Prion Protein Biosynthesis in Scrapie-Infected and Uninfected Neuroblastoma Cells

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Numerous studies have indicated that a modified proteinase K-resistant form of an endogenous brain protein, prion protein (PrP), is associated with scrapie infection in animals. This scrapie-associated PrP modification appears to occur posttranslationally in brain, but its molecular nature is not known. To learn about the normal PrP biosynthesis and whether it is altered by scrapie infection in vitro, we did metabolic labeling experiments with uninfected and scrapie-infected mouse neuroblastoma tissue culture cells. Pulse-chase labeling experiments indicated that, in both cell types, two major PrP precursors of 28 and 33 kilodaltons (kDa) were processed to mature 30- and 35- to 41-kDa forms. Endoglycosidase H, tunicamycin, and phosphatidilase treatments revealed that the 28- and 33-kDa precursors resulted from the addition of high-mannose glycans to a 25-kDa polypeptide containing a phosphatidylinositol moiety and that maturation of the precursors involved the conversion of the high-mannose glycans to hybrid or complex glycans. Treatments of the live cells with trypsin and phosphatidylinositol-specific phospholipase C indicated that the mature PrP species were expressed solely on the cell surface, where they were anchored by covalent linkage to phosphatidylinositol. Once on the cell surface, the major PrP forms had half-lives of 3 to 6 h. No differences in PrP biosynthesis were observed between the scrapie-infected versus uninfected neuroblastoma cells.

The causative agents of scrapie, kuru, Creutzfeldt-Jakob disease, and related transmissible degenerative neuropathies of mammals have not been conclusively identified. These agents appear highly resistant to treatments harmful to nucleic acids (1, 3, 12, 21, 32), and no agent-specific nucleic acids have been identified. Therefore, it was postulated that the infectious agents are devoid of genetic material and composed primarily of protein (1, 15, 30, 32). However, since the agents are also highly resistant to proteases under most conditions (25, 26), it has been difficult to draw accurate conclusions about their composition.

Attempts to purify the scrapie agent from brain led to the discovery of a particular protein, named prion protein (PrP) (5, 26, 33) or scrapie-associated fibril protein (13), as the major protein component of fractions containing scrapie infectivity. It was proposed that this protein, or an aggregate thereof, is the scrapie infectious agent (5, 13, 27). However, recent studies have shown that PrP is a normal endogenous protein of brain and a variety of other tissues (8, 10, 11, 18, 28, 29, 36). Scrapie infection does not appear to modify the endogenous PrP gene of brain (2) or the size and quantity of PrP mRNA (10, 29). Furthermore, expression of the PrP gene cloned from scrapie-infected mouse brain was not sufficient to cause the synthesis of the scrapie agent in mouse tissue culture cells (9). However, the disease results in the accumulation of a proteinase K-resistant form of PrP in brain which can aggregate into fibrils (11, 13, 18, 26, 28, 36). The chemical basis for the resistance to proteinase K is not known. Since no alteration of the mRNA encoding PrP has been detected, it would appear that the acquisition of proteinase K resistance is posttranslational. However, although several posttranslational modifications of PrP in hamster brain are known to occur (6, 16, 24, 37), none have been found to be scrapie specific. It is possible that proteinase K resistance does not require covalent modification but instead requires aggregation, conformational change, or association of the endogenous PrP with other components of scrapie-infected tissues. Whether a proteinase K-resistant form of PrP is the infectious scrapie agent itself, a component of the agent, or merely a secondary by-product of the disease in vivo remains to be determined.

