In Vitro Prion Protein Conversion in Detergent-Solubilized Membranes†

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Received October 21, 2003; Revised Manuscript Received January 7, 2004

ABSTRACT: A fundamental event in the pathogenesis of prion disease is the conversion of PrP^C, a normal glycophosphatidyl-anchored glycoprotein, into an infectious isoform designated PrP^{Sc}. In a modified version of the protein misfolding cyclic amplification (PMCA) technique [Saborio et al. (2001) *Nature 411*, 810−813], protease-resistant PrP^{Sc}-like molecules (PrPres) can be amplified in vitro in a species- and strain-specific manner from crude brain homogenates, providing a biochemical model of the prion conversion reaction [Lucassen et al. (2003) *Biochemistry 42*, 4127−4135]. In this study, we investigated the ability of enriched membrane subsets and detergent-solubilized membrane preparations to support PrPres amplification. Membrane fractionation experiments showed that purified synaptic plasma membrane preparations enriched in PrP^C but largely depleted of late endosomal and lysosomal markers were sufficient to support PrPres amplification. Detergent solubilization experiments showed that a small group of select detergents could be used to produce soluble preparations that contain PrP^C and fully support PrPres amplification. The stability of PrPres amplification ability in detergent-solubilized supernatants was dependent on detergent concentration. These results lead to the surprising conclusion that membrane attachment is not required for PrP^C to convert efficiently into PrPres in vitro and also indicate that biochemical purification of PrPres amplification factors from brain homogenates is a feasible approach.

Prion diseases such as Creutzfeldt−Jakob disease (CJD),[1] bovine spongiform encephalopathy (BSE), chronic wasting disease (CWD), and scrapie are a group of fatal neurological disorders whose etiology remains undetermined (*3*). A central event in the pathogenesis of prion diseases is the transformation of a normal neuronal glycoprotein, PrP^C, into a pathogenic conformation, PrP^{Sc}. PrP^{Sc} accumulates exponentially in the central nervous system during disease progression, and much evidence supports the unorthodox hypothesis that PrP^{Sc} is an infectious protein, which by itself is the infectious agent of prion disease (*4*).

Although PrP^C and PrP^{Sc} have identical amino acid sequences and posttranslational modifications, the two isoforms differ in terms of their secondary structures and biochemical properties. Circular dichroism measurements indicate that PrP^C is composed of 42% α-helix and 3% β-sheet. In contrast, PrP^{Sc} contains 30% α-helix and 43% β-sheet and is considerably more resistant to detergent solubilization and protease digestion than PrP^C (*5*−*7*).

PrP^C is predominantly localized at the synaptic terminal (*8*) and is likely attached to the extracellular leaflet of the plasma membrane through a glycophosphatidylinositol (GPI) anchor (*9*). However, less abundant topological variants of PrP^C, including transmembrane and cytoplasmic forms, also exist and may play significant roles in the pathogenic process (*10, 11*). Direct contact between uninfected cells and prion-infected surfaces is required for prion propagation, but whether PrP^{Sc} molecules need to incorporate into the same lipid membrane as PrP^C molecules to initiate conversion remains controversial (*12*−*14*). Some investigators have proposed that the formation of infectious prions requires specific lipid membranes (*15*−*18*), but firm evidence for this "membrane hypothesis" is lacking.

Several indirect lines of evidence suggest that factors other than PrP^C and PrP^{Sc} are required to propagate prions. First, by limiting dilution, it is possible to isolate clonal sublines of N2a neuroblastoma cells expressing PrP^C that are resistant to prion infection, indicating that these sublines lack a cellular factor required for prion propagation (*19*). Second, addition of Chinese hamster ovary (CHO) cell lysate to a mixture of immunopurified, epitope-tagged PrP^C and PrP^{Sc} in vitro improved the recovery of protease-resistant PrP (*20*). Third, transmission data in transgenic mice coexpressing endogenous and heterologous PrP molecules suggest the existence of a cellular factor other than PrP that facilitates prion propagation (*21*). Fourth, genetic studies in mice have identified several quantitative trait loci (QTL) distinct from the PrP gene that affect scrapie incubation time (*22*).

† This work was supported by the Burroughs Wellcome Fund Career Development Award, the Hitchcock Foundation, and an NIH Clinical Investigator Development Award (K08 NS02048-02).

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[1] Abbreviations: CJD, Creutzfeldt−Jakob disease; BSE, bovine spongiform encephalopathy; CWD, chronic wasting disease; GPI, glycophosphatidylinositol; PrP, prion protein; PrP^C, cellular isoform of the prion protein; PrP^{Sc}, scrapie isoform of the prion protein; PrPres, protease-resistant PrP^{Sc}-like molecules; SPM, synaptic plasma membrane; CHO, Chinese hamster ovary; QTL, quantitative trait loci; PMCA, protein misfolding cyclic amplification; PI-PLC, phosphatidylinositol-specific phospholipase C; MYL, synaptosomal myelin preparation; SPM, synaptic plasma membrane preparation; PEL, synaptosomal mitochondrial pellet; PBS, calcium- and magnesium-free phosphate-buffered saline; M_r, relative molecular mass; CHAPS, (cholamidopropyl)dimethylammonio-1-propanesulfonate; CHAPSO, (cholamidopropyl)dimethylammonio-2-hydroxy-1-propanesulfonate; NOG, *n*-octyl β-D-glucopyranoside; CMC, critical micellar concentration.

10.1021/bi035889l CCC: $27.50
Published on Web 02/10/2004

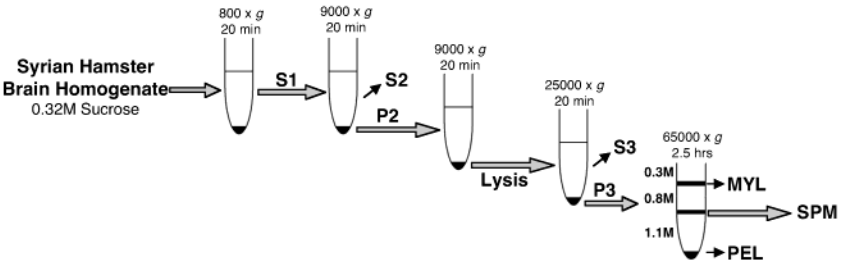

FIGURE 1: Schematic diagram of membrane fractionation. Synaptic plasma membranes were prepared from Syrian hamster brains by a series of differential centrifugation steps, hypotonic lysis, and discontinuous sucrose gradient centrifugation as described in Experimental Procedures. MYL = myelin; SPM = synaptic plasma membranes; PEL = mitochondrial pellet.

