Identification of Intermediate Steps in the Conversion of a Mutant Prion Protein to a Scrapie-like Form in Cultured Cells*

(Received for publication, January 14, 1997, and in revised form, February 18, 1997)

Nathalie Daude‡§, Sylvain Lehmann‡¶, and David A. Harris∥

From the Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

The central causative event in infectious, familial, and sporadic forms of prion disease is thought to be a conformational change that converts the cellular isoform of the prion protein (PrP(C)) into the scrapie isoform (PrP(Sc)) that is the primary constituent of infectious prion particles. To provide a model system for analyzing the mechanistic details of this critical transformation, we have previously prepared cultured Chinese hamster ovary cells that stably express mouse PrP molecules carrying mutations homologous to those seen in familial prion diseases of humans. In the present work, we have analyzed the kinetics with which a PrP molecule containing an insertion mutation associated with Creutzfeldt-Jakob disease acquires several biochemical properties characteristic of PrP(Sc). Within 10 min of pulse labeling, the mutant protein undergoes a molecular alteration that is detectable by a change in Triton X-114 phase partitioning and phenyl-Sepharose binding. After 30 min of labeling, a detergent-insoluble and protease-sensitive form of the protein appears. After a chase period of several hours, the protein becomes protease-resistant. Incubation of cells at 18 °C or treatment with brefeldin A inhibits acquisition of detergent insolubility and protease resistance but does not affect Triton X-114 partitioning and phenyl-Sepharose binding. Our results support a model in which conversion of mutant PrPs to a PrP(Sc)-like state proceeds in a stepwise fashion via a series of identifiable biochemical intermediates, with the earliest step occurring during or very soon after synthesis of the polypeptide in the endoplasmic reticulum.

Prion diseases are a group of unusual neurodegenerative disorders that includes Creutzfeldt-Jakob disease (CJD),

* This work was supported by grants (to D. A. H.) from the Alzheimer’s Association (Richard F. Bristor Investigator-initiated Research Grant) and the National Institutes of Health (Grant NS35496), and by a grant (to S. L.) from the Missouri Alzheimer’s Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ The first two authors contributed equally to this work.
§ Recipient of a grant from the Philippe Foundation.
¶ Recipient of a Postdoctoral Fellowship for Physicians from the Howard Hughes Medical Institute.
∥ To whom all correspondence should be addressed: Dept. of Cell Biology and Physiology, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-4690; Fax: 314-362-7463; E-mail: dharris@cellbio.wustl.edu.

The abbreviations used are: CJD, Creutzfeldt-Jakob disease; BFA, brefeldin A; CHO, Chinese hamster ovary; ECL, enhanced chemiluminescence; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; moPrP, mouse prion protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PIPLC, phosphatidylinositol-specific phospholipase C; PrP, prion protein; PrP(C), cellular isoform of the prion protein; PrP(Sc), scrapie isoform of the prion protein.
the prion phenomenon, cellular cofactors may function to enhance or modulate PrPSc formation, as suggested by the inefficiency of existing in vitro conversion systems (12, 13), the well-known role of chaperone molecules in protein folding (22), and by recent transgenic experiments that implicate a hypothetical protein "X" in prion replication (23).

To address these mechanistic issues, it is necessary to analyze prion formation in cultured cells that are amenable to experimental manipulations such as pulse labeling, microscopy, and subcellular fractionation. Scrapie-infected neuroblastoma cells and immortalized hamster brain cells have been invaluable in elucidating some of the cellular and biochemical events underlying the infectious manifestation of prion diseases (18-21, 24, 25). Until recently, however, there has been no cell culture model of familial forms of prion disease. We (26-28) and others (29) have now developed such a model. Our system utilizes transfected lines of Chinese hamster ovary (CHO) cells that stably express mouse PrP (moPrP) molecules carrying mutations homologous to those linked to inherited prion diseases of humans. We find that mutant PrPs synthesized in these cells display many of the biochemical properties of PrPSc, including detergent insolubility, protease resistance, slow metabolic generation and turnover, abnormally tight membrane attachment, and strain-like variations in glycosylation pattern and protease- cleavage site. We have now used this model system to examine the kinetics with which mutant PrP molecules acquire PrPSc-like properties and to draw inferences from this about the nature of the intermediates in the conversion process and the identity of the cellular compartments in which they are generated.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Cell culture reagents were from the Tissue Culture Support Center at Washington University. N-Glycosidase F was purchased from Boehringer Mannheim, sulfo-biotin-DX-NHS was from Calbiochem, brefeldin A (BFA) was from Sigma, phenyl-Sepharose CL-4B was from Pharmacia Biotech Inc., and [35S]-methionine (Pro-mix, 1,000 Ci/mmol) was from Amersham Corp. Phosphatidyllysinositol-specific phospholipase C (PIPLC) from Bacillus thuringiensis was prepared as described previously (30).

