Scrapie and Cellular Prion Proteins Differ in Their Kinetics of Synthesis and Topology in Cultured Cells

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Abstract. Both the cellular and scrapie isoforms of the prion protein (PrP) designated PrPc and PrPs are encoded by a single-copy chromosomal gene and appear to be translated from the same 2.1-kb mRNA. PrPc can be distinguished from PrPs by limited proteolysis under conditions where PrPc is hydrolyzed and PrPs is resistant. We report here that PrPc can be released from the surface of both normal-control and scrapie-infected murine neuroblastoma (N2a) cells by phosphatidylinositol-specific phospholipase C (PIPLC) digestion and it can be selectively labeled with sulfo-NHS-biotin, a membrane impermeant reagent. In contrast, PrPs was neither released by PIPLC nor labeled with sulfo-NHS-biotin. Pulse-chase experiments showed that [35S]methionine was incorporated almost immediately into PrPc while incorporation into PrPs molecules was observed only during the chase period. While PrPc is synthesized and degraded relatively rapidly (t1/2 ~ 5 h), PrPs is synthesized slowly (t1/2 ~ 15 h) and appears to accumulate. These results are consistent with several observations previously made on rodent brains where PrP mRNA and PrPc levels did not change throughout the course of scrapie infection, yet PrPs accumulated to levels exceeding that of PrPc. Our kinetic studies demonstrate that PrPs is derived from a protease-sensitive precursor and that the acquisition of proteinase K resistance results from a posttranslational event. Whether or not prolonged incubation periods, which are a cardinal feature of prion diseases, reflect the slow synthesis of PrPs remains to be established.

Over the last five years, there has been a remarkable convergence of information about the prion protein (PrP) (26, 39). Biochemical studies have shown that scrapie prion infectivity and a protease-resistant core of scrapie prion protein (PrPs) designated PrP 27-30 copurify (27, 28). Furthermore, prolonged exposure of purified prion fractions to proteases is accompanied by a concomitant degradation of PrP 27-30 and a loss of scrapie infectivity (21). Solubilization of scrapie prions into detergent-lipid-protein complexes (DLPCs) (13) made possible immunoaffinity chromatography with PrP monoclonal antibodies demonstrating the copurification of PrPs and scrapie infectivity (14). Neutralization of scrapie infectivity in DLPCs with PrP antiserum has also been observed. Human and hamster prions purified by limited proteolysis, detergent extraction, differential centrifugation and sucrose gradient sedimentation were found to aggregate into insoluble, rod-shaped structures indistinguishable from many purified amyloids (6, 28). This observation was extended to the brains of humans and animals dying of prion diseases where amyloid plaques were found to stain intensely with PrP antibodies (17, 32).

The foregoing studies argue persuasively that PrPs is a major and necessary component of the infectious prion. Molecular cloning studies established that PrPs is encoded by a single-copy, chromosomal gene and not by a putative nucleic acid hiding within the prion (4, 10, 25). This is an important feature distinguishing prions from viruses. Although the PrP gene is highly regulated in the developing brain, it is constitutively expressed during the adult life of rodents (22, 24, 25). The product of the PrP gene in normal animals and humans is a protein designated PrPc. Many lines of evidence suggest that PrPc and PrPs have the same amino acid sequence but differ in their properties because of an as yet unidentified posttranslational event (3, 4).

Until recently, cell culture systems have been disappointing with respect to extending our knowledge of prions since prion titers are low and there is no recognizable cytopathic effect associated with scrapie infection. The identification of PrPc on the surface of cultured cells (37) and PrPs in extracts of these cells (8) suggested that scrapie-infected cells in culture might be used to study PrPs synthesis. We report here that PrPc can be released from the surface of both normal-control and scrapie-infected murine neuroblastoma (N2a) cells by phosphatidylinositol-specific phospholipase C (PIPLC) digestion and it can be selectively labeled with sulfo-NHS-biotin, a membrane impermeant reagent. In contrast, PrPs was neither released by PIPLC nor labeled with

Abbreviations used in this paper: DLPC, detergent-lipid-protein complex; N2a, murine neuroblastoma; PIPLC, phosphatidylinositol-specific phospholipase C; PrP, prion protein; PrPc, cellular isoform of the prion protein; PrPs, scrapie isoform of the prion protein; Sarkosyl, sodium dodecyl sarcosinate; ScNa, scrapie-infected murine neuroblastoma.

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sulf-o-NHS biotin. Pulse-chase experiments showed that [35S]methionine was incorporated almost immediately into PrPc while incorporation into PrPs molecules was delayed for several hours. While PrPc is synthesized and degraded relatively rapidly, PrPs is synthesized slowly and appears to accumulate. The kinetic findings reported here demonstrate that PrPc and PrPs have different rates of synthesis and degradation and that these differences arise from posttranslational events.

