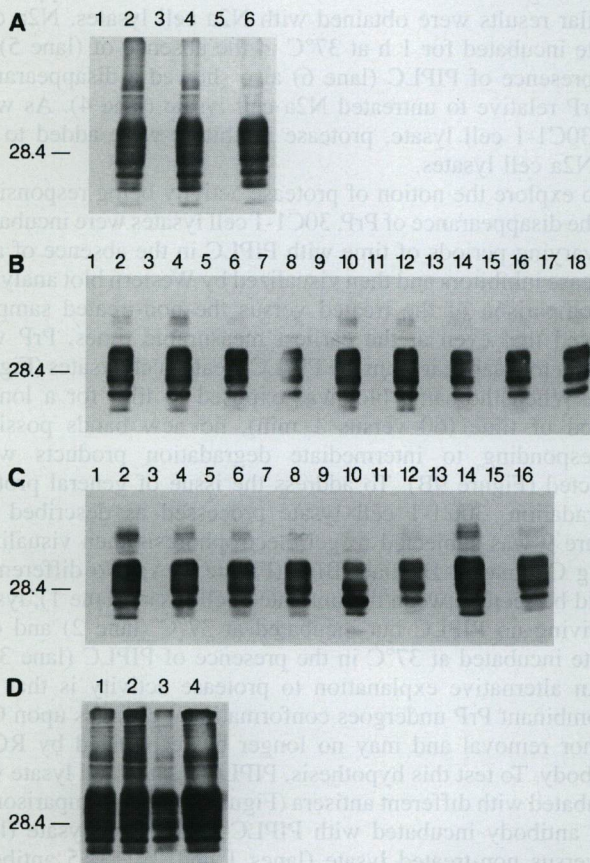


**Fig. 3.** Western blots of subclones from clone 30 incubated with RO73 antiserum. (**A**) The first generation. Lanes 1 and 2, subclone 30C1-1; lanes 3 and 4, subclone 30C1-2; lanes 5 and 6, subclone 30C1-3. (**B**) The second generation. Lanes 1 and 2, subclone 30C2-1; lanes 3 and 4, subclone 30C2-2; lanes 5 and 6, subclone 30C2-3; lanes 7 and 8, subclone 30C2-4; lanes 9 and 10, subclone 30C2-5; lanes 11 and 12, subclone 30C2-6; lanes 13 and 14, subclone 30C2-7; lanes 15 and 16, subclone 30C2-8; lanes 17 and 18, subclone 30C2-9. (**C**) The third generation. Lanes 1 and 2, subclone 30C3-1; lanes 3 and 4, subclone 30C3-2; lanes 5 and 6, subclone 30C3-3; lanes 7 and 8, subclone 30C3-4; lanes 9 and 10, subclone 30C3-5; lanes 11 and 12, subclone 30C3-6; lanes 13 and 14, subclone 30C3-7; lanes 15 and 16, subclone 30C3-8. For each of the above, the even-numbered lanes contain 30 µg of cell lysate protein. The odd-numbered lanes contain media corresponding to 300 µg of cell lysate protein. (**D**) Western blot comparison of clone 30 with the highest expressing subclones, incubated with RO73 antiserum. Lane 1, clone 30; lane 2, clone 30C1-1; lane 3, clone 30C2-8; lane 4, clone 30C3-1. 30 µg of cell lysate protein were loaded into each lane.



**Fig. 4.** Western blot comparison of clone 30C3-1 with other cell lines expressing PrP, incubated with RO73 antiserum. Lane 1, CHa brain homogenate; lane 2, SHa brain homogenate; lane 3, Mo brain homogenate; lane 4, CHO cell lysate; lane 5, HaB cell lysate; lane 6, N2a; lane 7, clone 30C3-1. 30 µg of cell lysate protein were loaded into each lane.
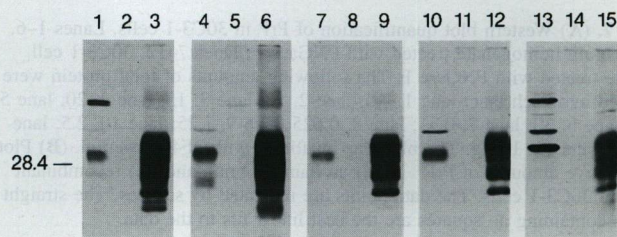


