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Purification and properties of the cellular prion protein from Syrian hamster brain

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Abstract

The cellular prion protein (PrP^C) is encoded by a chromosomal gene, and its scrapie isoform (PrP^{Sc}) features in all aspects of the prion diseases. Prior to the studies reported here, purification of PrP^C has only been accomplished using immunoaffinity chromatography yielding small amounts of protein. Brain homogenates contain two PrP^C forms designated PrP^C-I and -II. These proteins were purified from a microsomal fraction by detergent extraction and separated by immobilized Cu^{2+} ion affinity chromatography. PrP^C-II appears to be generated from PrP^C-I by limited proteolysis of the N-terminus. Fractions enriched for PrP^C-I were purified further by cation-exchange chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Greater than 90% of the final product migrated as a broad band of M_r 33-35 kDa as judged by silver staining after SDS-PAGE. Digestion of PrP^C-I with peptide-*N*-glycosidase (PNGase) compressed the band and shifted its mobility giving an M_r of 27 kDa. The protocol described should be amenable to large-scale preparation of PrP^C, enabling physical comparisons of PrP^C and PrP^{Sc}.

Keywords: glycoinositol phospholipid anchor; GPI protein; PrP^C; PrP gene; scrapie

The cellular prion protein (PrP^C) is a sialoglycoprotein (Bolton et al., 1985; Endo et al., 1989; Haraguchi et al., 1989), which is bound to the external surface of cells by a glycoinositol phospholipid (GPI) anchor (Stahl et al., 1987). PrP is encoded by a single-copy chromosomal gene and is highly conserved among mammals (Chesebro et al., 1985; Oesch et al., 1985; Basler et al., 1986; Westaway et al., 1987; Goldman et al., 1990, 1991; Lowenstein et al., 1990; Puckett et al., 1991). The entire open reading frame (ORF) is encoded within a single exon for all mammals examined offering no opportunity to generate alternatively spliced mRNAs (Oesch et al., 1985; Basler et al., 1986; Westaway et al., 1987; Hsiao et al., 1989).

PrP^C was discovered after PrP mRNA was found to be present at similar levels in the brains of normal and scrapie-infected Syrian hamsters (SHa) (Oesch et al., 1985). Cognate molecular clones were recovered from a complementary DNA (cDNA) library using an isocoding mixture of oligonucleotides corresponding to the N-terminal amino acid sequence of the protease-resistant core of the prion protein (PrP 27-30) isolated from scrapie-infected SHa brains (Prusiner et al., 1984). The function of PrP^C is unknown although a candidate PrP has been found in fractions highly enriched for acetylcholine receptor inducing activity (ARIA) from chicken brain (Harris et al., 1991). Whether the candidate chicken PrP or mammalian PrP molecules possess ARIA remains to be established.

Interestingly, PrP^C seems to be expressed constitutively in the adult brain but its levels in the developing brain are highly regulated and can be stimulated by nerve growth factor (Kretzschmar et al., 1986; Mobley et al., 1988). PrP expression is highest in neurons of the central nervous system but lower levels have been found in other tissues. The levels of $SHaPrP^C$ in transgenic (Tg) mice has been shown to correlate inversely with the length of the scrapie incubation period (Prusiner et al., 1990). The conversion of PrP^C to scrapie prion protein (PrP^{Sc}) may involve the formation of a heterodimeric intermediate between PrP^C and PrP^{Sc} (Prusiner, 1991). This conversion is thought to occur intracellularly after PrP^C or a precursor exits from the Golgi and transits to the cell surface

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but before PrP^{Sc} is deposited in lysosomes (Borchelt et al., 1990; Taraboulos et al., 1990; Caughey & Raymond, 1991; McKinley et al., 1991).

Whereas the conversion of PrP^{C} to PrP^{Sc} is clearly a posttranslational process (Basler et al., 1986; Borchelt et al., 1990), the molecular basis for this change is unknown. Attempts to demonstrate a unique posttranslational chemical modification have been unsuccessful, leading to the suggestion that the transformation of PrP^{C} or a precursor into PrP^{Sc} may be only conformational (Stahl et al., 1991). To facilitate studies designed to compare the properties of PrP^{C} with PrP^{Sc}, we set out to develop a purification protocol for PrP^{C} that could be performed on a large scale.

We report here on an isolation scheme that does not require an immunoaffinity step as utilized by others (Bendheim et al., 1988) and one of us (S.B.P.) in the past (Turk et al., 1988). Immunoaffinity purification has been problematic for large-scale preparation of PrP^{C} because of poor recoveries of the product and the leakage of anti-PrP antibodies. A crude microsomal fraction from SHa brains was obtained by low-speed centrifugation followed by precipitation with polyethylene glycol (PEG)-8000. PrP^{C} was solubilized from the membrane fraction with Zwittergent (ZW) 3-12, and fractionation was performed by immobilized metal-ion affinity chromatography (IMAC) and cation-exchange chromatography. The resulting fractions were further purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The procedure reported here should greatly facilitate future studies on the properties and structure of PrP^{C}.

Results

Because PrP^{C} is a GPI-anchored membrane protein, we attempted phosphatidylinositol-specific phospholipase C (PIPLC) digestion in combination with Triton X-114 phase partition. A microsomal preparation solubilized with Triton X-114 was prepared and digested with the PIPLC to release PrP^{C} from the microsomal membrane. After raising the temperature to 37 °C, two phases were formed with GPI-cleaved proteins partitioning into the aqueous phase and other membrane-bound proteins remaining in the detergent phase. However, the major protein in the aqueous phase was identified by sequence analysis as Thy-1, also a GPI-anchored protein abundantly present in the brain (Williams & Gagnon, 1982). Further separation of PrP from Thy-1 was not attempted.