Materials and Methods

Reagents
PIPLC was a gift of Dr. Martin Low (Columbia University, NY). One unit of PIPLC will digest 1 μmol of phosphatidylinositol in 1 min (19). Proteinase K used in digestion of cellular extracts is a product of Beckman Instruments Inc. (Palo Alto, CA). Reagents for cell culture were obtained from the University of California San Francisco Cell Culture Facility except for Opti-MEM I which is a product of Gibco Laboratories (Grand Island, NY). Detergents for the extraction of cells and the solubilization of cellular proteins were obtained from Sigma Chemical Co. (St. Louis, MO) as was PMSF and aprotinin. PAGE reagents were obtained from Bio-Rad Laboratories (Richmond, CA).

Cell Lines
The uninfected N2a cell line used in this study was obtained from American Type Tissue Culture Collection. A clonal cell line of scrapie-infected N2a cells (ScN2a) was found to produce ~10⁵ ID₅₀ U of prions/10⁷ cells, and protease-resistant PrPc (8). The ScN2a subclone reported here was in continuous passage for ~18 mo and continued to produce PrPsc at a constant level. Cell lines were maintained in DME with 10% FBS and penicillin/streptomycin in humidified incubators containing 5% CO₂. In general, scrapie-infected cultures were passaged every 7-10 d after a 1:5 or 1:10 split. Cells were terminated by addition of PMSF to 2-5 mM and methanol precipitation with 4 vol of methanol. EDTA was added to a final concentration of 5 mM before methanol precipitation to reduce the formation of insoluble Ca₃PO₄ precipitates.

Immunoblot Analysis
After PAGE separation of cellular proteins, PrP molecules were detected by electro-transfer to nitrocellulose membrane in 0.02 M Tris, 0.15 M glycine, 20% methanol, 0.02% SDS at 500 mA overnight at 4°C. After transfer, membranes were washed briefly in H₂O, then washed for 1 h in PBS with 5% nonfat dry milk (Carnation). After two additional washes in 0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl, 0.005% Tween 20 (TBST) (10 min each), membranes were further incubated in TBST with 1% horse serum for 45-60 min. After removal of the TBST serum, membranes were incubated overnight in TBST with anti-PrP sera as described in the figure legends. Complexes between antibodies and prion proteins were then revealed using Promega anti-rabbit Ig antibodies coupled to alkaline phosphatase as described by Promega Biotech (Madison, WI).

Immunoprecipitation
Methanol-precipitated cellular proteins were resuspended in DLPC buffer consisting of 0.5-2 ml of 0.05 M Tris-HCl pH 8.2, 0.15 M NaCl, 2% (wt/vol) Sarkosyl, 0.4% (wt/vol) phosphatidylcholine with 10² U/ml of aprotinin and sonicated for at least 5 min. This procedure forms DLPCs that enhance the solubility of PrPc and allows immunofluorescence purification (14).

Membrane Proteins for Immunoblot
Radiolabeled and nonradioactive cellular proteins were extracted from cells in detergent essentially as previously described (8, 34). Before extraction, cultures were washed 2-3 times in PBS, then harvested with a disposal scaper and pelleted by centrifugation. Cell pellets were then resuspended in 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.005 M EDTA (TNE) and lysed on ice by adding Triton X-100 and deoxycholate to 0.5%. Insoluble material was then removed by centrifugation at 3000 g before precipitation of protein extracts with at least 4 vol of methanol at ~20°C for 30 min. After centrifugation at 5000 g for 20 min, pellets were resuspended in TNE with 0.2% sodium dodecyl sarcosinate (Sarkosyl) and digested with 10-20 μg/ml proteinase K for 30-60 min at 37°C, where applicable. Proteinase K digestions were terminated by addition of PMSF to 2-5 mM and methanol precipitation (4 vol). We have recently modified this procedure and found that more efficient extraction was obtained when cells were treated directly on the culture vessel in 1-2 ml of ice-cold TNE, 0.5% Triton X-100, 0.5% deoxycholate. After removal of insoluble material by centrifugation, proteinase K digestion (10-20 μg/ml, 37°C, 60 min) can be carried out directly in the lysis buffer. All samples are then concentrated by methanol precipitation (4-10 vol) before immunoblot analysis or immunofluorimetry purification. This modified procedure was used for the experiments depicted in Figs. 5, 6 and 7. Each of these methods of extraction as well as proteinase K digestion yielded similar results.