We recently reported that a modified version of the protein misfolding cyclic amplification (PMCA) technique of Saborio and Soto (*1*) could be used to amplify protease-resistant PrP^{Sc}-like molecules (PrPres) from crude brain homogenates without sonication or SDS (*2*). Under these nondenaturing conditions, PrPres amplification was species- and strain-specific and correlated inversely with scrapie incubation times determined in vivo. PrPres amplification from mixtures of crude brain homogenates was more efficient than PrPres formation from mixtures of purified PrP^{C} and PrP^{Sc} molecules (*23*−*26*), suggesting that additional cellular factors may facilitate the process of PrPres formation in vitro. Biochemical purification is the most direct strategy to identify such cofactors, but this strategy first requires generating a soluble preparation capable of supporting PrPres amplification. In this report, we subjected normal brain homogenates to membrane fractionation and detergent solubilization techniques in order to (1) determine whether a specific membrane subset might contain all of the components required for PrPres amplification in vitro, (2) determine whether physical attachment of PrP^{C} to membranes is required for PrPres amplification, and (3) facilitate the eventual biochemical purification and identification of PrPres amplification cofactors.

EXPERIMENTAL PROCEDURES

Membrane Fractionation. Synaptic plasma membranes were prepared by a modified version of an established protocol (*8*). All procedures were performed at 4 °C. Six brains harvested from normal, specific-pathogen-free 3-week-old female golden Syrian hamsters (Charles River Laboratories, Wilmington, MA) were homogenized in 40 mL of buffer A [0.3 M sucrose, 5 mM Tris, pH 7.5, plus EDTA-free Complete protease inhibitor cocktail (Roche, Indianapolis, IN)] with 10 strokes of a motor-driven Teflon/glass homogenizer. The crude homogenate was centrifuged at 800*g* for 20 min. The supernatant (S1) was centrifuged at 9000*g* for 20 min. The resulting pellet (P2) was washed with 30 mL of buffer A, centrifuged again at 9000*g* for 20 min, and resuspended in 30 mL of buffer B (5 mM Tris, pH 8.1, plus EDTA-free Complete protease inhibitor cocktail). The sample was then incubated at 4 °C for 30 min with constant end-over-end rotation for hypotonic lysis and centrifuged at 25000*g* for 20 min. The resulting pellet (P3) was homogenized in 21 mL of buffer B using a glass/glass Potter homogenizer with 10 strokes each of the loose and tight pestles. The mixture was adjusted to 1.1 M sucrose, divided evenly to form the bottom of four sucrose gradients, and successively overlaid with 15 mL of buffer C (0.8 M sucrose, 5 mM Tris, pH 7.5, plus EDTA-free Complete protease inhibitor cocktail) and 5 mL of buffer A. After ultracentrifugation at 65000*g* for 2.5 h in an SW28 rotor (Beckman, Fullerton, CA), the following fractions were collected: MYL (myelin) at the 0.3/0.8 M sucrose interface, SPM (synaptic plasma membranes) at the 0.8/1.1 M sucrose interface, and the mitochondrial pellets (PEL). The pellets were resuspended in 20 mL of buffer D (0.25 M sucrose, 20 mM Tris, pH 7.5, plus EDTA-free Complete protease inhibitor cocktail). MYL and SPM were diluted into phosphate-buffered saline without calcium or magnesium (PBS) plus EDTA-free Complete protease inhibitor cocktail to reduce sucrose concentrations and centrifuged for 45 min at 170000*g* in a type 60 Ti rotor (Beckman, Fullerton, CA). The SPM pellets were resuspended in 10 mL of buffer D and MYL pellets in 3.5 mL of buffer D. Intermediate supernatant and pellet fractions were directly collected as illustrated in Figure 1. The total protein concentration in each fraction was determined with the Micro BCA protein assay reagent kit (Pierce, Rockland, IL) using bovine serum albumin as a standard reference.

Membrane Marker Analysis. Samples of membrane fractions S1 and SPM containing 50 μg of total protein were loaded onto a 12% polyacrylamide gel for electrophoresis and immunoblotting. Monoclonal antibodies from the Organelle sampler kit (Transduction Laboratories, Lexington, KY) were used according to the manufacturer's instructions. Densitometry was performed on Western blot signals as described below.

Preparation of Crude Brain Homogenates for Solubilization Studies. Whole brains, including cerebellum and brainstem, were harvested from normal, specific-pathogen-free 3-week-old female golden Syrian hamsters (Charles River Laboratories, Wilmington, MA). Brains were weighed and homogenized in 10 volumes of ice-cold PBS plus EDTA-free Complete protease inhibitor cocktail, using a glass/glass Potter homogenizer with 10 strokes each of the loose and tight pestles. Crude homogenates were centrifuged for 30 s at 200*g*, and aliquots of the postnuclear supernatant were frozen at −70 °C for solubilization studies.