In previous purification studies, organic extraction has been successfully applied to enrich Thy-1 preparations (Campbell et al., 1981). Upon extraction from either brain homogenates or microsomal fractions with a variety of organic solvents (like chloroform/methanol, acetone, or ether), only small amounts of PrP^{C} were released into the organic phase. On the contrary, most PrP^{C} was precipitated out of solution together with many other proteins. The PrP^{C} in the precipitated materials proved difficult to solubilize with a variety of detergents.

Identification of two forms of PrP^{C}

Using a monoclonal antibody (mAb) 13A5 raised against SHaPrP, two PrP-immunoreactive bands were identified from normal brain homogenate on the immunoblot (Figs. 1f, 2g). The upper band (PrP^{C}-I) has a molecular weight of 33–35 kDa and the lower band (PrP^{C}-II) about 25–27 kDa. An alternative mAb 3F4, directed against an epitope N-terminal to that recognized by mAb 13A5, only reacted with PrP^{C}-I but not PrP^{C}-II (Fig. 1e). Polyclonal antibodies raised against synthetic peptides corresponding to the N-terminal (αP1) and C-terminal (αP3) of PrP 27–30 were also used to probe the two PrP species. αP1 stained only PrP^{C}-I, whereas αP3 reacted with both PrP^{C}-I and PrP^{C}-II (Fig. 1a,c). Absorption of the αP1 or αP3 antisera with P1 or P3 peptide, respectively, resulted in the specific loss of PrP reactivities (Fig. 1b,d). These results argue that PrP^{C}-I and -II have the same C-termini but differ by an N-terminal segment.

Detergent-solubilized microsomal preparation

To purify PrP^{C}, a detergent-solubilized microsomal fraction was prepared from normal SHa brain homogenate. The homogenate was first centrifuged at 3,000 × *g* for 30 min. The supernatant contained about 50% of total PrP^{C} as determined by quantitative ELISA (Table 1).

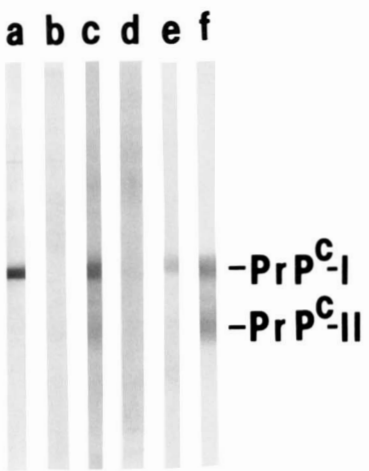

Fig. 1. Immunoreactivity of cellular prion protein (PrP^{C})-I and PrP^{C}-II with different antibodies. Hamster microsomal fraction was prepared and analyzed by immunoblotting assay as described in the Materials and methods. Lanes a, c, e, and f, immunostained with antisera from αP1, αP3, monoclonal antibody (mAb) 3F4, and mAb 13A5, respectively; lane b, αP1 antisera absorbed with excess synthetic peptides (PrP amino acids 90–102); lane d, αP3 antisera absorbed with the peptides (PrP amino acids 220–233).

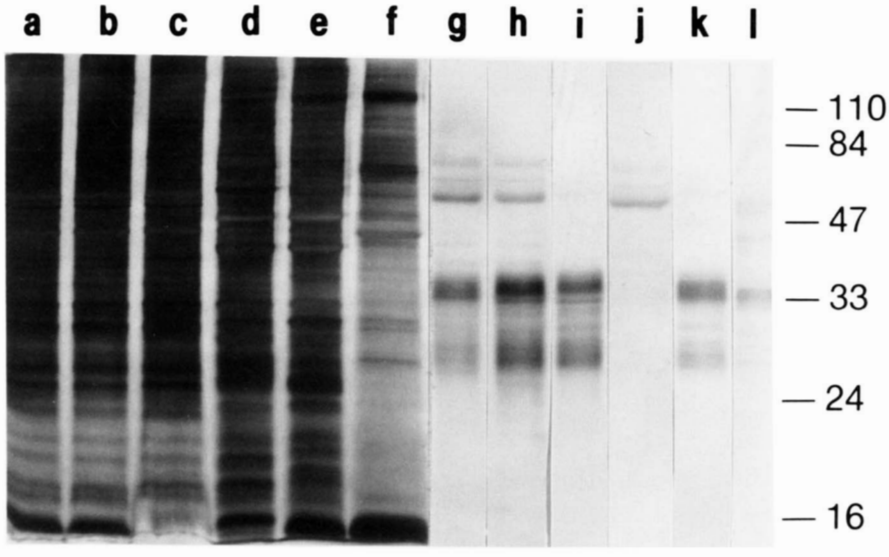

Fig. 2. Preparation of detergent-solubilized microsomal fraction from brain homogenate. Protein samples (5 μg each) were separated by SDS-PAGE, and analyzed by silver staining (lanes a–f) or transferred to an Immobilon membrane and immunostained with mAb 13A5 (lanes g–l). Lanes a and g, hamster brain homogenate; lanes b and h, supernatant of low-speed centrifugation; lanes c and i, pellet of low-speed centrifugation; lanes d and j, supernatant of PEG precipitation; lanes e and k, supernatant of ZW 3-12-solubilized fraction; lanes f and l, pellet of ZW 3-12-solubilized fraction. Molecular weights in kDa are indicated at the right.