Densitometry of [35S]-Met-Labeled PrP
Autoradiographic signals were quantitated by densitometric scanning using a model 620 instrument (Bio-Rad Laboratories). In each experiment, the relative amount of PrP present at any specific time during chase incubations is regarded as a percentage of the quantity of nascent PrP recovered immediately following the pulse-labeling period. The best fit to an exponential was calculated with a computer program that performs a nonlinear fit by minimizing the sums of the squares of the residuals between observed and calculated y-values.

Results
PIPLC Digestion Releases PrPc but not PrPs
The PIPLC-catalyzed release of PrPc from the surface of ScN2a cells is depicted in Fig. 1 A. Without PIPLC, little or no detectable PrP was found in the medium (lane j) and most of the PrP immunoreactivity remained cell associated (lane 2). Proteinase K digestion of ScN2a cell extracts revealed...
the characteristic pattern of PrP\(_{c}\) (lane 3), which is found in prion-infected N\(_2\)a cells (8). PrP\(_{c}\) was found in the medium of ScN\(_2\)a cells digested with PIPCLC (lane 4); however, this protein was degraded by proteinase K (lane 5) demonstrating that these molecules behave like PrP\(_{c}\). The band seen in lane 5 is proteinase K, which reacts with some of our antisera raised against PrP\(_{c}\) 27-30 as previously described (5). In contrast, PrP\(_{c}\) was not released by PIPCLC digestion, which released it into the medium (compare lanes 2 with 4). The poor immunostaining of PrP\(_{c}\) in cell extracts probably results from competition with cellular proteins for binding to the transfer membrane since mixing of a cell extract with PrP\(_{c}\) recovered after PIPCLC (lane 8) greatly reduced the immunostaining intensity (lane 9).

To assess the relative amounts of PrP\(_{c}\) and PrP\(_{sc}\) in ScN\(_2\)a cells, serial dilutions of the PrP\(_{c}\) released into the medium by PIPCLC were compared to proteinase K-digested extract (Fig. 1 B, lanes 1-8). Since the immunostaining of the two samples exhibited a parallel decrease with the same dilutions, we conclude that ScN\(_2\)a cells contain roughly similar amounts of PrP\(_{c}\) and PrP\(_{sc}\). This estimate must be tempered because we are uncertain about the relative efficiencies of PrP\(_{c}\) and PrP\(_{sc}\) transfer and binding to nitrocellulose as well as their immunoreactivity. PrP\(_{sc}\) immunostaining was diminished by no more than a factor of 2 if mixed with an equivalent amount of proteinase K-digested extract from uninfected N\(_2\)a cells (lanes 9-12).

All of the ScN\(_2\)a cells expressed PrP\(_{c}\) on their surface as assessed by indirect immunofluorescence (Fig. 2). The complete disappearance of immunostaining after PIPCLC digestion of ScN\(_2\)a cells indicates that PrP\(_{sc}\) cannot be visualized under the conditions used for these studies. Control N\(_2\)a cells before and after PIPCLC digestion (in Fig. 2, e and f) are indistinguishable from the ScN\(_2\)a cells in PrP immunostaining.

**PrP\(_{sc}\) in N\(_2\)a Cells**

The ScN\(_2\)a cell line used in this work is a clonal cell line originally isolated by Butler and colleagues (8). Other investigators have also reported long-term cultivation of ScN\(_2\)a cells (9, 30, 31). We have maintained this clonal line in continuous culture for over 18 mo. Occasionally cultures were found in which the PrP\(_{sc}\) content was substantially diminished. However, we observed no decrease in the level of PrP\(_{sc}\) production in most instances. 25 subclones were examined after \(\sim 6\) mo of cultivation. All were found to produce PrP\(_{sc}\) molecules. 10 additional subclones were isolated after \(\sim 12\) mo of continuous cultivation. All were found to produce PrP\(_{sc}\) molecules although one subclone produced significantly less than the parental population.

During the course of this work, we have made several observations on the cultivation of ScN\(_2\)a cells that warrant comment. We found that passage of the cultures every 7-10 d split at a 1:5 or 1:10 ratio enhanced the reproducibility of experiments as well as the stability of the cell line. Cells that were stored in liquid nitrogen became unstable after thawing and subcultivation, and produced less PrP\(_{sc}\). In fact, less than one-half of new subclones isolated from cells that had been stored in liquid nitrogen for 6 mo produced PrP\(_{sc}\) (data not shown).