Detergent-Solubilized Supernatants. The majority of the detergents tested were contained in the Anatrace master detergent kit (Maumee, OH). Zwittergent 3-12, Zwittergent 3-14, Zwittergent 3-16, cetyltrimethylammonium bromide, Big Chap, deoxy Big Chap, Zwittergent 3-8, NP-40, lauryldimethylamine oxide (LDAO), and Zwittergent 3-16 were obtained from Calbiochem (La Jolla, CA). Tween 20, FL-70, and Triton X-100 were obtained from Fisher (Pittsburgh, PA). Tween 80 was obtained from J. T. Baker (Phillipsburg,

NJ). Polyoxyethylene 10-lauryl ether, polyoxyethylene 10-oleyl ether, polyoxyethylene 23-lauryl ether, taurodeoxycholic acid, sodium deoxycholate, taurocholic acid, dodecyltrimethylammonium bromide, digitonin, and *N*-laurylsarcosine were obtained from Sigma (St. Louis, MO). Thesit and Tergitol TMN3 were obtained from Fluka (Milwaukee, WI). Lubrol WX is from Supelco (Belefonte, PA). 1-Dodecylpyridium cholate was obtained from Matheson Coleman & Bell (East Rutherford, NJ). All detergents were diluted in H_2O to generate a stock concentration of 10% (w/v) and kept at 4 °C prior to use. These stock solutions were added to 2% (w/v) normal brain homogenate in PBS for a 1% (w/v) final detergent concentration and incubated at 4 °C for 1 h unless otherwise specified. Following incubation, the samples were centrifuged either for 1 h at 100000*g* at 4 °C (Sorvall MS120E microultracentrifuge) for solubilization studies or for 1 h at 20800*g* at 4 °C (Eppendorf 5417C microcentrifuge) for stability studies. Solubilized supernatants were either added to an equal volume of SDS loading buffer or used in a PrPres amplification assay, as indicated.

In Vitro PrPres Amplification Assay. In vitro PrPres amplification was performed as previously described (*2*) with the following modifications for specific experiments. To assay membrane fractions, 50 μL of each fraction was mixed with 50 μL of 0.1−0.2% (w/v) Sc237 scrapie-infected brain homogenate in PBS plus 0.5% Triton X-100. To screen for detergent compatibility, 50 μL of 10% (w/v) normal brain homogenate was mixed with 50 μL of 0.2% (w/v) Sc237 scrapie-infected brain homogenate in PBS plus 1% test detergent. To assay detergent-solubilized supernatants, 100 μL of each supernatant was added to 25 μL of 0.25−0.5% (w/v) scrapie-infected brain homogenate in PBS with the same detergent used before. In all cases, the mixtures were incubated at 37 °C for 16 h with continuous shaking. Following incubation, 50 μg/mL proteinase K (Roche, Indianapolis, IL) was added to each sample and incubated for 1 h at 37 °C. After incubation, the sample was boiled in SDS sample buffer for 10 min before electrophoresis.

Purification of PrP 27−30. Sc237 infected brain homogenate was diluted to a 1% (w/v) concentration with PBS and 1% Triton-X-100 and digested with 50 μg/mL proteinase K (Roche) for 30 min at 20 °C. Digestion was terminated with 10 mM phenylmethanesulfonyl fluoride (Roche, Indianapolis, IL). The preparation was centrifuged for 30 min at 25000*g* at 4 °C, and the supernatant was removed. The pellet was resuspended in 1.35 mL of PBS and 1% Triton X-100 per 300 μL of 1% (w/v) starting brain homogenate and then sonicated using a Bandelin Sonopuls ultrasonicator (Amtrex Technologies, Saint-Laurent, Quebec, Canada) set for 10 × 1 s pulses (5 s/pulse).

Electrophoresis and Immunoblotting. SDS−PAGE was performed on 1.5 mm, 12% acrylamide gels with an acrylamide:bisacrylamide ratio of 29:1. Following electrophoresis, the proteins were transferred to ethanol-charged, buffer-equilibrated PVDF membranes (Millipore, Bedford, MA) using a Hoeffer TE 50X transfer unit set at 1.5 A for 2 h. Membranes were then treated with 0.2 N NaOH for 10 min, rinsed 3 × 5 min with TBST (Tris-buffered saline: 10 mM Tris, pH 7.2, 150 mM NaCl, 0.1% Tween 20), and blocked with 5% Carnation nonfat milk powder in TBST for 1 h. The membranes were then incubated with a 1:5000 dilution of 3F4 mAb (Signet Laboratories, Dedham, MA) in TBST, overnight at 4 °C, and washed 3 × 10 min with TBST, followed by a 1 h incubation at 4 °C of horseradish peroxidase-labeled anti-mouse IgG secondary antibody (Amersham, Piscataway, NJ) and 3 × 10 min washes with TBST. The blots were developed with ECL reagent (Pierce, Rockland, IL), sealed in plastic covers, and exposed to Super RX film (Fujifilm, Tokyo, Japan). The film was developed automatically in a Kodak M35A X-Omat film processor. Relative molecular mass values (M_r) in kilodaltons, based on the migration of prestained protein standards (Bio-Rad, Hercules, CA), are marked on the right side of each figure. Densitometric measurement of membrane marker film signals was performed through the analysis of multiple film exposures to ensure that comparisons were made within the linear range of the film. Signals within the linear range were quantified using the histogram functions in Adobe Photoshop and calibrated against the background signal. Serial dilutions of normal hamster brain were used to calibrate densitometric measurements.

RESULTS

PrPres Amplification in Synaptic Plasma Membrane Preparations. We used a standard protocol involving differential centrifugation, hypotonic lysis, and discontinuous density gradient fractionation in sucrose solutions to prepare synaptic plasma membranes (SPM) from normal Syrian hamster brains (Figure 1). As previously reported by Herms et al. (*8*), PrP^C detected by Western blot is enriched in SPM preparations (Figure 2A). Quantitation of the Western blot signals by densitometric scanning revealed that PrP^C was enriched ∼4-fold in SPM fractions, compared to the postnuclear supernatant (S1) (Table 1). To assess the purity of our SPM preparations, we determined the relative abundance of several specific membrane markers in SPM and S1 samples by quantitation of immunoblots (Table 2). Although quantification by densitometric scanning is potentially inaccurate, the results of these comparisons show qualitatively that membrane markers for caveolae, plasma membranes, and endoplasmic reticulum (ER) were present in high concentrations in SPM preparations. In contrast, SPM preparations had relatively low levels of contamination by membrane markers for nuclei, Golgi apparatus, mitochondria, endosomes, and lysosomes.