It was then subjected to PEG precipitation and all the PrP^C was recovered in the pellet as indicated by the lack of PrP^C in the PEG supernatant (Fig. 2d,j). The pellet was solubilized in 8% ZW 3-12 and centrifuged to remove insoluble materials. More than 90% of PrP^C became soluble after high-speed centrifugation (Fig. 2e,f,k,l).

Various detergents were used to solubilize PEG-precipitated pellets. Among them, SDS and Sarkosyl give almost complete solubilization of PrP^C together with many other proteins (Table 2). However, little protein, if any, was found to bind the IMAC column in the presence of either detergent. Other detergents like Triton, β-octyl glucopyranoside, or ZW 3-12 do not affect PrP^C binding to the IMAC column (Table 2). ZW 3-12 gives better solubilization of PrP^C than Triton and was used throughout in the study.

Immobilized metal ion affinity chromatography

The IMAC columns charged with either Cu^{2+}, Ni^{2+}, Zn^{2+}, or Co^{2+} were examined with small-scale preparations. Proteins were eluted from the column either by imidazole or pH gradient. PrP^C-I binds to the column more tightly and is eluted later than PrP^C-II with all metals (Table 3). The binding strength of PrP^C to the IMAC columns follows the order $Cu^{2+} > Ni^{2+} > Zn^{2+} > Co^{2+}$. Figure 3 shows the elution profile of ZW 3-12-solubilized supernatant chromatographed on an IMAC-Cu^{2+} column. Both PrP^C-I and PrP^C-II bound to the IMAC resin in the presence of 1 mM imidazole. A 10 mM imidazole wash eluted PrP^C-II from the column, whereas PrP^C-I remained bound (Fig. 4a,d). The low pH wash did not affect the binding of most of the PrP^C-I but did

Table 1. *Purification of cellular prion protein (PrP^C)*

Purification step	Total protein[a] (mg)	Total PrP^C[b] (μg)	Specific immunoreactivity PrP^C (μg/mg)	Purification factor	Recovery (%)
Brain homogenate	10,000	700	0.07	1	100
Supernatant of low-speed centrifuge	5,500	350	0.06	1	50
Pellet of PEG-8000 precipitation	2,500	350	0.14	2	50
Supernatant of ZW 3-12 extraction	2,000	315	0.16	2	45
EDTA eluate of IMAC	60	250	4.2	60	36
0.5 M NaCl eluate of cation exchanger	7	150	21.4	306	21
SDS-PAGE	0.13	120	923.1	13,186	17

[a] Mean of three determinations.
[b] Determined by a modified ELISA as described in Materials and methods.

Table 2. *Solubilization of cellular prion protein (PrP^C) by detergent extraction*

Detergent	% Soluble[a]	Binding to IMAC[b]
SDS	>99	No
Sarkosyl	>99	No
Triton X-100	60	Yes
Triton X-114	50	Yes
β-Octyl glucopyranoside	>90	Yes
ZW 3-12	>90	Yes

[a] PEG-precipitated pellets, prepared as described in the Materials and methods, were dissolved in various detergents with a weight ratio of 10 (detergent/protein). The mixtures were centrifuged at 100,000 × *g* for 1 h. PrP^C in the pellets and supernatants was estimated by ELISA as described in the Materials and methods.

[b] The IMAC-Cu^{2+} column was used to determine the binding of PrP^C to the column.

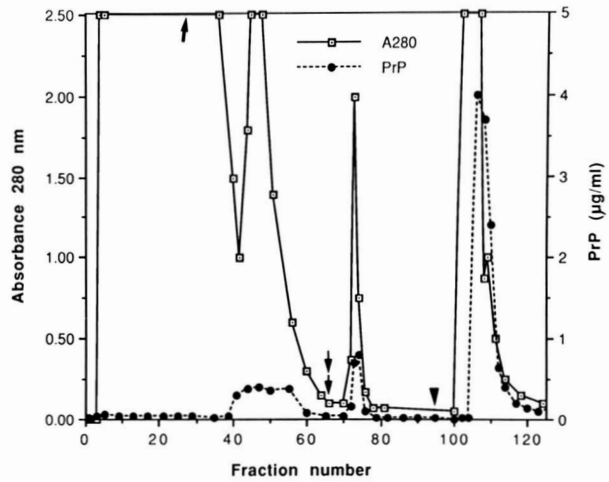

Fig. 3. Immobilized metal-ion affinity chromatography of ZW 3-12-solubilized microsomal fraction. The detergent-solubilized supernatant was applied onto an IMAC column, which was equilibrated in buffer A. Arrow stands for the start of 10 mM imidazole wash, double arrow for low pH wash (0.5 M NaCl, 0.2% ZW 3-12, and 0.1 M Na acetate, pH 5.0), and arrowhead for the beginning of EDTA elution. The flow rate was 2 mL/min, and 8-mL fractions were collected. The bars indicate the tubes pooled for each fraction. Fraction 1, tubes 40–60; fraction 2, tubes 72–76; fraction 3, tubes 103–120.

remove other proteins from the column (Fig. 4b,e). Finally, PrP^C-I was eluted by an EDTA wash (Fig. 4c,f). The strong absorption at 280 nm during the EDTA wash is caused by Cu^{2+}·EDTA complexes eluting from the column. At this step, PrP^C-I was separated from PrP^C-II and enriched by a purification factor of about 30-fold (Table 1). A second cycle of IMAC did not give any further purification of PrP^C-I. Further purification of PrP^C-II was not attempted.