To provide a system in which PrP and the scrapie agent can be studied in the absence of in vivo tissue pathology, we recently developed scrapie-infected mouse neuroblastoma cell clones with a high percentage of infected cells (34). In preliminary studies, we found that the mature PrP detected in the infected cells was neither resistant to proteinase K nor altered in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mobility by the presence of the scrapie agent. These results suggested that the scrapie agent is replicated in vitro in the absence of proteinase K-resistant PrP. However, it is also possible that scrapie infection is directly associated with biochemical alterations in normal PrP structure which might not result in resistance to proteinase K under tissue culture conditions. To learn more about PrP biosynthesis and whether scrapie infection alters the process in vitro, we extended our studies of these neuroblastoma cells. Here we identified the biosynthetic precursors of PrP in both scrapie-infected and uninfected neuroblastoma cells and monitored their maturation and transport to the cell surface.

MATERIALS AND METHODS

Neuroblastoma cells. The scrapie-infected (sc+) neuroblastoma cell clone 29-161, the scrapie-negative (sc−) clone 29-321, and the normal uncloned neuroblastoma cells used in

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this study have been described previously (34, 35). Clones 29-161 and 29-321 were derived from cultures infected with the Chandler mouse-adapted scrapie agent. By frequency analysis, it was found that approximately 88% of the cells in clone 29-161 cultures harbor scrapie infectivity.

Metabolic labeling, immunoprecipitation, and electrophoresis. For all studies, 10⁶ neuroblastoma cells were seeded in 25-cm² tissue culture flasks in minimal essential medium with 10% fetal bovine serum. The medium was changed after 2 days, and on day 3, the cells were used. Prior to labeling, the cells were preincubated for 30 min in methionine-free minimal essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 1% dialyzed fetal bovine serum. Cellular PrP was metabolically labeled with 100 to 200 μCi of L-[³⁵S]methionine (Dupont, NEN Research Products, Boston, Mass.) per ml, immunoprecipitated from detergent lysates of cells with an anti-PrP peptide antiserum, and analyzed by SDS-PAGE fluorography as described previously (34), except that the metabolic labeling time was varied. In all experiments, one-half 25-cm² flask equivalent of the immunoprecipitate was added to a single lane of a gel. In pulse-chase studies, the cells were incubated for the designated time between the labeling and lysis steps in complete minimum essential medium supplemented with 10% fetal bovine serum.

Tunicamycin treatment. Neuroblastoma cells were preincubated for 1 h in 25-cm² flasks each containing 4 ml of methionine-free minimal essential medium containing 10 μg of tunicamycin (T-7765; Sigma Chemical Co., St. Louis, Mo.) per ml. The tunicamycin was first dissolved in dimethyl sulfoxide at 10 mg/ml, and this stock was diluted 1:1,000 into the medium. After preincubation, half the medium was removed and 200 μCi of L-[³⁵S]methionine was added to the remainder for the labeling step. Preliminary experiments showed that tunicamycin concentrations of 0.1 and 1 μg/ml had no effect on the glycosylation of PrP and that 50 μg/ml inhibited protein synthesis generally (data not shown).

Endoglycosidase H treatments of PrP immunoprecipitates. Protein A-Sepharose-bound ³⁵S-labeled PrP immunoprecipitates representing one 25-cm² flask equivalent of neuroblastoma cells were washed with water and eluted by boiling for 5 min in 60 μl of 0.1% SDS–1% 2-mercaptoethanol–50 mM sodium phosphate (pH 6.1). The eluates were transferred to new tubes, and phenylmethylsulfonyl fluoride (0.1 M in isopropanol), pepstatin (1 mM in methanol), and 1,10-phenanthroline (0.1 M in methanol) were added to give final concentrations of 0.1 mM, 10 μM, and 1 mM, respectively. The eluates were then divided into two tubes, one of which received endoglycosidase H (Miles Laboratories, Inc., Elkhart, Ind.) at 0.3 IU/ml, and incubated for 12 h at 37°C. The digestion was stopped by cooling the tubes on ice and adding trichloroacetic acid to 10% to precipitate proteins. After 1 h on ice, the precipitated proteins were pelleted by centrifugation and washed once with ice-cold 10% trichloroacetic acid and once with diethyl ether before the pellet was redissolved by boiling in SDS-PAGE sample buffer.