**Topology of PrP\(_{c}\) Differs from PrP\(_{sc}\)**

One explanation for the inability of PIPCLC to release PrP\(_{sc}\) from ScN\(_2\)a cells could be that PrP\(_{sc}\) is not transported to the cell surface. To investigate the topology of PrP\(_{sc}\) in ScN\(_2\)a cells, we employed a membrane impermeant reagent (sulfo-NHS-biotin) that covalently couples biotin to free amino groups of cell surface proteins (16). In control experiments, crude microsome preparations of scrapie-infected mouse brain were exposed to sulfo-NHS-biotin, digested

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**Figure 1.** ScN\(_2\)a cells release PrP\(_{c}\) after exposure to PIPCLC while PrP\(_{sc}\) remains cell associated. This is an immunoblot that was developed with PrP antisera R073. (A) Confluent 10-cm dishes of ScN\(_2\)a cells were exposed to PIPCLC (0.2 U/ml) in PBS or PBS alone at room temperature for 3 h. Cells and medium were extracted or harvested, and digested with proteinase K as described in Materials and Methods. Lane 1 is culture medium without PIPCLC. Lanes 2 and 3 contain extracts of untreated cells incubated without and with proteinase K, respectively. Lanes 4 and 5 are PIPCLC-released proteins incubated without and with proteinase K, respectively. Lanes 6 and 7 are extracts of PIPCLC-treated cells incubated without (lane 6) or with proteinase K (lane 7). Lane 8 contains the PIPCLC-released PrP from one-half plate of ScN\(_2\)a cells while lane 9 contains a mixture of the other half of the sample in lane 8 and an extract of one-half a plate of ScN\(_2\)a cells. (B) Confluent cultures of ScN\(_2\)a cells were exposed to PIPCLC (0.2 U/ml) in PBS. Cells were harvested, extracted in detergent, and proteinase K digested before comparative immunoblot analysis of PIPCLC-released proteins and digested cell extracts. Lanes 1, 3, 5, and 7 contain serial 1:3 dilutions of proteins released from one 10-cm dish of ScN\(_2\)a cells by PIPCLC. Lanes 2, 4, 6, and 8 contain serial 1:3 dilutions of proteins found in proteinase K-digested extracts of one 10-cm dish of ScN\(_2\)a cells. Lanes 9-12 are a control that shows that N\(_2\)a cells do not normally produce proteins which interfere with PrP\(_{sc}\) immunoblot transfer and detection. Lane 9, proteinase K-digested ScN\(_2\)a extract mixed with an equivalent amount of proteinase K-digested extract of uninfected N\(_2\)a cells. Lanes 11 and 12 are serial 1:3 dilutions of sample electrophoresed in lane 9.
with proteinase K and analyzed by streptavidin shift PAGE/immunoblot analysis (Borchelt, D., A. Taraboulos, M. Scott, D. Bredesen, N. Stahl, and S. B. Prusiner, in preparation; N. Stahl, unpublished observations). PrP<sup>sc</sup> proteins extracted from nonbiotinylated microsomes did not bind streptavidin and their PAGE migration was unaffected (Fig. 3 A, lanes 1 and 2). In contrast, PrP<sup>sc</sup> molecules extracted from biotinylated microsomes exhibited a marked shift in PAGE migration in the presence of streptavidin (Fig. 3 A, lanes 3 and 4). In addition, large complexes of biotinylated PrP and streptavidin were found not to electrotransfer efficiently causing a reduction in immunostaining. These studies also showed that PrP<sup>sc</sup> retained its protease resistance after reacting with sulfo-NHS-biotin.

ScN<sub>2</sub>a cells were exposed to sulfo-NHS-biotin followed by PIPLC digestion. Cellular proteins were extracted and an aliquot digested with proteinase K. PrP<sup>c</sup> released from nonbiotinylated cultures did not bind streptavidin (Fig. 3 B, lanes 1 and 2). In contrast, the PAGE migration of PrP<sup>c</sup> released from cells exposed to sulfo-NHS-biotin was retarded in the presence of streptavidin (Fig. 3 B, lane 4), but not by streptavidin preincubated with excess d-biotin (Fig. 3 B, lane 3). Since PrP<sup>c</sup> released from biotinylated cultures quantitatively bound streptavidin, the amount of sulfo-NHS-biotin used in these experiments (1 mg/ml) was clearly sufficient to react with cell-surface PrP. PrP<sup>sc</sup> extracted from biotinylated ScN<sub>2</sub>a cultures did not quantitatively bind streptavidin (200 μg) (Fig. 3 C, lane 4), while the PAGE migration of a biotinylated control PrP<sup>sc</sup> added to the extract (Fig. 3 C, lanes 1 and 2) was completely retarded. The migration of neither PrP<sup>c</sup> nor PrP<sup>sc</sup> was affected by streptavidin (200 μg) preincubated with d-biotin (100 μg) (Fig. 3 C, lane 3). Similar analysis of extracts of biotinylated ScN<sub>2</sub>a cells without the addition of control biotinylated PrP<sup>c</sup> demonstrates that most PrP<sup>sc</sup> was not accessible to sulfo-NHS-biotin (Fig. 3 C, lanes 5 and 6). The PAGE migration of PrP<sup>sc</sup> extracted from nonbiotinylated cultures appeared unaltered by streptavidin (Fig. 3 C, lane 7). The inaccessibility of most PrP<sup>sc</sup> in ScN<sub>2</sub>a cells to sulfo-NHS-biotin suggests that the majority of PrP<sup>sc</sup> does not accumulate on the cell surface.

