Evidence for Synthesis of Scrapie Prion Proteins in the Endocytic Pathway*

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Infectious scrapie prions are composed largely, if not entirely, of an abnormal isoform of the prion protein (PrP) which is designated PrPSc. A chromosomal gene encodes both the cellular prion protein (PrPc) as well as PrPSc. Pulse-chase experiments with scrapie-infected cultured cells indicate that PrPSc is formed by a post-translational process. PrP is translated in the endoplasmic reticulum, modified as it passes through the Golgi, and is transported to the cell surface. Release of nascent PrP from the cell surface by phosphatidylinositol-specific phospholipase C or hydrolysis with dispase prevented PrPSc synthesis. At 18 °C, the synthesis of PrPSc was inhibited under conditions that other investigators report a blockage of endosomal fusion with lysosomes. Our results suggest that PrPSc synthesis occurs after PrP transits from the cell surface. Whether all of the PrP molecules have an equal likelihood to be converted into PrPSc or only a distinct subset is eligible for conversion remains to be established. Identifying the subcellular compartment(s) of PrPSc synthesis should be of considerable importance in defining the molecular changes that distinguish PrPSc from PrPc.

Many lines of investigation have converged to demonstrate that the scrapie isoform of the prion protein (PrPSc) mediates the transmission and pathogenesis of transmissible neurodegenerative diseases including scrapie, bovine spongiform encephalopathy, kuru, Gerstmann-Sträussler-Scheinker syndrome, and Creutzfeldt-Jakob disease (for reviews see Refs. 1 and 2). Both PrPSc and the cellular prion protein (PrPc) are encoded by a chromosomal, single copy gene (3). The two PrP isoforms are sialo-glycoproteins which possess glycoinositol phospholipid (GPI) anchors (4-6). In contrast to PrPc, PrPSc is resistant to digestion by proteases (4) and aggregates into amyloid rods after limited digestion in the presence of detergents (7). PrPSc is the only macromolecule, identified to date, that is consistently found in purified preparations of scrapie prions (1).

Although studies with transgenic mice expressing either foreign or mutant PrP genes have yielded important increments of knowledge about prions (8-11), scrapie-infected cultured cells have provided valuable insights into the synthesis of PrPSc (12-16). Many investigators have described cultured cells which are chronically infected with the scrapie agent (17-19). The finding that clones of scrapie-infected cells in culture produce both infectious prions and PrPSc (12, 14, 15) has stimulated research on the cellular biology of scrapie. While these scrapie-infected cloned cells produce relatively low levels of prions as measured by bioassays, the levels of PrPSc are sufficient for detection in radioimmunoassays (13).

Earlier studies established that PrPc synthesis begins with translation of the PrP mRNA in the endoplasmic reticulum, followed by modification of the N-linked carbohydrates in the Golgi and transport to the cell surface by the secretory pathway (6, 13, 20-22). At the cell surface, PrPc is attached by a GPI anchor which can be cleaved by phosphatidylinositol-specific phospholipase C (PIPLC) (5, 23).

Although transgenic mouse studies indicate that PrPc and PrPSc may form a complex during the synthesis of nascent PrPSc molecules (1, 8), the subcellular site(s) at which this interaction could occur is unknown. We report here that the recycling of PrP through the endosomal pathway is likely to be important for PrPSc synthesis. The release of nascent PrPSc molecules from the surface of scrapie-infected cells in culture by PIPLC digestion, or the hydrolysis of PrPc catalyzed by dispase, prevented PrPSc synthesis. While our studies were in progress, other investigators reported the blockade of PrPSc synthesis in cultured cells by digestion with trypsin or PIPLC (24). Although our conclusions are similar to those stated by others (24), our experimental results contrast sharply. For example, we were unable to prevent PrPSc synthesis using PIPLC at 37 °C. Only when the studies were performed at lower temperatures could we demonstrate an effect of PIPLC. Similarly, trypsin digestion gave uninterpretable results with respect to PrPSc synthesis. Only when we found more gentle conditions for hydrolyzing cell surface proteins using dispase were we able to obtain meaningful evidence. The reasons for these marked differences in experimental results remain to be established.

The results reported here argue that PrP transits to the cell surface before becoming PrPSc and are consistent with recent work that shows that brefeldin A reversibly inhibits...
PrP<sup>C</sup> transport to the plasma membrane and PrP<sup>Sc</sup> synthesis. Our observation that the conversion of cell-surface PrP into PrP<sup>Sc</sup> was reversibly arrested when cells were shifted to 18 °C implicates endosomal sorting in PrP<sup>Sc</sup> synthesis. Cooling cultured cells to 18 °C has been used by many investigators to inhibit the transport of membrane glycoproteins through the endosomal pathway. Endosomes are further implicated in recent work showing that PrP<sup>Sc</sup> accumulates in lysosomes (25, 26). However, the synthesis of PrP<sup>Sc</sup> appears to be completed before reaching lysosomes.

MATERIALS AND METHODS

Reagents, Antibodies, and Cell Lines—Cell culture reagents were obtained from the Cell Culture Facility of the University of California, San Francisco. Methionine-free MEM culture was made from reagents in the Select-amine kit from GIBCO. [<sup>35</sup>S]Methionine (SJ.1015) was obtained from Amersham Corp. Detergents for the extraction of cells and the solubilization of PrP were obtained from Sigma. L-<sup>α</sup>-Lecithin was purchased from Avanti Polar Lipids (Pelham, AL). Dispase was purchased from Boehringer Mannheim. FITC-labeled WGA and trypsin were purchased from Sigma.

Antibodies used in the immunopurification of PrP were produced in the laboratory. Rabbit PrP antisera R013 has been shown to react specifically with PrP<sup>C</sup> and PrP<sup>Sc</sup> molecules (12, 13, 15, 16, 27). Rabbit PrP antisera R066 and R073 are relatively new antisera that specifically recognize 33-35-, 30-, and 26-kDa protease-sensitive PrP molecules as well as 27-30-, 25-, and 19-kDa protease-resistant PrP<sup>Sc</sup> molecules (see Fig. 12). Preimmune sera from these rabbits do not react with these PrP molecules (15, 16, 27). Rabbit antisera R013, R002, and R009 were raised against synthetic PrP peptides P1 (amino acids 90–102), P2 (amino acids 15–40), and P3 (amino acids 220–233), respectively. These antisera have been described elsewhere and react specifically with both PrP<sup>C</sup> and PrP<sup>Sc</sup> molecules (13, 15, 16, 26, 28).