Next, we tested whether SPM preparations could support PrPres amplification. Our results indicate that SPM, as well as other intermediate fractions containing PrP^C, successfully amplified PrPres when mixed with 0.1% (w/v) crude scrapie brain homogenate (Figure 2B). To verify that the diluted scrapie brain homogenate did not contribute cellular components necessary for PrPres amplification but absent from SPM preparations, we also tested the ability of SPM to amplify purified PrP 27−30. The results show that PrP 27−30 can be amplified by SPM, albeit slightly less efficiently than by the crude S1 fraction (Figure 2C), confirming that SPM preparations alone contain all of the components required for PrPres amplification. Because the SPM preparations did contain some components of acidic organelles, indicated by the presence of 2% EEA1 and 6% LAMP-1 proteins (Table 2), we examined further the possibility that components of acidic organelles might stimulate PrPres amplification. Western blot analysis indicated that LAMP-1 protein was 4-fold enriched in the PEL fraction relative to

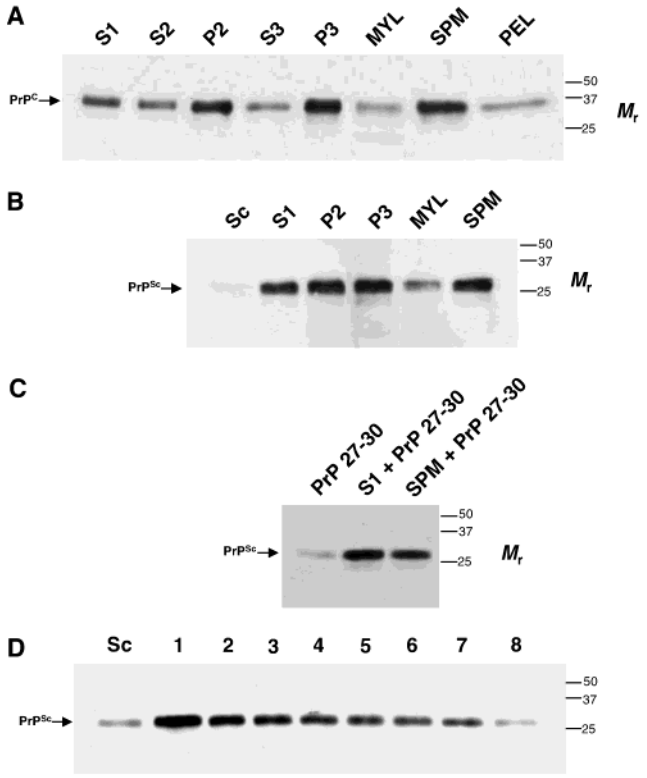

FIGURE 2: Brain membrane fractionation. Membrane fractions were prepared as described in Experimental Procedures and illustrated in Figure 1. (A) Samples of each fraction containing 50 μg of total protein were analyzed for PrP^C content by immunoblotting. (B) PrPres amplification by selected fractions. Samples were normalized for PrP^C content and tested for PrPres amplification as described in Experimental Procedures. (C) PrPres amplification driven by PrP 27−30. S1 and SPM membrane fractions were normalized for PrP^C content and tested for PrP 27−30 amplification as described in Experimental Procedures. (D) PrPres amplification with SPM and varying amounts of PEL. Each amplification reaction was done in the presence of 1% CHAPSO and 1 mM EDTA, pH 8.0, in PBS, and diluted scrapie brain homogenate was added to all samples for PrPres amplification. Lane 1 has 50 μL of 10% normal brain homogenate. Lanes 2−7 each have a constant 50 μL volume of SPM with increasing volumes of PEL: lane 2 = 0 μL of PEL, lane 3 = 10 μL of PEL, lane 4 = 20 μL of PEL, lane 5 = 30 μL of PEL, lane 6 = 40 μL of PEL, and lane 7 = 50 μL of PEL. Lane 8 has 50 μL of PEL and no SPM. Each sample was brought to a total volume of 100 μL with PBS. The total protein concentration of the SPM preparation used in this experiment was 1.2 mg/mL, and the total protein concentration of the PEL preparation was 0.15 mg/mL. Sc = diluted scrapie brain homogenate alone.

SPM (data not shown). Addition of various amounts of PEL to SPM did not stimulate, and may have even inhibited, PrPres amplification (Figure 2D), indicating that acidic organelles are not likely to contain factors required for PrPres amplification.

PrPres Amplification in Detergent-Solubilized Membranes. It is currently unknown whether membrane attachment is required for PrP^C to undergo efficient transformation into PrPres. To investigate this question, we initially attempted to release PrP^C from its GPI membrane anchor by treatment of SPM preparations with phosphatidylinositol-specific phospholipase C (PI-PLC). However, PrP^C molecules exposed to PI-PLC were rapidly degraded in SPM preparations (data not shown), preventing formation of PrPres (Figure 3A) and precluding the use of enzymatic release as a strategy to solubilize PrP^C for our studies.

Table 1: Enrichment of PrP^C by Membrane Fractionation[a]

fraction	total protein (mg)	total PrP^C (arbitrary units)	enrichment (*x*-fold)	yield (%)
S1	284	414	1	100
S2	127	134	0.7	32
P2	100	426	3	103
S3	27	27	0.7	6
P3	56	308	3.9	74
MYL	4	4	0.7	1
SPM	12	64	3.8	15
PEL	3	9	0.9	1

[a] Membranes were fractionated as described in Experimental Procedures and shown in Figure 1. Total protein measurements were determined by the Micro BCA assay (Pierce, Rockland, IL). Relative PrP^C content was determined by Western blotting and densitometric scanning of samples of each fraction containing 50 μg of total protein, as described in Experimental Procedures.

Table 2: Membrane Composition of SPM Preparations[a]

subcellular compartments	marker	relative marker concn [(SPM/S1) × 100] (%)
nucleus	nucleoporin p62	<1
endoplasmic reticulum	BiP/GRP78	75
Golgi	GM130	5
mitochondria	Mcl-1	<1
plasma membrane	annexin II	79
caveolae	caveolin 1	172
endosomes	EEA1	2
lysosomes	LAMP-1	6

[a] Samples of S1 and SPM fractions containing 50 μg of total protein were probed on Western blots with various monoclonal antibodies reactive against the indicated membrane markers. Densitometric scanning was performed as described in Experimental Procedures to determine the relative abundance of each marker in the SPM and S1 fractions.