**Fig. 5.** Immunoblot of clone 30C3-1. Lanes 1, 4, 7, 10 and 13, SHa brain homogenate; lanes 2, 5, 8, 11 and 14, CHO cell lysate; lanes 3, 6, 9, 12 and 15, 30C3-1 cell lysate. Lanes 1–3, N-10 antiserum; lanes 4–6, 13A5 antiserum; lanes 7–9 P1 antiserum; lanes 10–12, P2 antiserum; lanes 13–15, P3 antiserum. 30 µg of total protein were loaded into each lane.
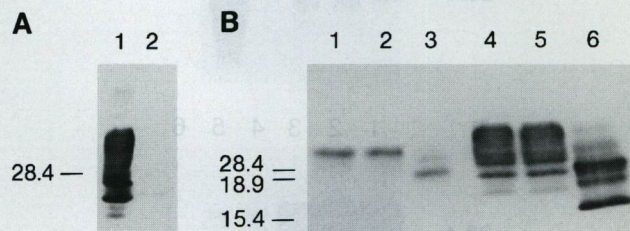


**Fig. 6.** (**A**) Western blot of 30C3-1 cell lysate treated with proteinase K. Lane 1, 30C3-1 cell lysate; lane 2, 30C3-1 cell lysate + proteinase K. (**B**) Western blot of SHa brain homogenate and clone 30C3-1 treated with PNGase F. Lane 1, SHa brain homogenate; lane 2, SHa brain homogenate, 37°C, 24 h; lane 3 SHa brain homogenate + PNGase F, 37°C, 24 h; lane 4, 30C3-1 cell lysate; lane 5, 30C3-1 cell lysate, 37°C, 24 h; lane 6, 30C3-1 cell lysate PNGase F, 37°C, 24 h. For both panels all lanes received 30 µg of total protein. Blots were incubated with 3F4 antiserum.

bands (17 and 15 kDa) were of a lower molecular weight than deglycosylated PrP$^C$ from brain and most likely represented degradation or processing products.

The SHaPrP$^C$ expression level of 30C3-1 cells was compared with PrP$^C$ expression in SHa brain by Western blot analysis (Figure 7A). Since PrP from the 30C3 cells contained more than one protein species, both the brain homogenate and cell lysate were treated with PNGase F and only the 19 kDa band corresponding to full-length, unglycosylated PrP$^C$ was quantified by densitometric scanning. Plots of the relative amounts of PrP$^C$ (densitometric units) versus the amount of total protein (µg) for both SHa brain (Figure 7B) and 30C3-1 cell lysate (Figure 7C) are shown. The ratio of the slopes of the best linear fits was employed to determine that the
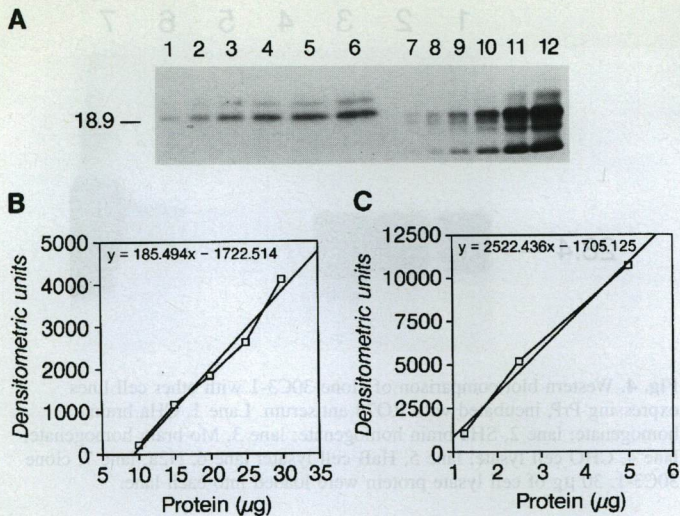
**A**