Trypsin treatment of live cells. Metabolically labeled neuroblastoma cells were washed with phosphate-buffered balanced salt solution and dislodged by 5 min of incubation in either 0.5 mg of trypsin (1:250; Difco Laboratories, Detroit, Mich.) per ml in 136 mM NaCl–5 mM KCl–5 mM glucose–7 mM NaHCO₃–0.6 mM EDTA or 2 mM EDTA in phosphate-buffered saline. To neutralize the effect of trypsin, we added fetal bovine serum to 20%. The cells were pelleted at 4°C, washed in ice-cold phosphate-buffered balanced salt solution, and lysed in lysis buffer (34) supplemented with 1 mg of soybean trypsin inhibitor per ml and 0.1 mM phenylmethanesulfonyl fluoride.

Phospholipase treatments of live cells. Labeled neuroblastoma cells were incubated in 1 ml of serum-free minimal essential medium with or without phosphatidylinositol-specific phospholipase C (PIPLC) from Bacillus thuringiensis (the generous gift of Martin Low of the Columbia University College of Physicians and Surgeons) at an activity of 2 μmol/min per ml with [³H]phosphatidylinositol as the substrate. Following incubation for 30 min at 37°C, the medium was removed and centrifuged at 11,000 × g for 5 min. Concentrated lysing buffer was added to the supernatant to give a final 1× concentration. The cells were washed and lysed as usual. PrP was immunoprecipitated from both the medium supernatants and the cell lysates. Higher activities of PIPLC did not increase the amount of PrP released from the cells (data not shown).

Phospholipase treatments of immunoprecipitates. PrP immunoprecipitates were treated with PIPLC by first washing one 25-cm² flask equivalent of PrP bound to protein A-Sepharose beads with water and then eluting the PrP by boiling it for 5 min in 30 μl of 0.1% SDS–50 mM Tris hydrochloride (pH 7.1) (37°C). The eluates were transferred into new tubes, and concentrated solutions of phenylmethylsulfonyl fluoride, pepstatin, and Nonidet P-40 were added to give final concentrations of 0.1 mM, 10 μM, and 1%, respectively. The eluates were divided into two parts, one of which received PIPLC at an activity of 33 μmol/min per ml. The tubes were incubated for 40 min at 37°C and prepared for SDS-PAGE by adding an equal volume of 2× SDS-PAGE sample buffer and boiling for 2 min.

RESULTS

Pulse-chase labeling of PrP. In a previous study, we showed that metabolically labeled PrP from sc+ and sc− neuroblastoma clones and normal uncloned neuroblastoma cells was composed of two major bands of apparent molecular sizes of 30 and 33 to 41 kilodaltons (kDa) on SDS-PAGE (34). To identify biosynthetic precursors of the mature PrP bands, we performed short pulse and pulse-chase ³⁵S]methionine labeling studies on the sc+ and sc− clones. For both clones, the earliest biosynthetic forms of PrP were bands at 25, 28, and 33 kDa, detected with labeling periods of 2 to 10 min. In a 15-min labeling period, the bands at 30 and 33 to 41 kDa began to appear (Fig. 1). Following a 15-min chase with medium containing unlabeled methionine, the 28- and 33-kDa bands disappeared and the 30- and 33- to 41-kDa bands became dominant. At this time, it appeared that the processing of all the molecules in the upper band of pulse-labeled PrP was not yet complete because, with the 30-min chase, the lower limit of the upper band shifted to a slightly higher apparent molecular size to become 35 to 41 kDa. No further change was observed with an additional 30 min of chase. Experiments with longer chase periods indicated that, once fully processed, the labeled 30- and 35- to 41-kDa PrPs had half-lives of approximately 3 to 6 h. The same sequence of events in PrP biosynthesis was observed in pulse-chase studies of the normal, uncloned neuroblastoma cells, except that an additional band at 26 kDa was present throughout the pulse-chase (data not shown). Since this additional band was not observed in clone 29-321, we concluded that its absence was not a scrapie-specific phenomenon and that it could have been due to the presence of multiple cell types in the uncloned neuroblastoma culture.