In an alternative approach, we screened 78 ionic, nonionic, and zwitterionic detergents for their ability to solubilize PrP^C and their compatibility with the PrPres amplification reaction (Table 3). On the basis of the results of these screens, we classified the detergents into four groups (Table 3 and Figure 3B,C). Eight ionic detergents solubilized PrP^C efficiently but were not compatible with the PrPres amplification reaction (group A). Forty-one of the detergents tested were compatible with the PrPres amplification reaction but failed to solubilize PrP^C efficiently; the majority of these detergents were uncharged (group B). Twenty-four detergents failed to solubilize PrP^C efficiently and also interfered with the PrPres amplification reaction (group C). Finally, five detergents successfully solubilized PrP^C and did not interfere with PrPres amplification (group D). These detergents were *n*-decyl β-D-thiomaltoside, *n*-undecyl β-D-thiomaltoside, (cholamidopropyl)-dimethylammonio-1-propanesulfonate (CHAPS), (cholamidopropyl)dimethylammonio-2-hydroxy-1-propanesulfonate (CHAPSO), and *n*-octyl β-D-glucopyranoside (NOG). Because CHAPS, CHAPSO, and NOG have high critical micellar concentration (CMC) values ($\geq$8 mM) and have been used extensively for the purification and reconstitution of other membrane proteins, we selected these detergents for further characterization.

We determined the optimal concentration of each detergent required to solubilize PrP^C from a 2% (w/v) normal brain homogenate in PBS at 4 °C. Under these conditions, concentrations ranging from 1% to 2% (w/v) of all three detergents were equally efficient at solubilizing PrP^C

Table 3: Detergent Screen for PrP^C Solubilization and PrPres Amplification[a]

group A	group B		group C	group D
n-decyl-*N*,*N*-dimethylglycine	Triton X-100	1-*S*-heptyl- β-D-thioglucoside	Tween 20	*n*-decyl β-D-thiomaltoside
n-dodecyl-*N*,*N*-dimethylglycine	CYMAL-4	1-*S*-octyl-β-D-thioglucoside	Tween 80	*n*-undecyl β-D-thiomaltoside
deoxycholate	CYGLU-3	1-*S*-nonyl-β-D-thioglucoside	polyoxyethylene 10-lauryl ether	*n*-octyl β-D-glucoside
taurocholic acid	C-HEGA-8	1-*S*-decyl-β-D-thioglucoside	polyoxyethylene 10-oleyl ether	CHAPSO
1-dodecylpyridium cholate	C-HEGA-9	*n*-hexyl β-D-glucoside	polyoxyethylene 23-lauryl ether	CHAPS
dodecyltrimethyl-ammonium bromide	C-HEGA-10	*n*-heptyl β-D-glucoside	Thesit	
cetyltrimethyl-ammonium bromide	C-HEGA-11	*n*-nonyl β-D-glucoside	Tergitol TMN3	
Sarkosyl	HEGA-8	*n*-decyl β-D-glucoside	FL-70	
	HEGA-9	*n*-dodecyl β-D-glucoside	Lubrol WX	
	HEGA-10	1-*S*-octyl-β-D-thiomaltoside	Myrj 59	
	HEGA-11	1-*S*-nonyl-β-D-thiomaltoside	digitonin	
	MEGA-8	*n*-dodecyl β-D-thiomaltoside	sodium cholate	
	MEGA-9	*n*-octyl β-D-galactoside	taurodeoxycholic acid	
	MEGA-10	Big Chap	dodecyldimethylamine oxide	
	n-hexyl β-D-maltoside	Deoxy Big Chap	sodium decanoylsarcosine	
	n-octyl β-D-maltoside	Zwittergent 3-8	FOS-choline-8	
	n-nonyl β-D-maltoside	NP-40	FOS-choline-9	
	n-decyl β-D-maltoside	LDAO	FOS-choline-10	
	n-undecyl β-D-maltoside		FOS-choline-12	
	n-dodecyl β-D-maltoside		FOS-choline-14	
	n-tridecyl β-D-maltoside		FOS-choline-16	
	n-tetradecyl β-D-maltoside		Zwittergent 3-12	
	n-hexadecyl β-D-maltoside		Zwittergent 3-14	
			Zwittergent 3-16	

[a] Detergent solubilization: Samples containing 2% (w/v) normal brain homogenate were mixed with each detergent as described in Experimental Procedures. Following incubation, samples were centrifuged for 1 h at 100000*g*, 4 °C. Supernatant fractions were assayed for PrP^C solubilization by Western blot. PrPres amplification: Reactions were performed as described in Experimental Procedures, testing each detergent at 1% (w/v) concentration. Detergents were classified according to their ability to solubilize PrP^C and their compatibility with the PrPres amplification reaction on the basis of the following criteria: group A, >40% PrP^C solubilization, <3-fold PrPres amplification; group B, <40% PrP^C solubilization, >3-fold PrPres amplification; group C, <40% PrP^C solubilization, <3-fold PrPres amplification; group D, >40% PrP^C solubilization, >3-fold PrPres amplification.

(Figure 4A), assessed by centrifugation for 1 h at 100000*g*. In contrast, detergent concentrations <0.5% failed to solubilize PrP^C (data not shown). Next, we tested whether detergent-solubilized brain homogenate supernatants could support PrPres amplification. The results show that CHAPS, CHAPSO, and NOG can all be used successfully to produce solubilized supernatants competent for PrPres amplification (Figure 4B).