The cell lines ScN<sub>a</sub> and ScHaB are mouse neuroblastoma N<sub>a</sub> and Syrian hamster HaB cells that are chronically infected with scrapie and have been described elsewhere (12, 15). We have recently determined that the N<sub>a</sub> cells possess Prn-p<sup>r</sup>-PrP<sup>gen</sup> genes (see (30) for a description of Prn-p<sup>r</sup>). The scrapie isolate used to infect the N<sub>a</sub> cell line is called RML (31), while the isolate used to infect HaB cells is called ROT3 as previously described (13).

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Both the undigested and protease-digested (GdnSCN-denatured) cellular proteins were precipitated in methanol and recovered by centrifugation before digestion in 0.5 ml of DLPC buffer (20 mM Tris-HCl, pH 8.2, 150 mM NaCl, 2% Sarkosyl, 0.4% L-<sup>α</sup>-lecithin). After 2 min of sonication (Lab Supplies Inc., Hicksville, NY) in a glass homogenizer, 2 ml of PrP<sup>Sc</sup> (R073) was added and the samples were incubated overnight at 4 °C. The resulting immunocomplexes were then bound to protein A-Sepharose (Pierce Chemical Co. or Pharmacia LKB Biotechnology Inc.) for 1 h at 4 °C, washed for 30 min in DLPC buffer, transferred to a clean Eppendorf tube, and washed three more times in 50 mM Tris-HCl, pH 8.2, 500 mM NaCl, 2% Sarkosyl. The immunopurified PrP<sup>Sc</sup> was then desorbed from immunocomplexes by boiling in SDS-PAGE loading buffer and electrophoresed on polyacrylamide gels (34).

Quantitation of Radiolabeled PrP<sup>Sc</sup>-To quantify the production of PrP<sup>Sc</sup> in our cell lines, we measured the amount of radiolabeled protein that could be immunopurified from metabolically radiolabeled cells. Immunopurified proteins were separated on polyacrylamide gels and quantitated in one of two ways. In initial experiments described in Fig. 2, we determined the optical density of autoradiographic bands created by radiolabeled PrP<sup>Sc</sup> molecules with a densitometer (Bio-Rad) (films were not preflashed before exposure). In later experiments (Figs. 2-12), we used a Molecular Dynamics PhosphoImager device (Sunnyvale, CA) to measure the amount of radiolabeled PrP<sup>Sc</sup> produced by the cell lines. Quantitation was performed as suggested by the manufacturer and employed Imagequant software.

Estimation of the Kinetics of PrP<sup>Sc</sup> Biosynthesis—The quantitative data (generated by the above procedures) was used to calculate the kinetics of PrP<sup>Sc</sup> biosynthesis in ScHaB and ScN<sub>a</sub> cells (see Fig. 2). Although three M<sub>i</sub>, species of PrP<sup>Sc</sup> were seen (see Fig. 2), we chose to sum the quantitative values for these three molecules and generated a single value for PrP<sup>Sc</sup> at each time point in the chase. To normalize for variations in different experiments, we determined the maximum value for PrP<sup>Sc</sup> in each experiment, then converted all the values for a given experiment to a percentage of that maximum value.

The percentage values for time points between 0 and 24 h of chase were entered into a computer program that calculates a best fit curve on the assumption that the production of PrP<sup>Sc</sup> follows single exponential kinetics. The program also generates a k constant for the kinetics of PrP<sup>Sc</sup> production that was used to calculate a t<sub>1/2</sub> for PrP<sup>Sc</sup> biosynthesis with the formula t<sub>1/2</sub> = ln 2/k.
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RESULTS

Studies of PrPSc biosynthesis require quantitative immunopurification of radiolabeled PrPSc. Because PrPSc resists solubilization in detergents, the immunopurification of PrPSc has proven to be difficult (35, 36). However, PrPSc can be functionally dispersed into DLPC (36), and this method was used in a previous study of PrPSc biosynthesis in ScN2a cells (13). By denaturing PrPSc in GdnSCN prior to dispersion in DLPC, we found that the solubilization of PrPSc could be increased considerably (Fig. 1).

To examine the solubility of PrPSc, we determined the sedimentation properties of the protein in DLPC during high speed centrifugation (60,000 \( \times \) g). We found that most or all of the protease-digested PrPSc from cell extracts that were denatured in 3 M GdnSCN remained in the supernatant fraction of DLPC after centrifugation (Fig. 1, A and B, lanes 5 and 6). In contrast, most of the PrPSc found in cell extracts that were dispersed directly in DLPC pelleted during centrifugation (lanes 1 and 2), indicating aggregation. PrPSc in GdnSCN-treated extracts lost its resistance to proteases as expected (27) (A and B, lanes 7 and 8), indicating complete denaturation (37). In contrast, most of the PrPSc that was dispersed directly into DLPC remained protease resistant (A and B, lanes 3 and 4). Based on these results, we chose to denature PrPSc in GdnSCN before dispersion in DLPC to achieve a more consistent and quantitative solubilization of PrPSc for subsequent immunopurification.

Kinetics of PrPSc Formation in ScN2a and ScHaB Cells—Previous studies in scrapie-infected cell cultures have shown that ScN2a and ScHaB cells produce 27-30-, 25-, and 19-kDa protease-resistant PrPSc molecules that are not found in their uninfected progenitors (N2a and HaB cells) (12, 13, 15). We have also reported that PrPSc molecules are derived from protease-sensitive prion proteins that slowly acquire protease resistance (13, 16). Using the improved methodology for PrPSc solubilization described above, we measured the kinetics of PrPSc formation in both ScN2a and ScHaB cells by pulse-chase radiolabeling experiments with [\(^{35}\)S]methionine. To identify radiolabeled PrPSc, cells were extracted in detergent, digested with protease K (40 \( \mu \)g/ml), and then denatured in 3 M GdnSCN, 20 mM Tris-HCl, pH 7.5, before dispersion into DLPC and immunopurification.