Rabbit polyclonal antibody P45-66, raised against a synthetic peptide encompassing moPrP residues 45-66, has been described (26). Rabbit polyclonal antibody ME7 and mouse monoclonal 3F4 antibodies, raised against PrP 27-30 from scrapie-infected mouse and hamster tissues, respectively, have been described (26).

Cell Lines—Stably transfected lines of CHO cells expressing wild-type, PG11, and E199K moPrPs have been described previously (26-28). The wild-type and PG11 constructs contained an epitope tag for the monoclonal antibody 3F4.

Cell Lines—Stably transfected lines of CHO cells expressing wild-type, PG11, and E199K moPrPs have been described previously (26-28).

Assay of Detergent Insolubility—Confluent cultures of CHO cells were harvested, washed in PBS, and resuspended at a concentration of about 2 x 10^6 cells/ml in Opti-MEM (Life Technologies, Inc.). Cells were then lysed in a buffer that contained 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.5% Triton X-100, and 0.5% sodium deoxycholate, supplemented with protease inhibitors (pepsin and leupeptin, 1 µg/ml; phenylmethylsulfonyl fluoride, 0.5 mM; EDTA, 2 mM). Lysates were first centrifuged for 5 min at 16,000 x g in a microcentrifuge, a procedure that removes debris but does not pellet significant amounts of PrP. The cleared lysates were then centrifuged at 265,000 x g for 40 min in the TLA 100.3 rotor of a Beckman Optima TL ultracentrifuge to separate detergent-soluble and detergent-insoluble protein. Immunoprecipitation of moPrP in pellet and supernatant fractions from the second centrifugation was performed using monoclonal antibody 3F4 antibody as described previously (26). In some experiments lysates were treated with N-glycosidase F (0.01 units/ml) for 16 h at 37 °C prior to immunoprecipitation to produce a single band of deglycosylated PrP that could be more easily quantitated (32). Immunoprecipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and radioactive gels were quantitated using a PhosphorImager (Molecular Dynamics).

Assay of Protease Resistance—Cells were metabolically labeled and chased as above. Proteins were methanol-precipitated from lysates, digested with proteinase K at 13.3 µg/ml for 10 min at 37 °C in Sarkosyl, methanol-precipitated a second time, and then dispersed in detergent-lipid-protein complexes for immunoprecipitation of moPrP using anti-ME7 antibody, all as described (19, 27, 28). Immunoprecipitated proteins were analyzed by SDS-PAGE.

For the experiment shown in Fig. 1C, lysates of unlabelled cells were centrifuged to assay detergent insolubility, after which proteins were methanol-precipitated from the pellet and supernatant fractions and subjected to proteinase K digestion as described above. Following digestion, proteins were concentrated by methanol precipitation and analyzed for moPrP by immunoblotting using 3F4 antibody.

Phase Partitioning in Triton X-114—Metabolically labeled cells were solubilized for 20 min at 4 °C in 1% Triton X-114 in phosphate-buffered saline (PBS) containing protease inhibitors; the detergent was diluted from a 12% stock solution that had been precondensed according to Bordier (33). After incubation at 37 °C for 10 min, aqueous and detergent phases were separated by centrifugation. The detergent phase was diluted to the initial volume with PBS, incubated with or without PIPLC for 2 h at 4 °C, and the phase separation repeated. PrP in each phase was immunoprecipitated with antibody 3F4 and separated on SDS-PAGE.

Binding to Phenyl-Sepharose—Metabolically labeled cells were lysed and subjected to an initial phase partitioning in Triton X-114 as described above, except that the final detergent concentration was 0.5%. The detergent phase was diluted to the original volume with PBS, incubated with or without PIPLC for 2 h at 4 °C, and then phenyl-Sepharose CL-4B (40 µl of beads, equilibrated in PBS plus 0.5% Triton X-114, per ml of sample) was added for 30 min at 4 °C. The phenyl-Sepharose beads were then collected by centrifugation and bound protein released by boiling in Triton X-100/deoxycholate lysis buffer containing 0.5% SDS. MoPrP in the bound and unbound fractions was analyzed by immunoprecipitation with 3F4 antibody.

Assay of Phospholipase C Release—For the experiments shown in Fig. 1, A-B, cells were biotinylated with sulfo-biotin-X-NHS as described by Shyng et al. (30) and were then incubated with PIPLC (1 unit/ml in Opti-MEM) at 4 °C for 2 h. MoPrP in incubation media and cell lysates was then assayed for detergent insolubility or phase partitioning in Triton X-114, as described above. Immunoprecipitates collected using antibody P45-66 were separated by SDS-PAGE, and biotinylated moPrP was visualized by developing blots of the gels with horseradish peroxidase-streptavidin and enhanced chemiluminescence (ECL) kit.

Sucrose Gradient Sedimentation—Lysates of metabolically labeled cells were cleared by centrifugation at 16,000 x g for 2 min and were then loaded on a 10-m1 linear gradient of 15-40% sucrose in lysis buffer. After centrifugation at 4 °C for 16 h at 274,000 x g, 1-ml fractions of the gradient as well as the entire pellet were collected, and moPrP was immunoprecipitated with 3F4 antibody.