**B** **C**



**Fig. 7.** (**A**) Western blot quantification of PrP in 30C3-1 cells. Lanes 1–6, SHa brain homogenate treated with PNGase F; lanes 7–12, 30C3-1 cell lysate treated with PNGase F. The following amounts of total protein were loaded into each lane: lane 1, 7.5; lane 2, 10; lane 3, 15; lane 4, 20; lane 5, 25; lane 6, 30; lane 7, 0.31; lane 8, 0.625; lane 9, 1.25; lane 10, 2.5; lane 11, 5; lane 12, 10 μg. The blot was incubated with 3F4 antiserum. (**B**) Plot of relative amounts of PrP$^C$ in Syrian hamster brain and (**C**) recombinant PrP in 30C3-1 cells. The data points are indicated by squares. The straight lines containing no squares are the best linear fits to the data.

**A**



**B**
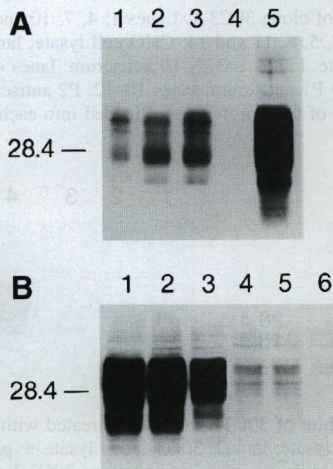


**Fig. 8.** Western blots of PrP after cells had been treated with PIPLC. (**A**) Western blot of 30C1-1 cells treated with PIPLC. Lane 1, medium from cells treated with PIPLC; lane 2, cell lysate from cells treated with PIPLC; lane 3, medium plus cell lysate from cells treated with PIPLC; lane 4, medium from cells not treated with PIPLC; lane 5, cell lysate from cells not treated with PIPLC. The blot was incubated with RO73 antiserum. (**B**) Western blot of 30C1-1 and N2a cell lysates treated with PIPLC. Lane 1, 30C1-1 cell lysate, no treatment; lane 2, 30C1-1 cell lysate at 37°C for 1 h; lane 3, 30C1-1 cell lysate + PIPLC at 37°C for 1 h; lane 4, N2a cell lysate; lane 5, N2a cell lysate at 37°C for 1 h; lane 6, N2a cell lysate + PIPLC at 37°C for 1 h. The blot was incubated with RO73 antiserum.

expression level of PrP in 30C3-1 cells relative to total protein was ~14-fold higher than PrP$^C$ in SHa brain.

Since full-length PrP$^C$ should accept the addition of a GPI anchor, 30C1-1 cells grown in culture were treated with the enzyme PIPLC and the medium was examined for released PrP (Figure 8A). A comparison of medium from cells treated with PIPLC (lane 1) and medium from an equal number of cells not treated with PIPLC (lane 4) clearly showed that PIPLC treatment of the cultured cells resulted in the release

of PrP into the medium. Subsequent examination of the cell lysate made from the PIPLC-treated cells (lane 2) showed that there was still some PrP remaining, but the majority was released, as was evidenced by comparison with cell lysate from non-treated cells (lane 5). However, the total PrP in the media and lysate of the PIPLC-treated cells (lane 3) was considerably less than that in the lysate from cells not treated with PIPLC (lane 5).

To address the possibility that extensive PrP loss might have occurred during the processing of the medium, 30C1-1 cell lysate was treated directly with PIPLC (Figure 8B). 30C1-1 cells were lysed in the presence of a cocktail of protease inhibitors and were either loaded directly on to a gel (lane 1) or incubated for 1 h at 37°C in the absence of (lane 2) or the presence of PIPLC (lane 3). Although the PIPLC was added directly to the cell lysate and the lysate was loaded directly on to a gel after incubation, much of the PrP disappeared. Similar results were obtained with N2a cell lysates. N2a cell lysate incubated for 1 h at 37°C in the absence of (lane 5) or the presence of PIPLC (lane 6) also showed a disappearance of PrP relative to untreated N2a cell lysate (lane 4). As with the 30C1-1 cell lysate, protease inhibitors were added to all the N2a cell lysates.