In both ScN2a and ScHaB cells, no protease-resistant PrPSc molecules were recovered when cells were harvested immediately after a 1-h labeling period; instead only protease-sensitive PrP was recovered (Fig. 2, A and B, lanes 1 and 2) as expected (13, 15). Radiolabeled protease-resistant PrPSc was detected in extracts of ScHaB cells by 1 h of chase in unlabeled medium (Fig. 2A, lane 3), with greater amounts detected after 2 and 4 h (lanes 4 and 5). No further increase in the amount of radiolabeled PrPSc was seen in cultures that were chased for 4, 6, 10, and 24 h (lanes 6–8). In the ScN2a cells, we found that increasing amounts of radiolabeled PrPSc were immunopurified at each chase time point between 1 and 6 h (Fig. 2B, lanes 3–6), while stable levels were seen in cultures chased for 6, 10, and 24 h (lanes 6–8).

 Autoradiographs shown in Fig. 2 suggested that the production of PrPSc reaches a maximum in 2–4 h in ScHaB cells and 6–8 h in ScN2a cells. To quantify the kinetics of PrPSc formation in the ScN2a cells and ScHaB cells, we measured the amount of radiolabeled PrPSc that was immunopurified after various intervals of chase with a Bio-Rad densitometer or a Molecular Dynamics PhosphoImager (see “Materials and Methods”). The quantitative values for four experiments with each cell line were fit to a single curve, generating a \( t_{1/2} \) for acquisition of protease resistance by PrP molecules of about 1 h for ScHaB cells (Fig. 2C) and 3 h for ScN2a cells (Fig. 2D). Of note, these values describe the behavior of a pool of radiolabeled PrP molecules that were translated during the 1-h pulse. We believe that the estimated \( t_{1/2} = 3 \) h value for ScN2a cells that we report here is more accurate than our initial estimate of \( t_{1/2} = 15 \) h (13) because of the improved methods used for solubilization (Fig. 1) and analysis of a larger data set.

In both cell lines, the amount of radiolabeled PrPSc recovered after a prolonged chase represented \( \sim 5-10\% \) of the amount of nascent PrP arguing that most of the nascent PrP behaves as PrPSc and is degraded (also see Fig. 3 and Refs. 13 and 16). Since both the ScN2a and ScHaB cell lines are clonal, and most of the cells in these cultures produce both PrPSc and PrPSc (13, 16), the foregoing results do not seem to be due to only a fraction of the cells in the culture producing PrPSc. Thus, only a minority of the PrP is converted into PrPSc. Whether all of the PrP molecules have an equal likelihood to
be converted into PrPSc or only a distinct subset is eligible for conversion remains to be established.

The Conversion of PrP into PrPSc Is a Temperature-dependent Process—PrPSc is a sialoglycoprotein that is bound to the cell surface by a GPI anchor (5, 6), and its transport through the secretory pathway is relatively rapid (13, 22). To determine if PrPSc biosynthesis involves membrane trafficking events, we studied the synthesis of PrPSc at 18°C. This temperature has been used by many investigators to examine the recycling and degradation of membrane proteins in cultured cells (38-45).

In both cell lines, the rate of PrP degradation was slowed at 18°C (Fig. 3, lanes 3 and 4). However, the two cell lines showed pronounced differences in their ability to synthesize PrPSc at 18°C. ScHaB cells metabolically radiolabeled at 37°C for 30 min produced as much PrPSc during a chase at 18°C as they did at 37°C (Fig. 3A, lanes 6 and 7), but ScN2a cells chased at 18°C could not synthesize PrPSc (Fig. 3B, lane 7).

Since the formation of PrPSc in ScHaB cells was unaffected at 18°C, we asked whether ScHaB cells produce PrPSc via a pathway that completely lacks a 37°C-dependent event. We found that ScHaB cells that were both labeled and chased at 18°C (Fig. 4) could not produce PrPSc (lane 6). Control ScHaB cultures that were labeled at 18°C but chased at 37°C did produce PrPSc (lane 4). One explanation for the lack of inhibition of PrPSc synthesis in ScHaB cells at 18°C after labeling at 37°C could be that PrP molecules undergo some 37°C-dependent processing before the end of the labeling period. This explanation is consistent with our observations that the kinetics of PrPSc biosynthesis in ScHaB cells are more rapid than those of the ScN2a cells (Fig. 2).

Characterization of the 18°C Block in PrPSc Formation in ScN2a Cells—To characterize further the 18°C block in PrPSc synthesis, confluent cultures of ScN2a cells were metabolically radiolabeled at 37°C for 30 min then chased for varied intervals (0, 1, 2, or 3 h) at 37°C before a shift to 18°C and an additional chase for 20 h. Cultures that were held at 37°C for 1 h after the initial labeling before a chase for 20 h at 18°C, produced twice as much PrPSc (Fig. 5A, lane 5) as control cultures which were chased for only 1 h at 37°C after the initial labeling (lane 6) or were directly placed at 18°C (lane 4). Similarly, cultures that were held at 37°C for 2 h then chased at 18°C were found to produce three to four times more PrPSc (lane 7) than cells that were chased for only 2 h at 37°C (lane 8) or chased at 18°C only (lane 4). These results, in conjunction with those from studies of ScHaB cells, indicate that the biosynthesis of PrPSc involves a temperature-dependent event that occurs shortly after the translation of PrP polypeptide chains, and suggests the possibility that two distinct events feature in the synthesis of PrPSc. The first event represents a commitment to the PrPSc pathway while the second involves the acquisition of protease resistance.

The inhibition of PrPSc production that occurred when ScN2a cells were chased at 18°C was reversible (Fig. 5B). Confluent cultures were metabolically radiolabeled at 37°C for 30 min then chased at 18°C for 20 h before a second chase at 37°C for varied intervals (1, 4, and 10 h). The kinetics of PrPSc formation after a shift from 18 to 37°C (lanes 7-9) were similar to those of control cells that were placed at 37°C.
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**Fig. 3.** ScHaB and ScN'a cells differ in their ability to produce protease-resistant PrP\(^{\text{Sc}}\) molecules when labeled at 37 °C and chased at 18 °C. Upper panel, ScHaB cells. Lower panel, ScN'a cells. Confluent 25-cm\(^2\) flasks of cells were metabolically radiolabeled for 30 min and prepared for chase as described in Fig. 2. Lane 1, no chase incubation. Lane 2, no chase incubation after digestion with proteinase K. Lane 3, chased at 37 °C for 20 h. Lane 4, chased at 18 °C for 20 h. To prevent overexposure of some lanes, the autoradiograph shown for lanes 1-4 are from a 2-day exposure, while lanes 5-7 were exposed for 10 days. Lane 5 is a 10-day exposure of lane 2. Lane 6, chased at 37 °C for 20 h and digested with proteinase K. Lane 7, chased at 18 °C for 20 h and digested with proteinase K.

immediately following the labeling (lanes 10-12). In both instances, there was little formation of PrP\(^{\text{Sc}}\) during the first hour at 37 °C (lanes 7 and 10) with greater amounts of PrP\(^{\text{Sc}}\) found after 4 h (lanes 8 and 11). While little or no synthesis of PrP\(^{\text{Sc}}\) occurred during the 20-h chase period at 18 °C, the pool of radiolabeled PrP did decrease by ~50% (lanes 1 and 2), demonstrating the turnover of PrP\(^{\text{Sc}}\). Under these conditions, a concomitant reduction in PrP\(^{\text{Sc}}\) synthesis was observed (lanes 7-9) as compared to controls (lanes 10-12). Since this 50% reduction was highly reproducible, these findings suggest that PrP destined to become PrP\(^{\text{Sc}}\) can be degraded if the acquisition of protease resistance is prevented.