**Nascent PrP on the Surface of N<sub>2</sub>a Cells**

Uninfected N<sub>2</sub>a (Fig. 4 A) and ScN<sub>2</sub>a (Fig. 4 B) cells were incubated in medium with 300 μCi/ml [<sup>35</sup>S]methionine for 2 h before exposure to PIPLC (Fig. 4, lanes 1 and 2) or sulfo-NHS-biotin (Fig. 4, lanes 3 and 4). The radiolabeled pro-
Figure 3. PrPc and PrPsc differ in their topology. (A) Initially, an examination of the ability of mouse PrPsc to react with sulfo-NHS-biotin and withstand proteinase K digestion was performed. Microsomal preparations of scrapie-infected mouse brain were exposed to 1 mg/ml sulfo-NHS-biotin in H2O after brief sonication. After 20 min, Tris-HCl (pH 7.5) was added to make a final concentration of 0.1 M before extraction in detergent, methanol precipitation, and digestion with proteinase K as described in Materials and Methods. Protein in each preparation was examined by streptavidin shift PAGE and immunoblot analysis with polyclonal anti-PrP 27-30 serum (R017). Biotinylated proteins bind streptavidin resulting in retarded migration on SDS-PAGE. Lanes 1 and 2 are nonbiotinylated microsomes without (lane 1) or with (lane 2) the addition of 50 μg of streptavidin. Lanes 3 and 4 are biotinylated microsomes without (lane 3) and with (lane 4) the addition of 50 μg of streptavidin. (B and C) Confluent cultures in 150 cm² flasks (1 per lane) were exposed to PBS containing 1 mg/ml sulfo-NHS-biotin, except where noted, for 20 min at room temperature. After two washes with 0.1 M Tris-HCl pH 7.5, 0.1 M NaCl, and 2 washes with PBS, the cells were exposed to PIPLC (0.2 U/ml) in PBS for 2 h at room temperature. The extent of PrP biotinylation in each fraction was then examined by streptavidin shift PAGE and immunoblot analysis with polyclonal anti-PrP 27-30 serum (R017). Biotinylated proteins bind streptavidin resulting in retarded migration on SDS-PAGE. Lanes 1 and 2 are PIPLC-released proteins from nonbiotinylated cultures electrophoresed in the presence of 20 μg of streptavidin (lane 1) or 20 μg of streptavidin (lane 2). Lanes 3 and 4 are PIPLC-released proteins from biotinylated cultures electrophoresed in the presence of 50 μg of streptavidin (lane 3) or 50 μg of streptavidin (lane 4).

Figure 4. Nascent PrPc in control and scrapie-infected N2a cells is transported to the cell surface and partially released by PIPLC. Confluent flasks (25 cm²) of normal and scrapie N2a cells were metabolically radiolabeled for 2 h with 300 μCi/ml [35S]methionine in methionine-free DME with 2.5% dialyzed FBS. One set of flasks was then exposed to 0.2 U/ml PIPLC in PBS for 3 h while a second set was exposed to sulfo-NHS-biotin in PBS for 20 min. Cells and medium were extracted in detergent or harvested, and immunoprecipitated as described in Materials and Methods. (A) Normal N2a cells. (B) ScN2a cells. Lanes 1 and 2 show released and cell-associated proteins after exposure to PIPLC, respectively. Lanes 3 and 4 are proteins immunoprecipitated from biotinylated cultures electrophoresed in the presence of 50 μg streptavidin preincubated with 25 μg d-biotin (lane 3) or 50 μg streptavidin (lane 4).
Synthesis and Degradation of PrPc

Metabolic pulse-chase radiolabeling with [35S]methionine was used to study PrP biosynthesis and degradation in normal and scrapie-infected N2a cells. Radiolabeled PrP molecules were immunoprecipitated after increasing periods of chase and analyzed by SDS-PAGE. Prominent 33-35-kD PrP bands were observed for both uninfected N2a (Fig. 5A) and ScN2a cells (Fig. 6A) immediately following a 2-h pulse of [35S]methionine. The radiogram shown above (A) (- - -) and one from a duplicate experiment (---) were densitometrically scanned and the values plotted as a function of the chase period. Values for PrPc are expressed as a percent of the signal detected at the end of the pulse period; i.e., no chase.