Tunicamycin treatment. To determine whether the multiple forms of PrP observed in the pulse-chase experiments...
were glycosylated and, if so, to identify the unglycosylated precursor of PrP biosynthesis, we labeled neuroblastoma cells in the presence of tunicamycin, a drug which prevents lipid carrier-dependent N-glycosylation of proteins (14). In the sc- and sc+ clones, tunicamycin reduced the synthesis of 30- and 35- to 41-kDa PrPs and greatly increased the label in the 25-kDa band, which was a minor component of PrP in control cells (Fig. 2). Tunicamycin did not influence the labeling of the nonglycosylated background proteins, indicating that the observed effects were due to the specific inhibition of glycosylation and not protein synthesis generally. Thus, the 25-kDa band was likely the PrP polypeptide lacking N-linked carbohydrate, though it remains possible that O-linked carbohydrate was attached. When normal uncloned neuroblastoma cells were treated identically with tunicamycin labeling was increased in both the 25- and 26-kDa PrP bands observed in the pulse-chase study described above.

Endoglycosidase treatments. To help identify the multiple glycosylated forms of PrP observed in the pulse-chase studies, we treated denatured PrP immunoprecipitates with endoglycosidase H (Fig. 3), which removes high-mannose glycans from glycoproteins (38). Endoglycosidase H treatment of the PrP precursors of both the sc- and sc+ clones labeled for 7 min resulted in the conversion of 28- and 33-kDa PrP bands to a band of ~26 kDa. This provided evidence that the larger two bands were the PrP polypeptide with differing amounts of high-mannose glycan attached. Upon further processing of PrP, it was apparent that the high-mannose glycans were replaced with complex glycans

since the mature forms labeled in the 2-h period were not altered by endoglycosidase H. Since there are two potential N-glycosylation sites in the mouse PrP sequence (22), the difference between the 28- and 33-kDa precursors could have been the glycosylation of one versus both sites, respectively. The presence of a residual N-acetylglucosamine residue normally left by the enzyme at one or both of the potential glycosylation sites may account for the deglycosylated PrP appearing slightly larger than 25 kDa. No differences between the sc- and sc+ clones were observed.

Transport of PrP to the cell surface. In a previous study, we found that PrP detected on the cell surface by immunofluorescence could be removed with trypsin and thus that PrP was an exofacial plasma membrane protein (9). To determine which of the forms of PrP were transported to the cell surface, sc- neuroblastoma cells labeled for 15 min or 3 h were treated with trypsin before lysis (Fig. 4). The PrP precursors labeled in the short pulse were protected from extracellular trypsin, which was consistent with their being associated with the endoplasmic reticulum or Golgi apparatus or both. On the other hand, the 30- and 35- to 41-kDa PrP bands labeled in 3 h were all digested and thus were expressed on the cell surface as final products of PrP biosynthesis. Tryptsin treatments of sc- cells pulse-chase labeled as described in the legend to Fig. 2 indicated that the bulk of 30- and 35- to 41-kDa PrP first became susceptible to trypsin after a 60-min chase; however, the 25-kDa PrP was still mostly resistant to trypsin at this time point (data not shown). Thus, although the 25-kDa form eventually appeared on the cell surface (see below), it was transported more slowly than the glycosylated forms.