To determine whether 18 °C slows the endocytosis and vesicular compartmentalization of membrane glycoproteins (38, 39) in our ScN'a cells, we incubated cells with FITC-labeled WGA, which binds to glycoproteins on the plasma membrane. When FITC-WGA was incubated with cells at 37 °C, well-defined vesicles of varying sizes appeared in the cytoplasm (Fig. 6, panel 1). In contrast, cells incubated with FITC-WGA at 18 °C displayed a diffuse staining in the cytoplasm as well as staining on the cell surface (panel 2). Control cells that were held at 4 °C with FITC-WGA were diffusely stained over the entire cell surface with no apparent internalization (panel 3). These results indicate that the endocytosis and transport of membrane glycoproteins is slowed at 18 °C, but not completely inhibited.

**PrP Transport to the Cell Surface Occurs Prior to Acquisition of Protease Resistance**—To determine whether PrP is transported to the cell surface before it is converted to PrP\(^{\text{Sc}}\) and acquires protease resistance, we took advantage of earlier observations which showed: 1) nascent PrP could be released from ScN'a cells by PIPLC (13), and 2) the formation of PrP\(^{\text{Sc}}\) is slowed at 18 °C (see Fig. 3). ScN'a cells were labeled at 37 °C and chased for 20 h at 18 °C in the presence of PIPLC, and then shifted to 37 °C for 10 h. Under these conditions almost no PrP\(^{\text{Sc}}\) was produced (Fig. 7A, lanes 6 and 8) in contrast to experiments without PIPLC (Fig. 5B). As shown, the 18 °C block of PrP\(^{\text{Sc}}\) production was reversible in the cultures not exposed to PIPLC (Fig. 7A, lanes 5 and 7). PIPLC was active at 18 °C since ~80% of the radiolabeled nascent PrP could be released into the medium (Fig. 7B, lanes 6-8; Fig. 7C). ScN'a cells that were preincubated at 18 °C for 20 h with PIPLC then labeled at 37 °C and chased at 37 °C produced as much PrP\(^{\text{Sc}}\) as control cells that were not preincubated with the enzyme (Fig. 7D). To determine if PrP remains accessible to PIPLC throughout a long incubation at 18 °C, ScN'a cells were labeled at 37 °C, then chased with or without PIPLC for 16 h at 18 °C, followed by a second chase for 5 h at 18 °C with or without PIPLC as noted (Fig. 8). We observed that 90% of the PrP\(^{\text{Sc}}\) in cells held for 16 h at 18 °C could still be released by PIPLC during the second 6-h chase (Fig. 8B, lane 3). We conclude that PrP\(^{\text{Sc}}\) remains in a PIPLC accessible compartment at 18 °C and that incubation of ScN'a cells with PIPLC at 18 °C (Fig. 7) prevents PrP\(^{\text{Sc}}\) synthesis. These results argue that the precursor of PrP\(^{\text{Sc}}\) is accessible to PIPLC digestion on the cell surface prior to the acquisition of protease resistance.

If similar experiments with PIPLC were performed at 37 °C, we found that the precursor of PrP\(^{\text{Sc}}\) was less accessible to PIPLC (Fig. 9A). Confluent cultures of ScN'a cells were radiolabeled with \[^{[\text{35}]}\text{S}\]methionine for 30 min at 37 °C in the presence of PIPLC, then chased for 1 h at 37 °C with PIPLC before an additional chase of 16 h at 37 °C. ScN'a cells that were labeled and chased in this manner produced substantial
amounts of PrPiso (lane 8) as compared to controls (lanes 4 and 9). No PrPiso was produced during the first 1.5 h of labeling and chase without or with PIPLC (lanes 3 and 7) as expected. Controls showed that radiolabeled nascent PrP could be released by PIPLC digestion into the culture medium at 37 °C (lane 6) while the medium from control cells that were not exposed to PIPLC contained no PrP (lane 2). These results indicate that the PrP which is destined to become PrPiso is largely insensitive to PIPLC digestion at 37 °C, suggesting that it may be rapidly endocytosed and protected from PIPLC-catalyzed hydrolysis. Our results contrast with those of other investigators who report that ScnNa cells incubated with PIPLC at 37 °C blocks the formation of PrPiso (24).

To determine whether all species of mature PrPiso possess a GPI anchor modification (Fig. 9B), radiolabeled PrPiso molecules were treated with aqueous HF, which hydrolyzes the phosphodiester bonds within GPI anchors leaving glycosidic bonds intact (46). Aqueous HF treatment increased the migration of all three species of PrPiso in SDS-PAGE compared to controls (Fig. 9B, lanes 1 and 2). This shift in SDS-PAGE migration indicates a loss of mass and suggests that all three PrPiso species possess GPI anchors. Unglycosylated PrPiso that was synthesized in cells in the presence of tunicamycin (16) showed a similar shift in SDS-PAGE migration after treatment with aqueous HF (lanes 3 and 4).