Figure 5. Turnover of PrPc in normal N2a cells. (A) Confluent N2a cells in 25-cm² flasks were metabolically radiolabeled for 2 h with 300 µCi/ml [35S]methionine in methionine-free DME with 2.5% dialyzed FBS after a 1-h preincubation in methionine-free medium. Cultures were then incubated in serum-free medium containing unlabeled methionine (Opti-MEM I) for increasing periods of time. Cell extracts were digested with proteinase K (even-numbered lanes only) followed by immunoprecipitation of DLPCs with immune R017 serum. Lanes 1 and 2, no chase incubation. Lanes 3 and 4, 2-h chase. Lanes 5 and 6, 6-h chase. Lanes 7 and 8, 24-h chase. (B) Degradation of PrPc in uninfected N2a cells after a 2-h pulse of [35S]methionine. The radioautograph shown above (A) (- - -) and one from a duplicate experiment (---) were densitometrically scanned and the values plotted as a function of the chase period. Values for PrPc are expressed as a percent of the signal detected at the end of the pulse period; i.e., no chase.

Kinetics of PrPsc Synthesis

Metabolic radiolabeling studies gave a strikingly different result when ScN2a cells were analyzed for proteinase K-resistant PrP molecules. Immediately following the 2-h pulse labeling period, no proteinase K-resistant PrP molecules were found (Fig. 6A, lane 2). With increasing intervals of chase, proteinase K-resistant PrP molecules became evident (Fig. 6A, lanes 4, 6, 8, 10, 12, and 14 correspond to 2 h, 4 h, 6 h, 10 h, 24 h, and 48 h of chase, respectively). This indicates that PrPsc is derived from a proteinase K-sensitive precursor. Lanes 15-17 are controls showing that the proteinase K-resistant PrPsc molecules are not immunopurified by preimmune monospecific antipeptide (PI) sera (3) (Fig. 6, lane 15) while immune serum (Fig. 6, lane 16) precipitates radiolabeled PrPsc. The immunoprecipitation of PrPsc with this antiserum was prevented by preincubation with the PI synthetic peptide (Fig. 6, lane 17), thereby confirming the identity of the putative PrP bands.

The total amount of PrPsc recovered in the nonproteinase K digested fractions of radiolabeled cell extracts declined with increasing chase time (Fig. 6A, odd-numbered lanes). This suggests that a significant fraction of the newly synthesized PrP molecules produced in ScN2a cells are destined for cellular degradation. Since we cannot distinguish the proteinase K-digestable precursor of PrPsc from PrPc, it is not possible, at present, to determine if the rate of PrPc degradation in ScN2a cells differs from that in uninfected N2a cells.

Release of PrPc from ScN2a Cells

To determine whether PrPc is secreted from ScN2a cells, aliquots of the culture medium were analyzed for PrP immunoreactivity. Figs. 7, A and B, show that similar amounts of PrP are released into the culture medium of uninfected N2a and ScN2a cells over a 16-h chase incubation (compare
Figure 6. Kinetics of PrPsc biosynthesis. (A) Confluent T25 flasks of ScN2a cultures were metabolically radiolabeled for 2 h as in Fig. 5. Cultures were then chase-incubated in Opti-MEM I before detergent extraction, proteinase K digestion, and immunoprecipitation in DLPCs as in Fig. 5. Lanes 1 and 2, no chase without (−) and with exposure to proteinase K (+). Lanes 3 and 4, 2-h chase (−) and proteinase K (+). Lanes 5 and 6, 4-h chase (−) and proteinase K (+). Lanes 7 and 8, 6-h chase (−) and proteinase K (+). Lanes 9 and 10, 10-h chase (−) and proteinase K (+). Lanes 11 and 12, 24-h chase (−) and proteinase K (+). Lanes 13-15 are controls to demonstrate the specificity of antisera to the proteinase K-resistant PrP-reactive molecules found in ScN2a cells. Lane 15, preimmune antipeptide P1 sera R013 (Gly-Gln-Gly-Gly-Thr-His-Gln-Trp-Asn-Lys-Pro-Gly-Gly-Cys). Lane 16, immune anti-P1 sera R013. Lane 17, immune anti-P1 sera R013 preincubated with 25 μg P1 peptide. (B) Accumulation of PrPsc in ScN2a cells after a 2-h pulse of [35S]methionine. The radioautograph shown above (−Δ−) and one from a similar experiment (−□−) were densitometrically scanned. The sum of the values for the 3 PrPsc species (a, even-numbered lanes) were plotted as a function of the chase period. Values for PrPsc are expressed as a percent of the 33-35-kD PrP signal detected at the end of the pulse period (a, lane 1).