We also measured the kinetics of PrP^C solubilization with each of the three test detergents. At 1% detergent concentration, all three detergents solubilized 40−50% of the PrP^C present within 15 min at 4 °C (Figure 4C). Solubilization of PrP^C with CHAPS and CHAPSO did not increase with longer detergent exposure times up to 1 h. We recovered noticeably smaller pellets from samples exposed to NOG for longer incubation periods, and the sample solubilized in NOG for 1 h had no visible pellet at all. However, specific recovery of PrP^C in the supernatant was again not increased by solubilization times longer than 15 min.

Stability of PrPres Amplification in Detergent-Solubilized Membranes. For the purposes of biochemical purification, it would be useful to generate detergent-solubilized preparations in which PrPres amplification activity remains relatively stable over time. Therefore, we characterized the stability of PrP^C and of PrPres amplification activity in crude brain homogenate preparations solubilized by various concentrations of CHAPS, CHAPSO, and NOG. We performed this set of experiments on detergent-solubilized preparations that were centrifuged at either 100000*g* or 20800*g* for 1 h or not subjected to centrifugation. We obtained similar results with solubilized preparations centrifuged at 100000*g* in an ultracentrifuge or at 20800*g* in a standard centrifuge, which would facilitate large-scale purification of PrP^C and potential

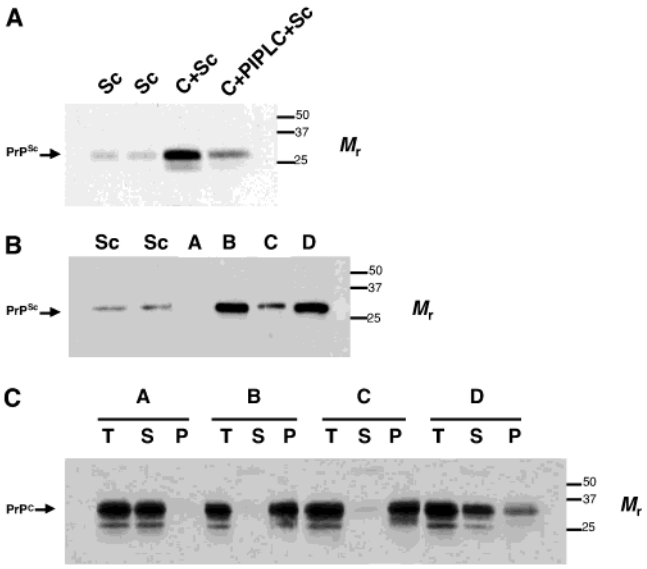

FIGURE 3: Enzyme and detergent treatments. (A) Enzymatic digestion with PI-PLC. The sample designated C + PIPLC + Sc contained normal brain homogenate which was pretreated with 0.1 unit of PI-PLC (Molecular Probes, Eugene, OR) for 1 h at 37 °C before addition of diluted scrapie brain homogenate. Sc = diluted scrapie brain homogenate alone. (B) Amplification with representative detergents. One detergent was picked for each group indicated in Table 3, demonstrating representative PrPres amplification ability. Each detergent was used at 1% concentration in PBS. A = DTAB, B = CYGLU-3, C = Tween 20, D = CHAPSO, and Sc = diluted scrapie brain homogenate alone. (C) Solubilization of PrP^C with representative detergents. The same detergents used in panel B were used to solubilize PrP^C at 1% (w/v) concentration. Except for lanes T, samples were centrifuged for 1 h at 100000*g*, and the supernatant and pellet fractions were assayed for the presence of PrP^C by immunoblotting. T = total 2% (w/v) brain homogenate not subjected to centrifugation, S = supernatant, and P = pellet.

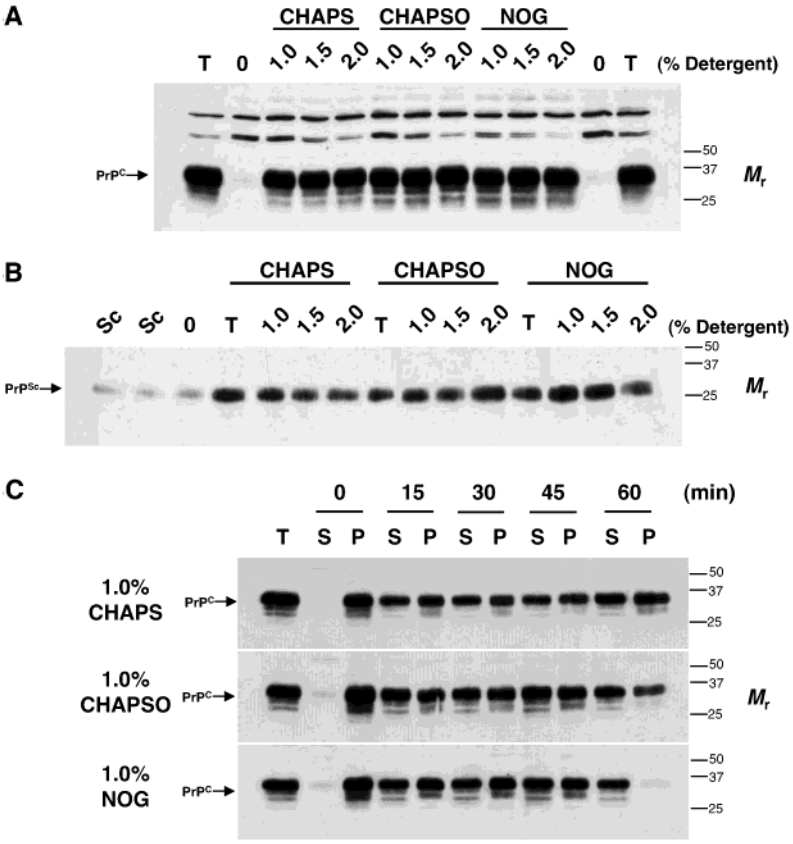

FIGURE 4: Optimization of detergent solubilization. CHAPS, CHAPSO, or NOG was used to solubilize PrP^C at the indicated concentrations. Except for lanes labeled T, all samples were centrifuged for 1 h at 100000*g*, and the supernatant was assayed for the presence of PrP^C by immunoblotting. T = total 2% (w/v) brain homogenate not subjected to centrifugation; 0 = no detergent added during solubilization. (A) Western blot of PrP^C. Note that, due to the saturation level of the film, the band intensities of detergent-solubilized samples appear to exceed 50% of the band intensity of the total PrP^C sample. A more accurate visual representation of solubilization efficiency is provided in panel C. (B) PrPres amplification with detergent-solubilized supernatants. Sc = diluted scrapie brain homogenate alone. (C) Kinetics of PrP^C solubilization. Following detergent solubilization for the indicated time periods, supernatant (S) and pellet (P) samples were collected and prepared for Western blot.