PrP Is Endocytosed Before Acquiring Protease Resistance—
Our observation that the PrP destined to become PrPiso is less accessible to PIPLC at 37 °C than at 18 °C suggests that PrP may be endocytosed at 37 °C before acquiring protease resistance. Consistent with this postulate, nonspecific endocytosis in our ScnNa cells was found to be slower at 18 °C (Fig. 6), and the formation of PrPiso was inhibited (Figs. 3–5). To provide additional evidence that PrP is transported to the cell surface and then endocytosed before acquiring protease resistance, we exposed labeled ScnNa cells to proteases at various intervals after the initial labeling period. While others have recently demonstrated that trypsin digestion of nascent PrPiso reduces PrPiso synthesis (24), we found that trypsin digestion did not readily digest PrPiso on the surface of living cells. In addition, we could not determine whether PrPiso synthesis was

* D. Borchelt and A. Taraboulos, unpublished observations.
with or without PIPLC

8, then identified by immunopurification. The cells were washed

shifted to 37 °C for 10 h at 35 °C. Radiolabeled PrP or PrPṣ molecules were identified

lane 1, without PIPLC. Lane 2, without PIPLC. Panel B, medium from the second

extract from cells chased for 20 h at 18 °C. Lane 2, with PIPLC. Lane 3, with PIPLC. Panel C, extracts of cells. Lane C, control cells that were not chased. Lane 1, chased without PIPLC. Lane 2, chased with PIPLC in the first chase only. Lane 3, chased with PIPLC in the second chase only. The sharp bands seen at 34 kDa in lanes 2 and 3 are not PrP specific and appear to bind immunoglobulin.

inhibited as a result of trypsin digestion of cell surface protein or as a result of nonspecific effects such as dislodging and reattachment of cells. In contrast, digestase digested PrP molecules on the cell surface, and it did not dislodge the ScNα cells from the surface of cell culture flasks. By using dispase which allowed the ScNα cells to remain attached to the surface of the cell culture flasks, we avoided the potential problems attendant with cell attachment, which might influence the synthesis of PrPṣ. A further advantage of dispase is that it is easily inhibited by EDTA.

When ScNα cells were labeled at 37 °C and chased for 30 min in the presence of 10 mg/ml disperse at 37 °C, PrP was degraded (Fig. 10, lane 5). Cells exposed to disperse under these conditions did not synthesize PrPṣ after removal of the

20 h at 37 °C. Lane 3, culture medium of cells from lane 2. Lane 4, extract of cells chased for 20 h at 18 °C in medium with PIPLC. Lane 5, extract of cells chased for 20 h at 18 °C in medium with PIPLC. Lane 6, culture medium from cells in lane 4. Lane 6, extract of cells chased for 20 h at 18 °C in medium with PIPLC. Lane 7, culture medium from cells in lane 6. Gel was autoradiographed for 5 days. Panel D, control experiment demonstrating that incubation of cells at 18 °C in the presence of PIPLC does not prevent PrPṣ synthesis in ScNα cells. Cells were preincubated at 18 °C for 20 h with PIPLC (lanes 4–6) and then labeled as described in Fig. 2. Lanes 1 and 4, extracts of cells not chased. Lanes 2 and 5, extracts of cells not chased but digested with protease K. Lanes 3 and 6, cells chased at 37 °C for 10 h followed by protease K digestion.

FIG. 7. The precursor of PrPṣ is transported to the cell surface before acquiring protease resistance. Confluent cultures of ScNα cells were metabolically radiolabeled for 30 min. After the labeling, cells were washed with PBS then immersed in Opti-MEM with or without PIPLC (200 milliliters/ml) as noted below, then chased at 18 °C for 20 h. The 18 °C-chase medium was harvested and soluble proteins were precipitated with at least 4 volumes of methanol (−20 °C) after the media was adjusted to 1×TNE from a 10×stock (see "Materials and Methods"). PrP molecules in the medium were then identified by immunopurification. The cells were washed with PBS then immersed in DMEM base medium with HEPES, and chased for 10 h at 37 °C. Radiolabeled PrP or PrPṣ molecules were identified as described in Fig. 2. Panel A, cell extracts were digested with proteinase K to identify radiolabeled PrPṣ. Lane 1, extract from cells not chased. Lane 2, extract from cells chased for 20 h at 18 °C. Lane 3, extract from cells chased for 20 h at 18 °C with PIPLC. Lane 4, extract from cells chased for 10 h at 37 °C. Lane 5, extract from cells that were chased for 20 h at 18 °C then shifted to 37 °C for 10 h. Lane 6, extract from cells chased for 20 h at 18 °C with PIPLC and then shifted to 37 °C for 10 h. Lane 7, repeat of experiment in lane 5. Lane 8, repeat of experiment in lane 6. Panel B, all lanes are PrP immunopurified from cell culture medium of cells shown in panel A, lanes 3−8. Lanes 5 and 7, culture medium from cells after 20 h at 18 °C without PIPLC. Lanes 6 and 8, culture medium from cells after 20 h at 18 °C with PIPLC. Gel was autoradiographed for 5 days. Panel C, lane 1, extract of cells not chased. Lane 2, extract of cells chased for