Discussion

Although a wealth of evidence argues persuasively that PrPsc is a major and necessary component of the infectious prion particle, some investigators continue to challenge this premise (1, 11, 30, 36). In general, these investigators have encountered difficulty in detecting PrPsc in a specific extract or fraction and have concluded that its apparent absence is evidence for a dissociation between PrPsc and scrapie infectivity. In part, these problems are quantitative since the bioassay is extremely sensitive while PrPsc detection relies on immunodetection of samples subjected to limited proteolysis. The propensity of PrPsc to form aggregates and the difficulties attendant with its solubilization create an additional level of complexity.

Contrasting Properties of PrPc and PrPsc

The studies described here on PrPsc biogenesis and topology are the first to report detection of radiolabeled PrPsc. These results complement those previously reported for the two PrP isoforms (Table I). Purified prions are composed largely, if not entirely, of PrPsc molecules. Limited proteol-
Table I. Properties of Cellular and Scrapie PrP Isoforms

|                          | PrP<sub>c</sub> | PrP<sub>s~</sub> |
|--------------------------|----------------|-----------------|
| Normal cells             | +              | -               |
| Scrapie-infected cells   | +              | +               |
| Purified prions          | -              | + (PrP 27-30)   |
| Protease resistance      | -              | + (PrP 27-30)   |
| Amyloid rods             | -              | + (PrP 27-30)   |
| Subcellular localization | Cell surface   | Primarily intracellular |
| PIPLC release            | +              | -               |
| Synthesis                | <2 h           | ~15 h           |
| Turnover (<t<sub>1/2</sub>) | ~5 h          | >24 h           |

Analysis degrades PrP<sub>s~</sub> by hydrolyzing only the NH<sub>2</sub> amino acids to produce PrP 27-30 (25, 29). Detergent extraction of membranes containing PrP 27-30 results in the polymerization of this protein in rod-shaped particles indistinguishable from amyloids (23, 28). Although both PrP isoforms possess glycosyl-phosphatidylinositol anchors, PrP<sub>c</sub> is localized primarily on the cell surface while PrP<sub>s~</sub> is found mainly within the cell. PrP<sub>c</sub> is released from the cell surface by PIPLC digestion but PrP<sub>s~</sub> is resistant to release. PrP<sub>c</sub> synthesis and maturation are rapid as evidenced by the large amount of radiolabeled PrP<sub>c</sub> found after a 2-h pulse. Other investigators suggest that the <t<sub>1/2</sub> for PrP<sub>c</sub> synthesis may be as small as 20 min (9). Our studies show that PrP<sub>c</sub> turns over with a <t<sub>1/2</sub> of 5.2 h. In contrast, PrP<sub>s~</sub> synthesis and maturation is slow with a <t<sub>1/2</sub> of 15.2 h. We have not been able to demonstrate the degradation of PrP<sub>s~</sub> in cultured cells; presumably, this process is extremely slow with a <t<sub>1/2</sub> > 24 h.

Subcellular Localization of PrP Isoforms

We found that ~50% of the PrP<sub>c</sub> molecules produced in either uninfected, or scrapie-infected N<sub>a</sub> cells could be released by PIPLC digestion (see Fig. 4). In both uninfected N<sub>a</sub> cells and ScN<sub>a</sub> cells, most of the newly synthesized PrP<sub>c</sub> molecules could be labeled with sulfo-NHS-biotin. This suggests that some cell surface PrP<sub>c</sub> molecules may be resistant to PIPLC digestion. However, it appears that it is not uncommon for cells to produce subpopulations of GPI anchored proteins that cannot be released by PIPLC digestion (18, 19). Most PrP<sub>s~</sub> produced in ScN<sub>a</sub> cells appears to be inaccessible to labeling with sulfo-NHS-biotin (see Fig. 3). This result is consistent with recent immunofluorescence studies of ScN<sub>a</sub> cells showing that PrP<sub>s~</sub> accumulates within the interior of cells (Taraboulos, A., D. Serban, and S. B. Prusiner, submitted for publication).

Posttranslational Processing

Our experiments have demonstrated that a protein indistinguishable from PrP<sub>c</sub> is present in ScN<sub>a</sub> cells. Since a high proportion of the cells also contain PrP<sub>s~</sub> and produce infectious prions, synthesis of PrP<sub>c</sub> and PrP<sub>s~</sub> are not mutually exclusive. Whereas PrP<sub>c</sub> may be observed in both normal and scrapie-infected N<sub>a</sub> cells following a brief (1-2 h) metabolic labeling period, detection of significant quantities of mature, protease-resistant PrP<sub>s~</sub> requires a prolonged chase period. Therefore, PrP<sub>s~</sub> is derived from a protease-sensitive precursor. Acquisition of protease resistance ensues slowly with an estimated half-time for synthesis of 15 h.