To explore the notion of protease activity being responsible for the disappearance of PrP, 30C1-1 cell lysates were incubated for varying periods of time with PIPLC in the absence of any protease inhibitors and then visualized by Western blot analysis. A comparison of the treated versus the non-treated samples showed that even at the earliest measurable times, PrP was starting to disappear from the PIPLC-treated cell lysates (Figure 9A). When the same blot was exposed to film for a longer period of time (60 versus 1 min), no new bands possibly corresponding to intermediate degradation products were detected (Figure 9B). To address the issue of general protein degradation, 30C1-1 cell lysate processed as described for Figure 9 was subjected to gel electrophoresis then visualized using Coomassie Brilliant Blue (Figure 10A). No differences could be seen between the untreated cell lysate (lane 1), lysate receiving no PIPLC but incubated at 37°C (lane 2) and cell lysate incubated at 37°C in the presence of PIPLC (lane 3).

An alternative explanation to protease activity is that the recombinant PrP undergoes conformational changes upon GPI anchor removal and may no longer be recognized by RO73 antibody. To test this hypothesis, PIPLC-treated cell lysate was incubated with different antisera (Figure 10B). A comparison of 3F4 antibody incubated with PIPLC-treated cell lysate (lane 3) versus non-treated lysate (lanes 1 and 2), 13A5 antibody incubated with treated lysate (lane 6) versus non-treated lysate (lanes 4 and 5) and N-10 polyclonal sera incubated with treated lysate (lane 9) versus nontreated lysates (lanes 7 and 8) showed a decrease in the amount of PrP being detected with all three antibodies. Since all antisera tested displayed large decreases in reactivity with the PIPLC-treated cell lysates, it is unlikely that the disappearance of PrP is due to lack of antibody recognition.

## Discussion

A conformational change in the structure of PrP seems to be the fundamental event underlying the prion diseases (Pan *et al.*, 1993). As such, structural studies of PrP are of utmost importance in deciphering the process whereby PrP$^C$ is transformed into PrP$^{Sc}$. Although the level of PrP$^C$ in rodent brain is high compared with that in other tissues, it still represents

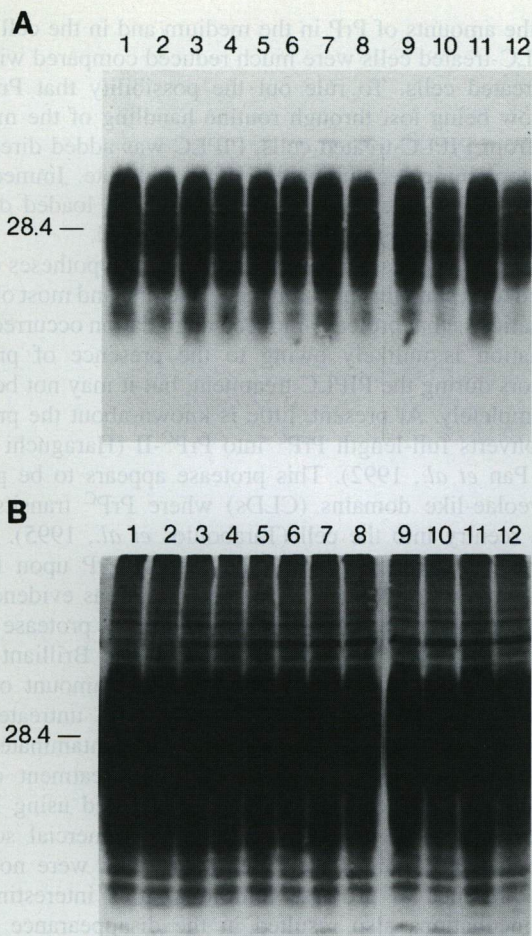**A**



28.4 —

**B**

28.4 —

**Fig. 9.** Western blot of 30C1-1 cell lysate treated with PIPLC for various periods of time. Lysates were incubated at 37°C for the following periods of time: lanes 1 and 2, 0 min; lanes 3 and 4, 2 min; lanes 5 and 6, 5 min; lanes 7 and 8, 10 min; lanes 9 and 10, 30 min; lanes 11 and 12, 60 min. Samples in odd-numbered lanes received no PIPLC. Samples in even-numbered lanes received PIPLC. The blot was incubated with RO73 antiserum. For (A) the film exposure time was 1 min and for (B) the blot was exposed to film for 1 h.