PrPres amplification factors (Supporting Information, Figure S1).

Unexpectedly, we found that varying detergent concentrations above CMC values affected the stability of PrP^C and of PrPres amplification activity in solubilized supernatant preparations. Specifically, our results indicate that the stability of PrP^C at 4 °C is enhanced when the detergent concentration is increased from 1% to 1.5% (Figure 5A, top two panels). In the presence of 1.5% detergent, PrPres amplification activity in samples of solubilized supernatant remains stable for nearly 24 h (Figure 5A, bottom panel).

We also examined the effect of detergent concentration on the freeze−thaw stability of PrP^C and PrPres amplification activity in solubilized preparations. We found that PrPres amplification activity was retained after four freeze−thaw cycles in 1% NOG or CHAPSO (Figure 5B, top two panels). In contrast, PrPres amplification activity in samples of 1% CHAPS-solubilized supernatant declined after two freeze−thaw cycles (Figure 5B, third panel, "spin"). Measurement of PrP^C levels showed that the loss of PrPres amplification activity in 1% CHAPS-solubilized supernatant samples could not be attributed to PrP^C degradation (Figure 5B, bottom panel). Furthermore, freeze−thaw instability of PrPres amplification activity was not observed in homogenates containing 1% CHAPS in which insoluble components had not been removed by centrifugation (Figure 5B, third panel, spin). One possible interpretation of these results is that an unidentified cofactor required for PrPres amplification has marginal solubility in 1% CHAPS. To test this possibility, we measured the stability of PrPres amplification activity in supernatants solubilized in 1.5% CHAPS. The results indicate that PrPres amplification activity is more resistant to repeated freeze−thaw cycles at 1.5% CHAPS, maintaining stability up to three cycles (Figure 5B, fourth panel).

DISCUSSION

In this study, we report that PrPres amplification in vitro can be supported by (1) an enriched membrane preparation and (2) detergent-solubilized preparations lacking lipid membranes. These two findings provide new clues about the mechanism of prion propagation and also represent significant methodological advances that will facilitate future detailed biochemical studies of the PrPres amplification reaction.

Our observation that purified SPM preparations support PrPres amplification suggests that PrP^{Sc} conversion in vivo may occur at the synaptic membrane. Immunolabeling studies demonstrate that PrP^C is localized to the synaptic terminal (*8*, *27*−*30*), and synapses are the only areas where a prion-infected neuron could directly contact uninfected neurons during spread of infection through the central nervous system. Membrane marker analysis of our SPM preparations showed