Cation chromatography

The EDTA eluate from the IMAC containing PrP^C-I was pooled and applied onto a cation exchanger with sulfonic groups. Figure 5 shows the elution pattern of this column. Most of the proteins eluted in the flow-through (Fig. 6a,c), whereas PrP^C-I was tightly bound to the column. PrP^C-I was then eluted by 0.5 M NaCl in the presence of 4 M urea. Attempts to elute PrP^C-I with higher ionic-strength solutions like 1 or 2 M NaCl in the absence of urea were unsuccessful. Upon silver staining after SDS-PAGE, PrP^C-I eluted by this procedure migrated as a well-separated, broad band (Fig. 6b,d).

Interestingly, an immunoreactive 30-kDa band, located just below PrP^C-I and often obscured by its intense

Table 3. *Influence of different metal ions on the binding of cellular prion protein (PrP^C) to IMAC*

	Release of PrP^C from IMAC (imidazole)	
Metal ion	PrP^C-I	PrP^C-II
Cu^{2+}	0.4 M[a]	10 mM
Ni^{2+}	>10 mM	>1 mM
Zn^{2+}	>10 mM	1 mM[b]
Co^{2+}	10 mM	1 mM[c]

[a] Even 0.4 M imidazole only partially eluted PrP^C-I from the column. In contrast, EDTA effectively released PrP^C-I by displacing metal ions from the column.

[b] Weakly bound to the column at 1 mM imidazole.

[c] Very weakly bound to the column at 1 mM imidazole.

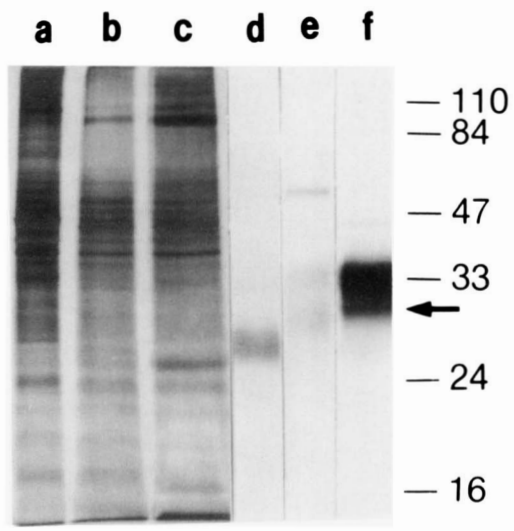

Fig. 4. Analysis of IMAC fractions. Fractions pooled as shown in Figure 3 were separated by SDS-PAGE, and stained with silver (lanes a–c) or transferred to an Immobilon membrane and immunostained with mAb 13A5 (lanes d–f). Arrow indicates 30-kDa protein band. Lanes a and d, fraction 1; lanes b and e, fraction 2; lanes c and f, fraction 3.

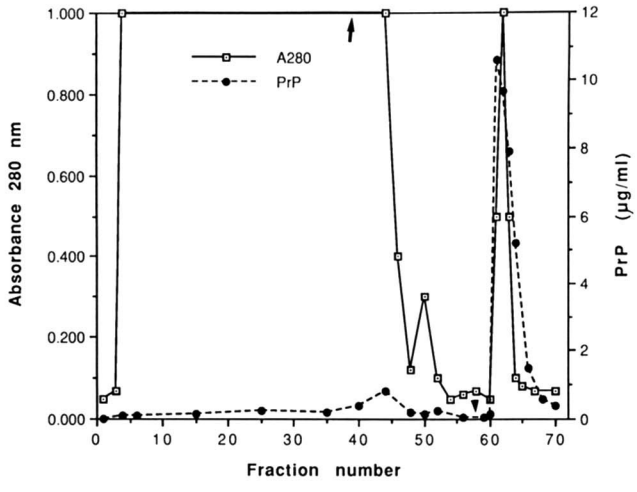

Fig. 5. Cation-exchange chromatography of EDTA eluant from IMAC. Fraction 3 of the IMAC was applied onto a cation exchanger consisting of two Econo-Pac S cartridges coupled in tandem, which was equilibrated with buffer B. Arrow indicates the wash with 0.25 M NaCl in buffer B and arrowhead the start of 0.5 M NaCl wash (in buffer B). The flow rate was 1 mL/min, and 4-mL fractions were collected. Tubes 5–50 were pooled as fraction 1 and tubes 61–65 as fraction 2.

staining, copurified with PrP^C throughout the purification (see Figs. 4f, 6d). This 30-kDa protein was barely detectable at earlier purification steps but became enriched and prominent by the IMAC. Furthermore, αP1 raised against a synthetic peptide homologous to the N-terminus of PrP 27–30, also reacted with this 30-kDa protein (data not shown).