FIG. 8. PrPṣ remains accessible to PIPLC at 18 °C. To determine whether PrPṣ remains on the cell surface during a long (16 h) chase at 18 °C, we metabolically radiolabeled ScNα cells for 30 min at 37 °C as described in Fig. 2, then chased cells at 18 °C in Opti-MEM with or without PIPLC as noted. After the first chase, the medium was harvested, and cells were chased a second time at 18 °C for 5 h with or without PIPLC as noted. PrP molecules from the medium and cell extracts were immunopurified with R053 in DLPC as described under "Materials and Methods." Panel A, medium from the first chase at 18 °C for 16 h. Lane 1, without PIPLC. Lane 2, with PIPLC. Lane 3, without PIPLC. Panel B, medium from the second chase at 18 °C for 5 h. Lane 1, without PIPLC. Lane 2, without PIPLC. Lane 3, with PIPLC. Panel C, extracts of cells. Lane C, control cells that were not chased. Lane 1, chased without PIPLC. Lane 2, chased with PIPLC in the first chase only. Lane 3, chased with PIPLC in the second chase only. The sharp bands seen at 34 kDa in lanes 2 and 3 are not PrP specific and appear to bind immunoglobulin.
The labeling medium was harvested and prepared for immunopurification as described in Fig. 7 before the cells were washed in PBS then chased for 1 h at 37 °C in Opti-MEM with PIPLC (200 milliunits/ml). The chase medium was harvested, prepared for immunopurification, and combined with the labeling medium. Some cultures were washed with PBS after the first 1-h chase then chased for an additional 16 h at 37 °C. Radiolabeled PrP and PrP-sc molecules were identified as described in Fig. 2. Lanes 3 and 4 and 7–9, which contain radiolabeled nascent PrP, were autoradiographed for 2 days. Lanes 3 and 4 and 7–9, which contain radiolabeled PrP-sc, were autoradiographed for 10 days. Lane 1, extract of cells chased for 1 h. Lane 2, culture medium from cells in lane 1. Lane 5, extract of cells chased for 1 h followed by proteinase K digestion. Lane 4, extract of cells chased for 16 h followed by proteinase K digestion. Lane 5, extract of cells labeled for 50 min and chased for 1 h with PIPLC present. Lane 6, culture medium from cells in lane 5. Lane 7, extract of cells treated as in lane 5 with proteinase K. Lane 8, extract of cells labeled for 30 min and chased for 1 h with PIPLC present followed by an additional chase for 16 h; extracts digested with proteinase K. Lane 9 is a repeat of lane 4. Panel B, all species of PrP-sc that are produced by ScN2a cells possess a GPI anchor modification. Confluent cultures of ScN2a cells were preincubated in methionine-free medium in the absence or presence of 30 μg/ml of tunicamycin with 300 μCi/ml [35S]methionine for an additional hour. The cells were then chased in unlabeled medium overnight, detergent extracted, digested with proteinase K, and precipitated with 10 volumes of methanol. After centrifugation (3,000 × g for 25 min), the pellets were resuspended in DLPC buffer (no sonication) and centrifuged at 60,000 × g for 1 h. The pellet, which was highly enriched for PrP-sc, was denatured in Gdn-SCN and reprecipitated in methanol as described under “Materials and Methods.” This protocol partially purifies the PrP-sc molecules (not shown) and is required to reduce the formation of insoluble aggregates after treatment in aqueous HF. The partially purified PrP-sc was resuspended in aqueous HF (48% v/v) and divided into 2 aliquots. One aliquot served as a control and was immediately diluted with 4 volumes of H2O and desiccated in a Savant speed vacuum until dry. The other aliquot was incubated on ice for at least 36 h but not more than 48 h, then diluted, and dried. PrP-sc molecules were then immunopurified in DLPC as described under “Materials and Methods.” Lane 1, control extract. Lane 2, extract incubated in aqueous HF for 36 h. Lane 3, extract of cells labeled in the presence of tunicamycin. Lane 4, same as lane 3 after treatment with aqueous HF for 36 h.

**Fig. 9. PrP-sc and its precursor PrP molecules possess GPI anchors.** Panel A, the precursor of PrP-sc rapidly becomes inaccessible to PIPLC at 37 °C. ScN2a cells were metabolically radiolabeled for 30 min at 37 °C in the presence of PIPLC (200 milliunits/ml). The labeling medium was harvested and prepared for immunopurification as described in Fig. 7 before the cells were washed in PBS then chased for 1 h at 37 °C in Opti-MEM with PIPLC (200 milliunits/ml). The chase medium was harvested, prepared for immunopurification, and combined with the labeling medium. Some cultures were washed with PBS after the first 1-h chase then chased for an additional 16 h at 37 °C. Radiolabeled PrP and PrP-sc molecules were identified as described in Fig. 2. Lanes 1, 2, 3, 5, and 6, which contain total radiolabeled nascent PrP, were autoradiographed for 2 days. Lanes 5 and 4 and 7–9, which contain radiolabeled PrP-sc, were autoradiographed for 10 days. Lane 1, extract of cells chased for 1 h. Lane 2, culture medium from cells in lane 1. Lane 3, extract of cells chased for 1 h followed by proteinase K digestion. Lane 4, extract of cells chased for 16 h followed by proteinase K digestion. Lane 5, extract of cells labeled for 50 min and chased for 1 h with PIPLC present. Lane 6, culture medium from cells in lane 5. Lane 7, extract of cells treated as in lane 5 with proteinase K. Lane 8, extract of cells labeled for 30 min and chased for 1 h with PIPLC present followed by an additional chase for 16 h; extracts digested with proteinase K. Lane 9 is a repeat of lane 4. Panel B, all species of PrP-sc that are produced by ScN2a cells possess a GPI anchor modification. Confluent cultures of ScN2a cells were preincubated in methionine-free medium in the absence or presence of 30 μg/ml of tunicamycin with 300 μCi/ml [35S]methionine for an additional hour. The cells were then chased in unlabeled medium overnight, detergent extracted, digested with proteinase K, and precipitated with 10 volumes of methanol. After centrifugation (3,000 × g for 25 min), the pellets were resuspended in DLPC buffer (no sonication) and centrifuged at 60,000 × g for 1 h. The pellet, which was highly enriched for PrP-sc, was denatured in Gdn-SCN and reprecipitated in methanol as described under “Materials and Methods.” This protocol partially purifies the PrP-sc molecules (not shown) and is required to reduce the formation of insoluble aggregates after treatment in aqueous HF. The partially purified PrP-sc was resuspended in aqueous HF (48% v/v) and divided into 2 aliquots. One aliquot served as a control and was immediately diluted with 4 volumes of H2O and desiccated in a Savant speed vacuum until dry. The other aliquot was incubated on ice for at least 36 h but not more than 48 h, then diluted, and dried. PrP-sc molecules were then immunopurified in DLPC as described under “Materials and Methods.” Lane 1, control extract. Lane 2, extract incubated in aqueous HF for 36 h. Lane 3, extract of cells labeled in the presence of tunicamycin. Lane 4, same as lane 3 after treatment with aqueous HF for 36 h.

**Fig. 10. The PrP that will become PrP-sc becomes inaccessible to detergent-solubilized proteinase K.** Confluent cultures of ScN2a cells were metabolically radiolabeled for 30 min and prepared for chase as described in Fig. 2. The labeled cells were then immediately exposed to dispose (10 mg/ml in Opti-MEM) for 30 min at 37 °C, or allowed to chase for 2 h at 37 °C before exposure to the protease. After dispose digestion, the cells were washed twice in PBS then harvested immediately, or allowed to chase for 16 h at 37 °C in complete medium. PrP molecules were immunopurified from detergent extracts of cells as described in Fig. 2. The top panel is from a 3-day autoradiograph while the lower panel was exposed for 30 days. Lane 1, extract of cells not chased. Lane 2, extract of cells not chased followed by proteinase K digestion before immunopurification. Lane 3, extract of cells chased for 16 h. Lane 4, extract of cells chased for 16 h followed by proteinase K digestion before immunopurification. Lane 5, extract of cells that were digested with dispose immediately following the labeling period. Lane 6, same as lane 5 except extracts were digested with proteinase K before immunopurification of PrP. Lane 7 is from cells that were digested with dispose immediately following the labeling period then chased for 16 h at 37 °C before harvest and digestion with proteinase K. Lanes 8 and 9 are from cells that were held at 37 °C for 2 h then digested with dispose without and with proteinase K digestion before immunopurification. Lane 10 is from cells that were held at 37 °C for 2 h then digested with dispose then chased for 16 h at 37 °C before harvest and digestion with proteinase K. Lane 11 is from cells that were chased for 16 h then digested with dispose before harvesting and proteinase K digestion.