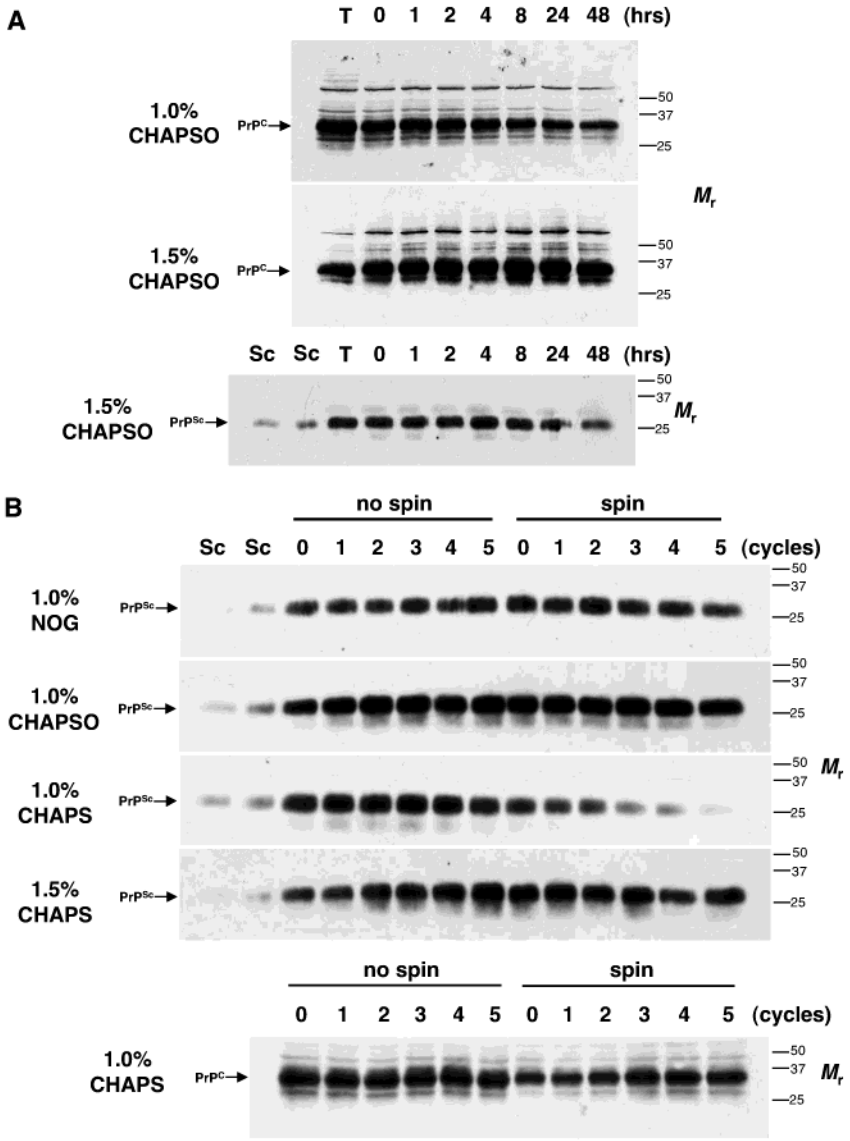

FIGURE 5: Stability of PrPres amplification activity in detergent-solubilized supernatants. (A) Stability of CHAPSO-solubilized supernatants at 4 °C. PrP^C was solubilized from brain membranes with CHAPSO and centrifuged at 20800*g* for 1 h. Aliquots of the supernatant were incubated at 4 °C for the time periods indicated before being assayed for PrP^C content (top two panels) or PrPres amplification (bottom panel). T = total 2% (w/v) brain homogenate not subjected to centrifugation; Sc = diluted scrapie brain homogenate alone. (B) Freeze−thaw stability of detergent-solubilized supernatants. Samples were solubilized with detergents as indicated and were either subjected to centrifugation at 20800*g* for 1 h (spin) or not (no spin). Supernatant (spin) or total (no spin) samples were subjected to repeated cycles of freezing and thawing, as indicated. In each cycle, samples were frozen at −70 °C for 30 min and thawed at 37 °C for ~1 min. The top four panels show PrPres amplification experiments, and the bottom panel shows PrP^C content. Sc = diluted scrapie brain homogenate alone.

a relative paucity of endosomal and lysosomal components, suggesting that the membranes of these acidic organelles may not play a significant role in PrP^C to PrPres conversion, even though they accumulate PrP during infection (*31*). Such a conclusion is consistent with measurements of the optimal pH ~7 for PrPres formation in vitro (*2*, *13*), as well as the observation that cultured scrapie-infected cells can be cured by the addition of surface-binding anti-PrP antibodies (*32*, *33*). Our membrane fractionation studies alone cannot rule out the possibility that some factors required for PrPres amplification are peripherally associated with SPM membranes through noncovalent interactions with integral SPM components. However, we also found that stripping S1 membrane preparations with 1 M KCl did not diminish PrPres amplification (data not shown). An important limitation of our membrane fractionation study is that SPM preparations are heavily contaminated with ER membranes (Table 2). Therefore, our studies cannot distinguish whether PrPres formation occurs in ER membranes or in plasma membranes. Our experiments also do not distinguish whether PrP^{Sc} formation occurs on the cytoplasmic or extracytoplasmic side of these membranes.

Our observation that detergent-solubilized preparations containing PrP^C but lacking membranes support PrPres amplification indicates that intact lipid membranes are not essential for efficient PrP conversion. These results contradict the membrane hypothesis of prion production, which proposes that prions are necessarily generated in lipid membranes (*15*−*18*). Our results also indicate that physical attachment of PrP^C to a lipid membrane is not absolutely required for its conversion into PrPres and imply that membrane-detached PrP^C molecules might retain full efficiency as conversion substrates. Using a radiolabeled in vitro conversion technique, Baron et al. previously showed

that PI-PLC-mediated release of PrP^C from raft membranes facilitates PrPres formation in the absence of detergents (*13*). Taken together, these findings indicate that PrP molecules not attached to membranes are good in vitro conversion substrates and raise the possibility that unattached PrP molecules might play a role in the spread of prion infection from cell to cell across discontinuous membranes in vivo.

Currently, there is substantial interest in identifying factors other than PrP that may be required for prion propagation, and other investigators have used protein−protein interaction and chemical cross-linking techniques to identify potential PrP binding partners. The laminin receptor, Bcl-2, Hsp60, and the neuronal cellular adhesion molecules (N-CAMs) are some of the candidate PrP-binding molecules identified by these techniques (*34*−*39*). However, it remains uncertain whether any of these putative PrP-binding molecules participate in the pathogenic process of PrP conversion.

The in vitro PrPres amplification technique offers an opportunity to identify PrP conversion factors biochemically using a functional assay. However, to determine the molecular mechanism of prion propagation with this approach, it will ultimately be necessary to use purified components to reconstitute the process of PrPres amplification in vitro. Here, we fulfill a critical prerequisite for this strategy by describing conditions that solubilize all of the components in brain homogenate required to maintain PrPres amplification activity in stable form. Having this soluble preparation will enable chromatographic isolation of PrPres amplification factors in future studies. Our ability to enrich PrP^C and PrPres amplification activity ~4-fold by preparing SPM samples will also facilitate future purification schemes, because SPM can be used as a starting material for solubilization and chromatographic steps. Taken together, our results suggest that a biochemical approach to purifying PrPres amplification factors is feasible.

A limitation of our work is that PrPres generated by in vitro amplification may not be identical to PrP^{Sc}; therefore, equivalence of PrPres and PrP^{Sc} can only be proven by infectivity studies. However, it is worth noting that the processes of PrPres amplification in vitro and prion propagation in vivo share many specific features (*2*): (1) In vitro PrPres amplification is both species- and strain-specific, consistent with the specificity of prion propagation in vivo, and the level of PrPres amplification in vitro is inversely proportional to scrapie incubation times in three hamster species susceptible to Syrian hamster prions. (2) In vitro PrPres amplification is dependent upon time, temperature, and pH. Amplification is optimized under physiologic conditions and proceeds at a rate similar to the rate of PrP^{Sc} formation in vivo. (3) Several compounds that inhibit PrP^{Sc} formation in living scrapie-infected cells also inhibit in vitro PrPres amplification. (4) Under nondenaturing conditions, a small amount of PrP^{Sc} can stimulate the conversion of stoichiometric amounts of PrP^C, indicating that the reaction is efficient. Moreover, it is likely that the biochemical identification of PrPres amplification factors will yield valuable insights into the mechanism of prion formation even if PrPres is not identical to PrP^{Sc}.

ACKNOWLEDGMENT

The authors thank Drs. William Wickner, John Hwa, and Harry Higgs for helpful advice, Drs. Ta Yuan Chang and Nancy Speck for use of their equipment, and Dr. Judy Rees for help in manuscript preparation.

SUPPORTING INFORMATION AVAILABLE

One figure showing freeze−thaw stability of detergent-solubilized supernatant samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI035889L