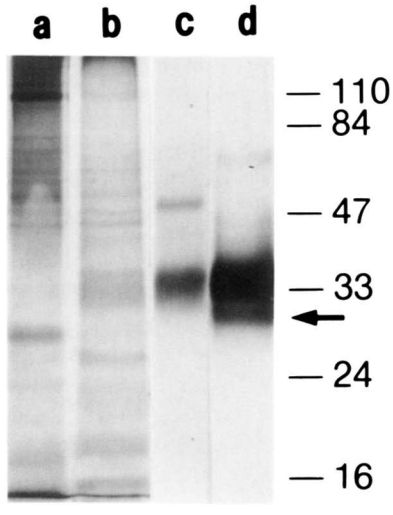

Fig. 6. Analysis of cation-exchange fractions. Fractions pooled as shown in Figure 5 were separated by SDS-PAGE, and silver-stained (lanes a, b) or transferred to an Immobilon membrane and immunostained with mAb 13A5 (lanes c, d). Arrow indicates 30-kDa protein band. Lanes a and c, fraction 1; lanes b and d, fraction 2.

Gel purification of PrP^C-I

Gel-purified PrP^C-I appeared as a broad band on the silver-stained gel and was strongly immunoreactive with mAb 13A5 (Fig. 7a,c). We estimated the recovery of PrP^C-I to be about 80%, resulting in a ~40-fold purification in this step. The series of purification procedures yielded a ~13,000-fold purification overall for PrP^C-I, with a recovery of 17% (Table 1).

Enzymatic deglycosylation

PNGase F digestion of gel-purified PrP^C-I yielded two species: one with an M_r of 30 kDa and the other with an M_r of 27 kDa (Fig. 7b,d). The 27-kDa and 30-kDa bands appeared more compact than the undigested one on the silver-stained gel. This result suggests that deglycosylation diminished the size heterogeneity of PrP^C-I and resulted in the singly and the nonglycosylated form of M_r 27 kDa. Treatment of PrP^C-II-enriched fractions with PNGase F resulted in two species having an M_r of 22 kDa and an M_r of 17 kDa (Fig. 8b). These results indicate that both PrP^C-I and PrP^C-II are diglycosylated and can be deglycosylated by PNGase F.

Discussion

The discovery of PrP^C evolved from studies on PrP^{Sc}. Enriching fractions for scrapie infectivity was an onerous task because each fraction had to be evaluated by murine bioassays requiring almost 1 year and 60 mice (Chandler,

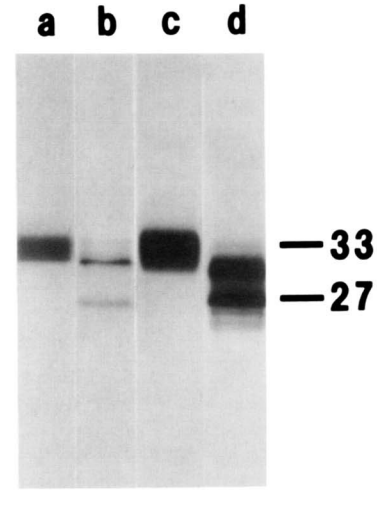

Fig. 7. Gel purification and PNGase F digestion of cellular prion protein (PrP^C)-I. Fraction 2 of the cation exchanger was collected for gel purification of PrP^C-I and prepared as described in the Materials and methods. Lanes a and c, gel-purified PrP^C-I; lanes b and d, gel-purified PrP^C-I digested with PNGase F (5 mU/mL) in 40 mM Tris, pH 8.5, 0.1% SDS, 0.25% ZW 3-12, and 1 mM EDTA. Lanes a and b, silver-stained gel; lanes c and d, immunoblots stained with 13A5 monoclonal antibody.

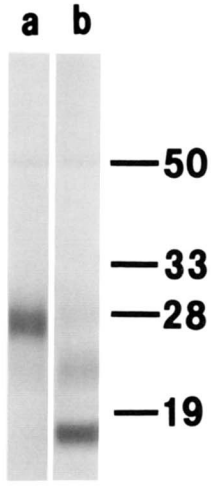

Fig. 8. Enzymatic deglycosylation of cellular prion protein (PrP^C)-II. PrP^C-II enriched fraction 1 (2.5 mg/mL) of the IMAC column was digested with PNGase F (50 mU/mL) at 37 °C for 24 h and stopped by boiling in SDS sample buffer, immunostained with 13A5 monoclonal antibody. Lane a, before PNGase F digestion; lane b, PNGase F treated.

1961; Prusiner et al., 1978a,b). The development of an incubation time assay using Syrian hamsters greatly facilitated purification of the scrapie agent (Prusiner et al., 1980) and led to the discovery of the protease-resistant core of PrP^{Sc} designated PrP 27–30 (Prusiner et al., 1982). Determination of the N-terminal sequence of PrP 27–30 (Prusiner et al., 1984), and subsequent molecular cloning of PrP cDNA allowed measurement of PrP mRNA levels that were similar in the brains of normal and scrapie-infected animals (Chesebro et al., 1985; Oesch et al., 1985). This observation prompted a search for the product of the PrP gene in the brains of normal animals. The PrP gene product was detected by immunoblotting and was designated PrP^C (Oesch et al., 1985; Meyer et al., 1986).

Using subcellular fractionation and immunoaffinity chromatography to purify PrP^C was reported to give low recoveries of the protein (<5%) and about a 3,000-fold purification (Bendheim et al., 1988; Turk et al., 1988). In contrast, our nonimmunological protocol is able to achieve ~13,000-fold purification with a recovery of 17% and makes large-scale preparation feasible. Furthermore, the recovery of PrP^C purified by this method is comparable to that of PrP^{Sc} (20–30%) (Table 4). For the purpose of structural comparison between PrP isoforms, it is important to employ a procedure allowing purification of a substantial quantity of PrP molecules that are probably representative of all PrP^C molecules.