**A**

**B**

**Fig. 10.** (A) Coomassie Brilliant Blue-stained gel of 30C1-1 cell lysate treated with PIPLC. Lane 1, 30C1-1 cell lysate, no treatment; lane 2, 30C1-1 cell lysate at 37°C for 4 h; lane 3, 30C1-1 cell lysate + PIPLC at 37°C for 4 h. (B) Western blot analysis of 30C1-1 cell lysate treated with PIPLC. Lanes 1, 4 and 7, 30C1-1 cell lysate, no treatment; lanes 2, 5 and 8, 30C1-1 cell lysate, 37°C, 1 h; lanes 3, 6 and 9, 30C1-1 cell lysates + PIPLC, 37°C, 1 h. The blot was incubated with the following antibodies: lanes 1–3, 3F4 antibody; lanes 4–6, 13A5 antibody; lanes 7–9, N-10 antibody.

**Table I.** Summary of systems employed for recombinant PrP expression

| Cell type | Protein expressed | Ref. |
|---|---|---|
| *Escherichia coli* | SHaPrP[a] | Weiss *et al.*, 1995; Mehlhorn *et al.*, 1996 |
| | MoPrP[b] | Hornemann and Glockshuber, 1996 |
| *Spodoptera frugiperda* | SHaPrP[a] | Scott *et al.*, 1988; Weiss *et al.*, 1995 |
| *Drosophila melanogaster* | SHaPrP$^C$ | Raeber *et al.*, 1995 |
| Xenopus oocytes | SHaPrP$^C$ | Hay *et al.*, 1987 |
| Mouse C127 | MoPrP$^C$ | Caughey *et al.*, 1988 |
| Mouse N2a | SHaPrP$^C$ | Scott *et al.*, 1988; Chesebro *et al.*, 1993 |
| | ChPrP$^C$ | Harris *et al.*, 1993; Gorodinsky and Harris, 1995 |
| | Mo/ChPrP$^C$ | Shyng *et al.*, 1995 |
| | Mo/SHaPrP$^C$ | Chesebro *et al.*, 1993 |
| Mouse ScN2a | Mo/MoPrP$^C$ | Scott *et al.*, 1992; Rogers *et al.*, 1993 |
| Mouse NIH3T3 | SHaPrP$^C$ | Scott *et al.*, 1988; Kocisko *et al.*, 1994 |
| Mouse Sc+-MNB | SHaPrP$^C$ | Priola *et al.*, 1994 |
| | SHa/MoPrP$^C$ | |
| | MoPrP$^C$ | |
| Mouse MNB | MoPrP$^C$ | Kocisko *et al.*, 1995 |
| | Mo/SHaPrP$^C$ | |
| Hamster HJC | SHaPrP$^C$ | Scott *et al.*, 1988 |
| Hamster CHO | SHaPrP$^C$ | Borchelt *et al.*, 1993 |
| | MoPrP$^C$ | Lehmann and Harris, 1996 |
| | Mo/SHaPrP$^C$ | |
| Rat RAT-1 | SHaPrP$^C$ | Scott *et al.*, 1988 |
| Monkey COS-7 | SHaPrP$^C$ | Scott *et al.*, 1988 |
| Monkey CV-1 | SHaPrP$^C$ | Rogers *et al.*, 1991 |
| | SHa/MoPrP$^C$ | |
| | MoPrP$^C$ | |

[a]Fusion protein formed from PrP and glutathione-S-transferase.
[b]C-terminal fragment corresponding to mouse residues 111–231.