**Dispase** and a further chase at 37 °C for 16 h in complete medium (lane 7). Cells chased at 37 °C for 2 h (lanes 8–10) prior to digestion with dispose for 30 min did produce PrP-sc which was measured after a further chase at 37 °C for 16 h (lane 10). Of note, PrP was largely degraded after the dispose treatment (lane 8), and little PrP-sc formation was detected at 37 °C (lane 11) and was inactive against newly radiolabeled PrP in lysis buffer containing detergent and EDTA (not shown). These observations suggest that some PrP may be generated from the dispase prior to the acquisition of protease resistance during PrP-sc formation and that PrP may be endocytosed before acquiring protease resistance.

**Synthesis of Unglycosylated PrP-sc**—The extent of glycosylation of membrane proteins serves as a marker for protein transport through the endoplasmic reticulum and Golgi. Both ScHaB and ScN2a cells produce PrP-sc molecules with a molecular mass of 27–30, 25, and 19 kDa. The 19-kDa form of PrP-sc comprises ~25% of the total amount of PrP-sc produced in ScN2a cells and is unglycosylated (16, 47). We found that all glycoforms of mature PrP-sc appear to be derived from molecules that are accessible to PIPLC and dispose, and thus probably reach the cell surface. To investigate the origin of the various PrP-sc glycoforms, we compared the rate of PrP synthesis and glycosylation in infected cell lines (ScN2a and ScHaB) to that in uninfected controls (N2a and HaB) (Fig.
PrP molecules were rapidly synthesized and glycosylated to yield molecules of 30 and 33 kDa, with a broad smear up to 40 kDa in both the infected and uninfected cell lines (unglycosylated full-length PrP has a mass of 26 kDa, see (16, 21, 22)). These results argue that unglycosylated PrP$^{Sc}$ is not produced as a result of altered glycosylation in scrapie-infected cells.

That prion infection does not alter the glycosylation of PrP was also demonstrated using antibody binding of newly radiolabeled PrP and mature protease-digested PrP$^{Sc}$ (Fig. 12). We consistently recovered glycosylated 33-35-kDa species of newly radiolabeled PrP from ScN₂a cells with antisera that were raised against any one of three PrP peptides (P₁, residues 90102; P₂, residues 15-40; P₃, residues 220-233) (29) (Fig. 12A, lanes 1-6), and with three polyclonal PrP antisera denoted R017, R073, and R066 that were raised against the protease-resistant core fragment of Syrian hamster PrP$^{Sc}$ designated PrP 27-30 (lanes 7-12). All of the antisera described above, except P2 antiserum, immunopurified all three species of mature protease-digested PrP$^{Sc}$ (Fig. 12B). This was expected, since the P2 epitope is located at the NH₂ terminus of full-length PrP and is not part of the protease-resistant core of PrP$^{Sc}$ (3, 29, 48, 49). We conclude that the 27-30-, 25-, and 19-kDa species of PrP$^{Sc}$ share similar NH₂ and COOH termini but differ in their degree of N-linked glycosylation, with the 19-kDa species being unglycosylated (16, 21).

In summary, our results show that the predominant species of newly radiolabeled PrP in ScN₂a cells have masses of 33-35 and 30 kDa and appear to be glycosylated, but the mature forms of radiolabeled PrP$^{Sc}$ tend to be less glycosylated. Although PrP$^{Sc}$ could be deglycosylated as it accumulates, we do not see any evidence of this in the pulse-chase experiments shown in Fig. 2. All three species of PrP$^{Sc}$ maintain a relatively constant ratio even after a prolonged chase of 48 h (not shown). That unglycosylated PrP can acquire protease resistance is in accord with earlier studies demonstrating PrP$^{Sc}$ in ScN₂a cells treated with tunicamycin or expressing recombinant PrP with mutated N-linked glycosylation sites (16).

**DISCUSSION**

Radiolabeled PrP$^{Sc}$ molecules do not appear until hours after synthesis of the full-length polypeptide, which is complete within a few minutes (Table I). These results argue that PrP$^{Sc}$ molecules acquire their protease resistance during post-translational processing events. PrP chains transit from the endoplasmic reticulum through the Golgi and are transported to the cell surface before PrP$^{Sc}$ is formed. Those PrP molecules that are destined to become PrP$^{Sc}$ appear transiently on the cell surface and can be released with PIPLC (Fig. 7) (24) or hydrolyzed by dispase (Fig. 10). Consistent with these results are other studies showing that brefeldin A reversibly inhibits PrP$^{Sc}$ synthesis.² Brefeldin A has been found to inhibit the transport of proteins out of the endoplasmic reticulum and Golgi apparatus as well as modifying the endosomal pathway (50-54). Clearly, PrP$^{Sc}$ synthesis requires nascent PrP chains to transit from the endoplasmic reticulum through the Golgi and onto the cell surface.

Most of the studies described here involved immunopurification of radioactive PrP$^{Sc}$ from metabolically labeled ScN₂a cells. Quantitative comparisons of PrP$^{Sc}$ synthesis were made...
between replicate cultures that were separately radiolabeled, extracted, protease digested, and immunopurified. Some differences between samples probably arose from variability in the efficacy of one or more of these procedures. For example, we sometimes observed 2-fold differences in the amount of radiolabeled PrPSc that was recovered from replicate experimental cultures. The variability in PrPSc radiolabeling is illustrated in Fig. 2 where data on the kinetics of PrPSc synthesis in four separate experiments are plotted. In general, 4–5-fold changes in the amount of PrPSc synthesis were convincing and judged as meaningful. We found that the synthesis of PrPSc was dramatically reduced when nascent PrPSc was digested with PIPLC at 18°C, but digestion of nascent PrPSc with PIPLC at 37°C did not reproducibly diminish the synthesis of PrPSc by more than a factor of 2. We also observed that digestion of nascent PrPSc with dispase significantly reduced the synthesis of PrPSc and that this phenomenon could be partially prevented by delaying the exposure of cells to dispase. While trypsin digestion (2 mg/ml for 20 min at 37°C) of nascent PrPSc did on occasion cause a large decrease in PrPSc synthesis (not shown), these conditions were so harsh that we could not determine if the reduction in PrPSc synthesis was due to digestion of cell surface protein or to nonspecific effects of the treatment, such as the dislodging and reattachment of the cells. Dispase proved to be a more gentle protease that did not dislodge cells from culture vessels.