As shown in Table 4, estimated concentrations of PrP^C vary widely among three previous reports. In the quantitation of PrP^C involving two antibody incubations, the secondary antibody (anti-mouse) used to detect the primary antibody (mouse) can cross-react with endogenous antibodies present in the brain tissues producing artificially high signals. Perhaps endogenous antibodies account for high PrP^C concentrations reported by Bendheim et al. (1988) (Table 4). We have overcome the problem by first blocking endogenous biotin activities and then incubating with the biotinylated monoclonal antibody and peroxidase-conjugated streptavidin. Our ELISA results indicate that PrP^C is a rare protein because only ~70 μg/g protein is found in the brain tissues. Thus, PrP^C represents ~0.01% of the proteins in SHa brain.

IMAC is a purification method exploiting the interaction between biomolecules and immobilized metal ions (Porath et al., 1975). In principle, only three amino acid residues—histidine, tryptophan, and cysteine—can function as binding sites for metal ions. Extensive studies on several selected model proteins have recognized histidine as the dominant electron donor (Sulkowski, 1985; Hemdan et al., 1989). Based upon the observation that SHaPrP has nine histidine residues and thus might be purified by IMAC (Sulkowski, 1989), we investigated the efficacy of this procedure. As shown in Figures 3 and 4, IMAC allows the separation of PrP^C-I from PrP^C-II and enriches PrP^C-I by ~30-fold.

The N-terminal segment, which is missing in PrP^C-II, contains six histidines, four of which are located within the octarepeat region. It is well documented that the retention of proteins on IMAC columns depends upon the number of histidine residues available for coordination. The presence of vicinal histidine residues or clustering can result in strong binding to the column due to the cooperative interaction of histidine residues with metal ions (Sulkowski, 1989). Therefore, the clustering of histidines in the octarepeats of PrP^C-I molecules may account for the tight binding of PrP^C-I to the IMAC column. Sec-

Table 4. *Comparison of prion protein (PrP) purification from previous reports*

PrP isoform[a]	Concentration (μg PrP/g protein)	Purification (-fold)	Recovery (%)	Reference
PrP^{Sc}	72	10,000	20–30	Prusiner et al. (1983, 1990)
PrP^C	180	3,400	4	Turk et al. (1988)
PrP^C	200–400	2,700	2	Bendheim et al. (1988)
PrP^C	70	13,000	17	From Table 1

[a] Sc, scrapie; C, cellular.

ondary structure analysis has suggested that this proline- and glycine-rich region may form a flexible coiled structure and participate in intermolecular interactions (Bazan et al., 1987).

Epitope mapping of PrP with different antibodies has revealed that PrP^C-I and PrP^C-II differ by an N-terminal segment, derived by proteolytic cleavage between amino acids 112 and 138 (Fig. 1). The deduced amino acid sequence of PrP reveals a hydrophobic segment between amino acids 112 and 136 (Oesch et al., 1985). The protease(s) responsible for this cleavage is unknown but seems likely to have a substrate specificity directed toward amino acids having nonpolar side chains. Among various protease inhibitors tested, tosylphenylalanylchloromethyl ketone (TPCK) included in the purification decreased the degradation of PrP^C-I into PrP^C-II. Using the IMAC to separate PrP^C-I from PrP^C-II, 80–85% of PrP^C in the detergent-solubilized microsomal preparations was found to be PrP^C-I as compared to 40–60% PrP^C-I if no protease inhibitor was used. Moreover, brain homogenate freshly prepared in the presence of protease inhibitor cocktail, still contained about 10–20% PrP^C-II (data not shown). The inhibitory effect of TPCK suggests that chymotrypsin-like activity in brain is responsible for converting PrP^C-I to PrP^C-II.

Many co- or posttranslational modifications of PrP^C and PrP^{Sc} have been identified. A signal peptide of 22 amino acids is cleaved from the N-terminal of both PrP isoforms (Basler et al., 1986; Hope et al., 1986; Turk et al., 1988). The only two cysteine residues in mature PrP form an intramolecular disulfide bond (Turk et al., 1988). By N-terminal sequencing, possible modifications at Arg 25 and 37 in PrP^{Sc} were found (Hope et al., 1988; Turk et al., 1988). These were manifest as the absence of signals in Edman degradation cycles. Evidently these modifications are labile, since other investigators have detected Arg residues during amino acid sequencing of PrP^{Sc} (Bolton et al., 1987; Safar et al., 1990), and no modifications of Arg were detected by mass spectrometry (N. Stahl et al., in prep.). N-terminal amino acid sequencing showed a similar Arg 25 modification but no data were available for Arg 37 due to limited quantities of PrP^C purified by immunoaffinity chromatography (Turk et al., 1988).