less than 0.1% of the total CNS protein (Turk *et al.*, 1988; Pan *et al.*, 1992, 1993). To overcome this difficulty, many investigators have tried to express recombinant PrP$^C$ at high levels in cultured cells (Table I). The need for such systems has been emphasized by recent reports on the NMR structure of fragments of PrP synthesized and labeled in bacteria (Riek *et al.* 1996; James *et al.*, 1997). In one case, a PrP fragment of 111 amino acids likely to correspond to PrP$^C$-II, a degradation product of PrP$^C$ that appears to be formed in caveolae (Pan *et al.*, 1992), was prepared from *Escherichia coli* for structural studies (Hornemann and Glockshauber, 1996). In another case, a PrP fragment of 142 amino acids that corresponds in sequence to PrP 27–30, the protease-resistant core of PrP$^{Sc}$ (Prusiner *et al.*, 1984), was prepared from *E.coli* for structural studies (Mehlhorn *et al.*, 1996). Such studies with *E.coli*-derived proteins suffer from the lack of post-translational modifications which may substantially alter the structure of PrP. In most of the mammalian expression systems where the PrP$^C$ was post-translationally modified by addition of Asn-linked oligosaccharides and a GPI anchor, the quantity of recombinant PrP$^C$ was low and purification was not reported. In the studies presented here, we chose a glutamine synthetase gene amplification system to establish a mammalian cell system capable of generating PrP$^C$ at high levels of expression (Bebbington *et al.*, 1987).

The Asn-linked oligosaccharides of PrP$^C$ are thought to modify the structure of the protein and in turn alter the rate

at which it is converted into PrP$^{Sc}$ (DeArmond *et al.*, 1997). Differences in the rates of conversion of PrP$^C$ into PrP$^{Sc}$ may be responsible for the strain-specific patterns of PrP$^{Sc}$ accumulation (Hecker *et al.*, 1992; DeArmond *et al.*, 1993). The GPI anchor of PrP$^C$ is required for PrP$^{Sc}$ formation (Taraboulos *et al.*, 1995; Vey *et al.*, 1996; Kaneko *et al.*, 1997; Naslavsky *et al.*, 1997). The anchor directs the trafficking of PrP$^C$ to the caveolae; redirecting PrP$^C$ to clathrin coated pits or preventing its egress to the cell surface by brefeldin A abolished PrP$^{Sc}$ formation in ScN2a cells (Taraboulos *et al.*, 1991). These findings emphasize the importance of post-translational glycosylation of PrP$^C$ with respect to PrP$^{Sc}$ formation. As such, determining the structure of glycosylated and glycolipidated PrP is of utmost interest. Hence the development of a mammalian system for the high-level expression of PrP$^C$ as described here should greatly facilitate structural studies.

To test the involvement of the GPI anchor in the structural and functional aspects of PrP, we decided to express and compare the full-length SHaPrP protein and a SHaPrP mutant that lacked the carboxyl terminal addition site for a GPI moiety (Rogers *et al.*, 1993). The full-length SHaPrP would receive its GPI anchor and be deposited on the outer cell surface. In this cell type, the structure and function of PrP$^C$ might be preserved if they depended upon the presence of the GPI anchor. Without the GPI anchor, the PrP would not be attached to the outer cell surface and thus most likely would be secreted into the medium. An advantage of not having an anchor would be to facilitate the purification of the recombinant protein.

All of the GPI$^-$ clones secreted PrP that was partly of lower molecular weight than native PrP$^C$, which could be due to underglycosylation and/or protein degradation. Non-glycosylation of SHaPrP lacking a GPI anchor has also been reported for recombinant SHaPrP expressed in NIH 3T3 cells (Kocisko *et al.*, 1994). We sought to obtain PrP that was structurally similar to native PrP$^C$, but at higher levels than in brain. We therefore chose clone 30, which expressed full-length PrP, and subcloned it to derive clone 30C3-1. In contrast to GPI$^-$ clones, the full-length recombinant PrP protein was hyperglycosylated and migrated as a diffuse band between 19 and 40 kDa. PNGase F treatment of the 30C3-1 lysate resulted in three bands of 19, 17 and 15 kDa. The 19 kDa species corresponded to the correct size of unglycosylated, full-length PrP and had also been reported to PNGase F-treated PrP in ScN2a cells (Taraboulos *et al.*, 1990). The 17 and 15 kDa species may represent degradation products caused by possible proteases present in the PNGase F (Tarentino *et al.*, 1985; Taraboulos *et al.*, 1990). Lower molecular weight products were also seen with the PNGase F-treated ScN2a cells. Quantification of the 19 kDa band revealed a 14-fold over-expression of recombinant PrP relative to PrP$^C$ from SHa brain homogenate.