Release of PrP from cell surface with phospholipase. Hamster brain PrP has been shown to have a covalently bound glycolipid containing phosphatidylinositol which can serve...
to anchor the protein in membranes (37). Other studies have indicated that hamster PrP can exist as an integral membrane protein which spans the phospholipid bilayer twice (16, 17). To distinguish between these two possibilities for PrP associated with the surface of the mouse neuroblastoma cells, we treated labeled cells with PIPLC, an enzyme that has been shown to specifically release proteins with phosphatidylinositol anchors from the cell surface (19). Treatment with PIPLC resulted in the complete loss of the 25-, 30-, and 35- to 41-kDa PrP bands from the sc + neuroblastoma cell lysates (Fig. 5). Concurrently, these PrP bands appeared in the culture medium. Thus, each of these PrP forms was anchored to the plasma membrane of these cells by phosphatidylinositol. The electrophoretic mobility of the PrP bands was decreased slightly by the loss of the diacylglycerol moiety, and this may be attributed to a reduction in the number of SDS molecules bound to the protein (37). Similar results were obtained with the sc − clone and normal uncloned neuroblastoma cells.

**Addition of phosphatidylinositol to PrP precursors.** Studies of the biosynthesis of other proteins containing glycosylphosphatidylinositol anchors have indicated that the anchor is added cotranslationally or soon after translocation of the polypeptide into the endoplasmic reticulum (23). To determine whether the same is true of PrP biosynthesis, we treated the early PrP precursors with PIPLC after immunoprecipitation (Fig. 6). As was the case for the mature forms of PrP on the cell surface, the electrophoretic mobilities of the 25-, 28-, and 33-kDa precursors decreased with PIPLC.
treatment. This indicated that early PrP precursors contained phosphatidylinositol and, therefore, that the addition of the phospholipid anchor occurred before or concurrent with high-mannose glycan addition.

**Release of PrP from cells.** Previous studies have indicated that PrP may be targeted both as a plasma membrane protein and as a secretory protein (9, 17). To test whether a proportion of pulse-labeled PrP was released from normal neuroblastoma cells and the sc− and sc+ clones, we immunoprecipitated PrP from the cell lysate and the chase medium. By scanning densitometry of fluorograms, it was estimated that a maximum of 3 to 10% of the labeled 35- to 41-kDa PrP was lost from the cells and was detected in the medium following chases of 4 and 16 h, the latter being long enough to effectively eliminate labeled PrP from the cells (data not shown). This suggested that catabolism, not secretion or release from the cell surface, was the major fate of PrP lost from these cells.

**DISCUSSION**

**PrP biosynthesis in neuroblastoma cells.** Based on the present knowledge of PrP and plasma membrane proteins in general, we can provide the following synopsis of PrP biosynthesis (Fig. 7). Since mouse PrP has a hydrophobic amino-terminal signal sequence (22) and is targeted as a plasma membrane protein, its biosynthetic processing presumably begins within the endoplasmic reticulum. The earliest PrP precursors detected in the sc− and sc+ neuroblastoma clones are the 25-kDa PrP polypeptide with phosphatidylinositol and its 28- and 33-kDa derivatives carrying differing amounts of high-mannose glycan. The simultaneous labeling of these three forms of PrP is consistent with studies of other proteins showing that the addition of glycosyl-phosphatidylinositol (23) and high-mannose glycans (20) can occur concurrently or soon after translation and translocation of polypeptides into the lumen of the endoplasmic reticulum. Since the 25-kDa PrP appears smaller in molecular size than the theoretical value of 27.8 kDa calculated for the full-length PrP polypeptide (22), the nascent PrP polypeptide may be rapidly truncated in the endoplasmic reticulum. This truncation may result from the removal of an amino-terminal signal peptide by signal peptidase, as has been shown to occur with hamster PrP in brain (4, 18). Also, a carboxy-terminal peptide sequence may be removed prior to the addition of phosphatidylinositol, as described for other proteins containing glycosyl-phosphatidylinositol groups (23).

Within the next 10 to 30 min, the high-mannose glycans on the 28- and 33-kDa PrPs are processed to yield complex or hybrid glycans, presumably during passage through the Golgi apparatus (31). At this point, two major forms of PrP migrating at 30 and 35 to 41 kDa can be detected which are then shuttled to the plasma membrane along with a minor amount of the 25-kDa form. All the PrP species in the plasma...
membrane are anchored in an exofacial orientation by phosphatidylinositol. Once in the plasma membrane, 35- to 41-kDa PrP has a half-life of approximately 3 to 6 h, and only a minor proportion is released from the cells into the medium.