Two consensus sites for Asn-linked glycosylation in PrP were deduced from the translated sequence of a PrP cDNA clone (Oesch et al., 1985). Both chemical and enzymatic deglycosylations have demonstrated the presence of two Asn-linked oligosaccharides in both PrP forms (Endo et al., 1989; Haraguchi et al., 1989). As shown in Figure 7, digestion of PrP^C-I with PNGase F produced two proteins of M_r 30 kDa and 27 kDa. Haraguchi et al. (1989) reported that PNGase F digestion of denatured PrP^C yielded two species of 28 kDa and 26 kDa. The small apparent molecular weight differences between these two results might be due to the different molecular weight markers as well as SDS-PAGE systems used. Two proteins of M_r 22 kDa and 17 kDa were formed during digestion of PrP^C-II. Our results indicate that both PrP^C-I and PrP^C-II are diglycosylated and the structural difference between two molecules is probably not within the region containing N-linked glycosylation sites. These findings are consistent with the conclusion from antibody staining of PrP that PrP^C-I differs from PrP^C-II at the N-terminus.

The discovery of GPIs attached at the C-termini of both PrP^C and PrP^{Sc} was important in elucidating the structure of both isoforms (Stahl et al., 1987). Recent structural studies show that the GPI anchors attached to PrP^{Sc} have at least six different glycan structures (Baldwin et al., 1990; Stahl et al., 1992). The glycan structure of PrP^C is unknown except for the presence of sialic acid. Capillary electrophoresis has been used to demonstrate the presence of sialic acid attached to some of the GPI anchors of both PrP^{Sc} and PrP^C (Stahl et al., 1992). The PrP^C was purified according to the protocol described here.

Although structural analyses of PrP^{Sc} have been extensive, no candidate scrapie-specific covalent modification has been found. This situation makes the need for purified PrP^C all the more acute since it is possible that only conformational differences distinguish PrP^{Sc} from PrP^C. Fourier transform infrared spectroscopy of PrP 27–30 has confirmed the presence of a β-sheet and turns (Caughey et al., 1991) as predicted from the primary structure (Bazan et al., 1987). Another approach using attenuated total reflectance Fourier transform infrared spectroscopy to detect PrP 27–30 under various conditions reveals that the dispersion of prion rods into phospholipid vesicles or phospholipid-detergent micelles does not produce significant changes in PrP 27–30 structure (Gasset et al., 1992). The conservation of PrP 27–30 secondary structure is consistent with the retention of scrapie infectivity upon dispersion of prion amyloid rods into detergent–lipid–protein complexes or liposomes (Gabizon et al., 1987).

In summary, the development of an effective protocol for purification of PrP^C using a nonimmunologic method is an important advance. IMAC provides a useful method not only for separating PrP^C-I from PrP^C-II but also for enriching PrP^C-I. Further purification of PrP^C-I was accomplished by a combination of cation-exchange chromatography and SDS-PAGE.

Materials and methods

Chemicals

PEG-8000, 2-(*N*-morpholino)ethanesulfonic acid, TPCK, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, Missouri). ZW 3-12 was purchased from Calbiochem (San Diego, California). Chelating Sepharose Fast Flow was purchased from Pharmacia (Uppsala, Sweden). Econo system and Econo-Pac S

cartridges were purchased from Bio-Rad (Richmond, California). Nitrocellulose membrane and manifold filtration unit were bought from Schleicher and Schuell (Keene, New Hampshire). Immobilon-P transfer membrane was purchased from Millipore (Bedford, Massachusetts). PNGase F was purchased from Boehringer-Mannheim (Indianapolis, Indiana).

Animals

Syrian golden hamsters (Lak:LAV) were purchased from Charles River Laboratories (Lakeview, New Jersey). The animals were sacrificed at 6 weeks of age by CO_2 asphyxiation. Brains were removed immediately, frozen in liquid nitrogen, and stored at −80 °C until use.

PrP antibodies

Antibodies used in the study were prepared as described previously (Barry & Prusiner, 1986; Kascsak et al., 1987; Barry et al., 1988). Monoclonal antibody 13A5 was directed against an epitope including hamster PrP amino acid 139 (Rogers et al., 1991), and the other mAb 3F4 reacted with an epitope including PrP amino acids 109–112 (Bolton et al., 1991), which was provided by Richard Kascsak and Richard Carp (Kascsak et al., 1987). Polyclonal antibodies αP1 and αP3 were raised in rabbits against synthetic peptides homologous to PrP amino acids 90–102 and 220–233, respectively.

Purification of PrP^C

Normal hamster brains (100 g) were homogenized in 9 volumes (v/w) of 0.25 M sucrose, TPCK (0.1 mg/mL), 1 mM PMSF, 10 mM sodium phosphate (pH 7.0), and 0.15 M NaCl with a Polytron for four 10-s periods with a 15-s break between each period. After centrifugation at 3,000 × *g* for 30 min, the supernatant was collected. PEG-8000 was added to make a final concentration of 4%. The suspension was stirred at 4 °C for 15 min and centrifuged at 14,000 × *g* for 10 min. The precipitates were solubilized in a solution of 0.15 M NaCl, 10 mM sodium phosphate (pH 7.0), 1 mM PMSF, TPCK (0.1 mg/mL), and 8% ZW 3-12 (to achieve a weight ratio of detergent/protein = 10). The mixture was stirred at 4 °C for 1 h and then centrifuged at 100,000 × *g* for 1 h. The final supernatant was applied onto a chelating Sepharose Fast Flow column (5 × 5 cm), which was charged with Cu^{2+} (Andersson et al., 1987) and equilibrated in 0.15 M NaCl, 10 mM sodium phosphate (pH 7.0), and 0.2% ZW 3-12 (buffer A). The column was washed in succession with 5 volumes of 10 mM imidazole in buffer A, and 0.5 M NaCl, 0.2% ZW 3-12, and 0.1 M sodium acetate (pH 5.0). Finally, PrP^C was eluted with 50 mM EDTA, 0.2% ZW 3-12, 10 mM sodium phosphate (pH 7.0), and 0.2 M NaCl.