Characterization of the GPI anchor of recombinant PrP revealed some interesting features. PIPLC treatment of the PrP-expressing CHO cells resulted in the release of the majority of PrP. These results are similar to those of Lin *et al.* (1990), who reported that PIPLC treatment of CHO cells expressing recombinant T cell antigen receptor released ~90% of the receptor from the cell surface. The residual cell-associated PrP may be located inside the cell and thus inaccessible to the PIPLC. An alternative explanation may be that a subset of PrP molecules exist that are resistant to PIPLC release due to acylation of inositol hydroxyl groups (Mayor *et al.*, 1990).

Also, the amounts of PrP in the medium and in the cell lysate of PIPLC-treated cells were much reduced compared with that of untreated cells. To rule out the possibility that PrP was somehow being lost through routine handling of the medium taken from PIPLC-treated cells, PIPLC was added directly to tubes containing recombinant CHO cell lysate. Immediately after the PIPLC incubation, the lysates were loaded directly on to gels but a loss of PrP was still observed.

The loss of PrP is perplexing but several hypotheses can be invoked to explain this phenomenon. The first and most obvious explanation is that protease-induced degradation occurred. This explanation is unlikely owing to the presence of protease inhibitors during the PIPLC treatment, but it may not be ruled out completely. At present, little is known about the protease that converts full-length PrP$^C$ into PrP$^{C}$-II (Haraguchi *et al.*, 1989; Pan *et al.*, 1992). This protease appears to be present in caveolae-like domains (CLDs) where PrP$^C$ transits as it begins reentry into the cell (Taraboulos *et al.*, 1995). Had a protease been responsible for the loss of PrP upon PIPLC treatment, it was not specific for CHO cells, as evidenced by results obtained with N2a cell lysates. Also, the protease would have been specific for PrP since Coomassie Brilliant Blue-stained gels revealed no differences in the amount of total cellular protein between PIPLC-treated and untreated cell lysates. It is not likely that the PIPLC was contaminated with a protease. Additional studies of PIPLC treatment on the recombinant CHO cell lysates were conducted using PIPLC samples purchased from two different commercial sources. Although these studies were preliminary and were not done in the presence of protease inhibitors, it is interesting that these incubations also resulted in the disappearance of the majority of the PrP (data not shown). A second explanation for the disappearance of PrP may be that removal of its diacylglycerol leads to its instability and thereby renders it susceptible to degradation. However, Western blot analysis of samples taken from PIPLC treatment of the recombinant CHO cell lysates at different times showed a gradual disappearance of PrP without the generation of any new lower molecular weight products. A third possibility, that the removal of the GPI anchor may have altered the antibody recognition of PrP, was examined. Both the two monoclonal antibodies 3F4 and 13A5 (which recognize different epitopes) and the two polyclonal sera RO73 and N-10 displayed large decreases in reactivity with the PIPLC-treated cell lysates; therefore, this explanation seems improbable. A fourth and unexplored possibility is that PIPLC-treated PrP was not detected owing to inefficient electrophoretic transfer on to membranes upon GPI anchor removal.

The data reported here on recombinant PrP stability and PIPLC release are particularly notable since the GPI anchor of PrP is now known to play an important role in PrP$^{Sc}$ formation (Taraboulos *et al.*, 1995; Vey *et al.*, 1996; Kaneko *et al.*, 1997; Naslavsky *et al.*, 1997). The formation of PrP$^{Sc}$ is thought to occur in CLDs where GPI anchored proteins are known to congregate (Smart *et al.*, 1995; Vey *et al.*, 1996). In CLDs, PrP$^C$ is either initially degraded by N-terminal proteolytic cleavage to generate PrP$^{C}$-II or it is converted into PrP$^{Sc}$ (Taraboulos *et al.*, 1995). Hence, the production of large quantities of recombinant PrP$^C$ with a mammalian GPI anchor may prove essential for dissecting the mechanism of PrP$^{Sc}$ formation.

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