Since the 25-kDa PrP arrives at the cell surface, albeit at a slower rate than 30- and 35- to 41-kDa PrPs, N-linked glycosylation must be essential for the proper trafficking through the intracellular biosynthetic apparatus. This is also the case for several other plasma membrane and secreted proteins (14). The reason for the smallest PrP having a slower rate of transport to the plasma membrane is unknown. However, studies of the constitutive transport of other proteins to the plasma membrane have indicated that the rate-limiting step is export from the endoplasmic reticulum (14, 31). Furthermore, an important prerequisite for this step appears to be the proper folding of nascent polypeptide chains and/or their assembly into oligomers (31). Thus, the addition of N-linked high-mannose sugars to the nascent PrP polypeptide may facilitate the preparation of PrP for export from the endoplasmic reticulum.

Comparison of scrapie-infected and uninfected neuroblastoma cells. The sequence of events in PrP biosynthesis outlined above occurred in both scrapie-infected and uninfected neuroblastoma cells. Thus, we have no evidence that scrapie infection modifies PrP biosynthesis in these cells. It remains conceivable that subtle scrapie-specific modifications of the oligosaccharide structure or other posttranslational modifications could have occurred without being detected by the techniques used. Another possibility is that scrapie infection results in the modification of a small proportion of the total PrP below the level of detection. This would be consistent with our previous study showing that none of the PrP detected in this cell species clone had the proteinase K resistance typical of most of the PrP extracted from the brains of scrapie-infected animals (34). However, an alternative explanation might be that a modified form of PrP is not a necessary component of the infectious scrapie agent but arises as a pathological by-product of the disease in vivo (7) under conditions which were not duplicated in tissue culture.

Comparison of live-cell biosynthesis to cell-free translation. In studies of the synthesis of hamster PrP in cell-free translation systems containing microsomal membranes, PrP species of 25, 28, and 33 kDa were observed (16). The 28-kDa form of the hamster PrP was interpreted as being the unprocessed full-length PrP polypeptide and the 33- and 25-kDa species as being glycosylated and unglycosylated forms lacking an amino-terminal signal peptide. Despite the apparent correspondence of molecular masses of these hamster PrP species to the early PrP precursors we observed in mouse neuroblastoma cells, there are two ways in which the events in the cell-free translation system and the live cells appear to have differed. First, the 28-kDa mouse PrP precursor we observed in neuroblastoma cells was clearly not the unprocessed full-length mouse PrP polypeptide, but instead was a 25-kDa polypeptide with phosphatidylinositol and high-mannose glycan attached. Second, the 25- and 33-kDa PrP forms of the hamster cell-free translation system were shown to be integral membrane proteins which twice span the microsomal membrane. Whether this is true of the early precursors of PrP biosynthesis in neuroblastoma cells is not yet known. However, the polypeptide backbones of final products of PrP biosynthesis in these cells do not appear to span the plasma membrane bilayer, since the proteins can be released from the membrane solely by the action of PIPLC. This has also been shown to be the case for plasma membrane PrP in primary cultures of neonatal hamster brain cells (37).

Comparison of mouse neuroblastoma and epithelial cells. In our preliminary studies of PrP biosynthesis in mouse epithelial cells (9), several PrP intermediates were observed in the epithelial cells which, according to their SDS-PAGE mobilities, were distinct from those identified here in neuroblastoma cells. When the level of PrP expression was greatly increased in the epithelial cells by transformation with a bovine papillomavirus expression vector containing the mouse brain PrP gene, additional distinct PrP bands were observed. Thus, the posttranslational processing of PrP can vary with cell type and the level of PrP expression within the cells. Since PrP appears to be expressed in numerous other cell types as well (8), it will be of interest to compare the biosynthetic processing of PrP in these cells with their ability to replicate the scrapie agent.

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LITERATURE CITED