The PrP-containing EDTA eluate was collected, 36 g of urea was added per 100 mL, and the pH was adjusted to 6.2 with 2 N HCl. The mixture was loaded onto a cation exchanger consisting of two Econo-Pac S cartridges (Bio-Rad) coupled in tandem, which was equilibrated in 4 M urea, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.2, and 0.12% ZW 3-12, and 0.2 M NaCl (buffer B). After washing with 0.25 M NaCl in buffer B, PrP^C was eluted from the column by buffer B containing 0.5 M NaCl. PrP^C-enriched fractions were pooled and stored at −20 °C until use.

The 0.5 M NaCl eluate from the cation exchanger was used for gel purification of PrP^C. The pooled fractions were precipitated with 10 volumes of ethanol and separated by SDS-PAGE. With prestained molecular weight standards (Bio-Rad) running along the sides of the gel as a guide, a PrP^C-containing gel strip was excised and incubated overnight in 10 mM ammonium bicarbonate, 1 mM EDTA, and 0.1% SDS.

Quantitation of PrP^C

To monitor PrP^C purification, a modified ELISA was carried out as previously described (Prusiner et al., 1990). Briefly, protein samples (5 μL) were applied onto a nitrocellulose membrane (NC) via a manifold filtration unit (Schleicher and Schuell). After treating with 5% nonfat dry milk to block protein binding sites and avidin to saturate endogenous biotin activities, the NC was incubated with biotin (2 μg/mL) in phosphate-buffered saline–Tween, biotinylated mAb 13A5, and peroxidase-conjugated streptavidin sequentially. Each dot was then cut out with a punch and placed in the well of a 96-well plate. Color development with *o*-phenylenediamine was performed as previously described (Ambler & Peters, 1984). The final solution was transferred to an empty 96-well plate and checked for absorbance at 492 nm with a Titertek Multiskan II spectrophotometer (Flow Laboratories, Rockville, Maryland).

Gel-purified SHaPrP 27–30 was used to construct a standard curve for correlating optical intensities of protein samples to their PrP contents (Fig. 9). Protein fractions from chromatographic columns were quantitated by the above method and the PrP^C concentration was evaluated in terms of micrograms per milliliter.

SDS-PAGE

Protein samples were analyzed by discontinuous SDS-PAGE according to Laemmli (1970). Each was denatured in sample buffer by boiling at 100 °C for 3 min and electrophoresed onto a 12% polyacrylamide gel. Resolved proteins on the gel were visualized by silver staining (Merril et al., 1981).

Protein determination

Protein concentration was estimated by the method of Bradford (1976) with bovine serum albumin as the standard.

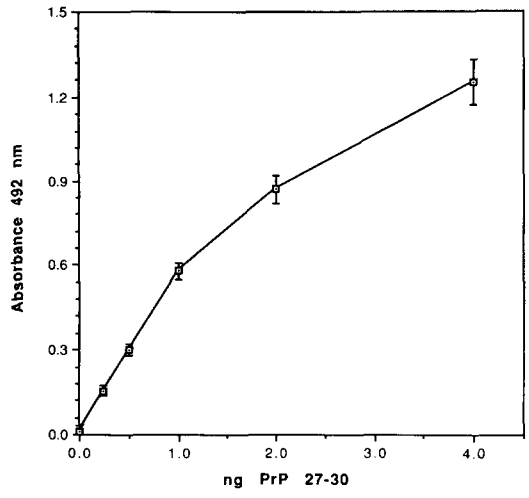

Fig. 9. The ELISA standard curve generated from known amounts of gel-purified prion protein (PrP) 27–30 for the quantitation of PrP^C in protein samples. Each data point is the mean of three determinations. Vertical error bars represent standard deviations of the mean.

Immunoblotting assay

Protein samples were applied to SDS-polyacrylamide gels using the method described above. The separated proteins were then electrophoretically transferred onto Immobilon-P membrane (Millipore). The procedure for transfer was essentially as described by Towbin et al. (1979). After transfer, the membrane was blocked at 37 °C for 1 h with 5% nonfat dry milk in 0.15 M NaCl, 10 mM Tris HCl, pH 8.0, and 0.05% Tween-20 (TBST). Subsequently, it was incubated with anti-PrP antibody in TBST overnight and then alkaline phosphatase-conjugated secondary antibody (Promega Biotech, Madison, Wisconsin) for 90 min at room temperature. Immunoreactive staining was developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitro blue tetrazolium (NTB) as described by Promega Biotech. All immunoblots shown in the Results were probed with MA 13A5 except those specified in Figure 1.

Absorption of antisera

The antigen specificity of the rabbit antiserum was analyzed by the immunoblotting assay following absorption of antibody activity with synthetic peptides. The antiserum (1:100 dilution in TBST) was mixed with synthetic peptides (2.5 mg/mL) and incubated at room temperature for 1 h. After another 1:10 dilution, the preabsorbed antibody was used in the assay under the same conditions as unabsorbed antibody.

Enzymatic deglycosylation

Fifty microliters of protein samples was incubated with PNGase F at 37 °C for 24 h. After adding an equal volume of SDS sample buffer, the mixture was boiled for 3 min, separated by SDS-PAGE, and analyzed by the immunoblotting assay as described above.

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