Figure 7. Release of PrP<sub>c</sub> into cell culture medium from N<sub>a</sub> and ScN<sub>a</sub> cells. (A) N<sub>a</sub> and (B) ScN<sub>a</sub> cells were labeled with [35S]methionine for 2 h followed by a chase period of 2 h (lanes 1 and 2) or 16 h (lanes 3 and 4). Cells (lanes 1 and 3) and media (lanes 2 and 4) were harvested and analyzed for PrP molecules. Immunoprecipitations were performed with R017 antisera. (C) Time course for release of PrP<sub>c</sub> into culture medium from ScN<sub>a</sub> cells. The labeling and chase medium from cultures used in Fig. 6A were harvested and examined. Lane 1, labeling medium. Lane 2, 2-h chase medium. Lane 3, 4-h chase medium. Lane 4, 6-h chase medium. Lane 5, 10-h chase medium. Lane 6, 24-h chase medium. Lane 7, 48-h chase medium. (D) Radioautograph above in C was analyzed densitometrically and the values (∆-) plotted as a function of the chase time.
A significant fraction of PrP chains eventually acquire protease resistance. We estimate that ~30% of the PrP chains acquire protease resistance based on the data presented in Figs. 1 and 6. Our estimates suffer from uncertainties about the efficiencies of PrP c and PrP s~ transfer to nitrocellulose membranes as well as our assumption that virtually all PrP c is released by PIPLC digestion. In addition, the relative efficiency of immunoprecipitation of radiolabeled PrP molecules in DLPCs is unknown. In spite of these uncertainties, immunoblot analysis and metabolic labeling studies give similar estimates.

Both normal and Scn2a cells spontaneously release a protease-sensitive form of PrP. We note that the sum of the estimated rate constants for production of the extracellular form (0.066h−1) and for formation of the protease-resistant form (0.046h−1) approximates the rate constant for disappearance of PrP c in normal cells (0.13h−1). It follows that a large proportion of PrP molecules destined for intracellular degradation in uninfected cells might serve as substrates for conversion to PrP s~ in infected cells.

The organization of the PrP gene argues that the cellular and scrapie isoforms do not arise from alternative splicing since the entire open reading frame is contained within a single exon (4). Furthermore, there is no evidence for DNA rearrangements of the PrP gene during scrapie infection. Based on these observations, we suggested that PrP c and PrP s~ differ because of some posttranslational event (4).

We are unable to determine from our present experiments whether the precursor of PrP s~ is identical to or distinct from PrP c. PrP s~ might be derived directly from PrP c via a subsequent posttranslational event, perhaps an abrogation of a normal cellular process. Alternatively, a distinct protease-sensitive precursor to PrP s~ could be synthesized which would be committed toward formation of the protease-resistant form at an early stage of biosynthesis. In either event, it is clear from our present study that the biochemical property of protease-resistance is acquired posttranslationally.

**Slow Accumulation of PrP s~ and the Scrapie Incubation Period**

Although scrapie has been successfully transmitted to cultured cells on many occasions (8, 20, 30, 31, 33), the low prion titer observed (usually 10^4 to 10^5 ID50/ml of extract) contrasts markedly with those found in the brains of scrapie-infected hamsters exhibiting clinical signs of scrapie. In brain, titers in excess of 10^8 ID50/ml of homogenate and correspondingly high concentrations of PrP s~ are found. Our data suggest a simple explanation for this paradox. A continuous culture of scrapie-infected cells such as that described here may be unable to accumulate high titers of PrP s~ (and by inference, infectious particles) because of constant dilution by ongoing cell division. In contrast, high titers in the brain may be achieved because prions replicate primarily in nondividing neurons. The extreme stability of PrP s~ compared to PrP c would allow accumulation to high concentrations during the extremely protracted incubation period that is characteristic of prion diseases.

**New Approaches Arising from these Studies**

The identification of conditions for the radiolabeling of PrP s~ in scrapie-infected cultured cells and its detection after SDS-PAGE should open many new approaches to the study of prions. Learning which inhibitors of posttranslational processing and transport prevent PrP s~ synthesis may elucidate the posttranslational event responsible for PrP s~ formation. For example, numerous inhibitors have become available which block specific steps in the synthesis and processing of Asn-linked complex oligosaccharides. Both PrP c and PrP s~ are known to be modified by complex oligosaccharides (7, 12, 15). Elucidating the nature of these chemical reactions as well as identifying the rate limiting step will add significantly to our understanding of prion diseases. Thus, dynamic studies involving inhibitors of cellular functions and a more detailed exploration of precursor-product relationships in Scn2a cells may elucidate the nature of the PrP s~ precursor and its relationship to PrP c.

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