While there seems to be agreement, in general, on the sequence of events that occur during the synthesis of both PrPSc and PrPSc+, we remain concerned about substantial differences between our studies employing PIPLC and protease digestions to block PrPSc synthesis and those reported by Caughey and Raymond (24). These investigators also reported experiments in which they used iodogen-coated coverslips to catalyze the radioiodination of PrPSc on the surface of cells and followed its conversion into PrPSc during a 24-h chase. However, approximately 50% of the total amount of iodinated PrPSc was found immediately after labeling for 5 min indicating that some PrPSc was accessible to the labeling procedures (24). We previously reported that ~10% of steady-state PrPSc in ScN2a cells was labeled with sulfo-NHS-biotin, a reagent thought to label only cell surface proteins (55). Whether PrPSc was labeled with these reagents because they enter cells through free-flow endocytosis, because some PrPSc is exposed to the cell surface, or because these cultures contain a significant number of dead permeable cells, is unknown. The reasons for the discrepancies between the experimental results reported by us here and those of others (24) are unclear. Whether these differences can be attributed to variations in ScN2a subclones remains to be established.

**Is PrPSc Derived from a Specific Precursor Pool?**—Whether PrPSc is synthesized from a subset of PrP molecules or all PrPSc molecules are eligible for conversion remains to be established. Less than 10% of the radiolabeled nascent PrP molecules that have been synthesized by the end of a 1-h metabolic radiolabeling pulse are converted into PrPSc (Fig. 2). At present, we have not been able to identify any differences between those PrP molecules which are destined to become PrPSc and the pool of PrPSc molecules. Both the PrPSc precursor molecules and PrPSc can be released from the surface of cells by PIPLC digestion or hydrolyzed by dispase (Figs. 7–10). Furthermore, both PrPSc and those PrP molecules destined to become PrPSc appear to be susceptible to cellular degradation (Fig. 5).

**Synthesis of PrPSc from Unglycosylated PrPSc—**ScN2a cells produce three species of PrPSc that differ in their degree of N-linked glycosylation (Fig. 12, Ref. 16). In our experiments, we have been unable to uncouple the formation of one particular glycoform of PrPSc from another. These results suggest that all three species of PrPSc traverse the same biosynthetic pathway.

Earlier studies showed that unglycosylated PrP produced in the presence of tunicamycin was converted to unglycosylated PrPSc (16), but unglycosylated PrPSc is not readily detected at the cell surface (22). Recombinant PrP molecules lacking consensus sites for N-linked glycosylation were not detected on the cell surface (56) but could be converted to unglycosylated PrPSc in ScN2a cells (16). Although these results suggest that unglycosylated PrP need not transit to the cell surface before conversion into PrPSc, we cannot eliminate the possibility that a small fraction of PrP was transported to the cell surface in these experiments and was converted to PrPSc upon re-entry into the cell. Alternatively, in the presence of tunicamycin, PrP may be transported directly from the endoplasmic reticulum to Golgi to the endocytic pathway where it may be converted to PrPSc prior to entering the lysosomes (16, 57).

**Where Does PrP Acquire Protease Resistance?**—Since infectious prions are composed largely, if not entirely, of PrPSc molecules (1, 8), it is important to identify the site of PrPSc synthesis and define the molecular events involved in this process. Our observations argue that PrPSc synthesis occurs within the endocytic pathway. Exogenous prions could initiate infection by entry through the endocytic pathway to stimulate the conversion of PrPSc molecules from cell surface to PrPSc.

Multiple lines of evidence suggest that PrP may acquire protease resistance in the endocytic pathway. First, when ScN2a cells were exposed to dispase immediately after the labeling pulse, no PrPSc was formed during the chase at 37°C (Fig. 10). In contrast, when ScN2a cells were chased for 2 h at 37°C prior to dispase exposure, some PrP became inaccessible to the dispase in the media and subsequently acquired resistance to proteinase K (Fig. 10). Second, when ScN2a cells were chased at 18 but not at 37°C, PrP was released by PIPLC digestion and PrPSc synthesis was abolished (Figs. 7–10).

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**TABLE I**

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>Normal cells</th>
<th>ScN2a</th>
<th>ScHaB</th>
</tr>
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<tr>
<td>Synthesis of full length 28-kDa PrP</td>
<td>N2a</td>
<td>HaB</td>
<td>ScN2a</td>
</tr>
<tr>
<td>Acquisition of PrP protease resistance</td>
<td>None</td>
<td>None</td>
<td>3 h</td>
</tr>
<tr>
<td>Degradation of PrPSc</td>
<td>6 h</td>
<td>6 h</td>
<td>&gt;24 h</td>
</tr>
</tbody>
</table>

a Since radiolabeled PrP can be detected in these cells in 5-min pulse radiolabelings, the t1/2 for the synthesis of PrP must be no more than 2.5 min. We cannot be certain that the precursor to PrPSc is synthesized at the same rate since so little of the radiolabeled pool of PrP molecules present after 1 h can eventually acquire protease resistance.

b Ref. 13.

c Refs. 15, 16.

d These values pertain to PrPSc in uninfected cells and PrPSc+ in the infected cell lines. The degradation rate of PrPSc in scrapie-infected cell lines was labeled with these reagents because they enter cells by PIPLC digestion or hydrolyzed by dispase (Figs. 7–10). Furthermore, both PrPSc and those PrP molecules destined to become PrPSc appear to be susceptible to cellular degradation (Fig. 5).

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D. Borchelt, M. McKinley, and A. Taraboulos, unpublished observations.
Scrapie Prion Protein Synthesis

and 9A). In our hands, PIPLC catalyzes the release of PrP∗∗
called caveolae (61, 62). Alternatively, PrP∗∗ could enter the endocytic pathway via free flow endocytosis (63). While each of these requires further resolution, the current studies argue that the conversion of PrP∗ into PrP∗ is likely to occur in the endocytic pathway.

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