RESULTS

Operational Properties of Mutant PrPs—We have previously shown that moPrP molecules carrying disease-related mutations display several biochemical properties characteristic of PrPSc when expressed in CHO cells (26-28). These properties include (a) detergent insolubility, (b) protease resistance, (c) aberrant membrane attachment, as manifested by retention of the mutant protein on the cell surface after treatment with the enzyme phosphatidylinositol-specific phospholipase C (PIPLC), and (d) hydrophobicity, as revealed by partial retention of the PIPLC-treated protein in the detergent phase of Triton X-114 lysates. Our objective was to use pulse-chase metabolic labeling to analyze the kinetics with which each of these properties was acquired following synthesis of the protein. In this way, we hoped to define a sequence of steps in the transformation of mutant PrPs to a PrPSc-like state.

An important assumption underlying this strategy is that the same population of molecules eventually acquires all four properties. To test this assumption, we assayed the properties in pairs-wise fashion using biotinylation or Western blotting to visualize the steady-state population of PrP molecules. For

2 N. Daude, S. Lehmann, and D. A. Harris, unpublished data.
these experiments we utilized CHO cell lines expressing two different moPrP mutants whose human homologues are associated with familial CJD (4): PG11, which contains six additional copies of the N-terminal octapeptide repeat which is found in five copies in the wild-type protein, and E199K which contains a single amino acid substitution.

E199K moPrP was particularly useful for experiments involving PIPLC treatment, since ~50% of this protein is releasable by the phospholipase (compared with <10% for the other mutants), allowing us to analyze both released and non-released fractions (27). We have previously shown that the pool of E199K moPrP that is retained on the cell surface after PIPLC treatment is protease-resistant, whereas the released pool is protease-sensitive (27). To test the correlation between PIPLC releasability and detergent insolubility, we treated surface-biotinylated cells with the phospholipase and then subjected the released and cell-associated fractions to centrifugation at 265,000 × g to separate detergent-soluble from detergent-insoluble molecules. Proteins in supernatant (S) and pellet (P) fractions were then either left untreated (− lanes) or were digested with proteinase K (+ lanes), after which moPrP was detected by immunoblotting. Five times as many cell equivalents were loaded in the + lanes as in the − lanes. The bracket beside lane 8 marks the position of PrP 27–30. The band at 30 kDa in lanes 3, 4, and 7 is due to nonspecific reactivity of the antibody with proteinase K; a PrP-specific band at this position in lane 8 is superimposed on the nonspecific band.

We conclude from these experiments that mutant PrP molecules that are protease-resistant, detergent-insoluble, and hydrophobic are also PIPLC non-releasable. In addition, molecules that are protease-resistant are also detergent-insoluble. Although we have not assayed all four properties simultaneously in the same experiment, our pairwise correlations indicate that at steady-state the same population of mutant moPrP molecules possesses the four biochemical properties of PrP\textsuperscript{Sc}

Intermediate Stages of Aggregation of Mutant PrP—The centrifugation conditions we have used to assay detergent insolubility would pellet PrP aggregates having a sedimentation coefficient >40 S. To more accurately characterize the size distribution of mutant PrP, we pulse-labeled cells expressing PG11 moPrP and then fractionated detergent lysates by velocity sedimentation on 15–40% sucrose gradients. Consistent with earlier results showing that acquisition of detergent insolubility is primarily a posttranslational process (28), PG11 moPrP sedimented as a single peak at ~4 S (corresponding to the size of monomeric protein) immediately following pulse labeling for 20 min (Fig. 2, C–D). After a 2-h chase, the majority of the protein was found in two peaks at 4 S and >16.6 S but a substantial amount was also broadly distributed throughout the gradient (fractions 5–10). Wild-type moPrP sedimented near 4 S at both time points (Fig. 2, A–B). The broad size distribution of the PG11 protein at the 2-h chase point indicates that intermediate stages of aggregation of mutant PrP exist and suggests that acquisition of detergent insolubility may be a gradual process in which PrP aggregates grow in size over time. Based on a comparison with molecular size standards, the smallest aggregates consist of only a few molecules of PrP, whereas the largest contain over 10.

Detection of Detergent-insoluble, Protease-sensitive PrP—In a previous study we showed that following pulse labeling PG11 moPrP becomes maximally detergent-insoluble at 1 h and maximally protease-resistant at 6 h (28). This result suggests that acquisition of detergent insolubility may precede acquisition of protease resistance and raises the possibility that a detergent-insoluble but protease-sensitive form of PrP might be detectable at early times after pulse labeling. To test this possibility, we labeled cells expressing PG11 moPrP for 30 min, chased them for either 0 or 3 h, and then tested the protease resistance of pellet and supernatant fractions derived from a 265,000 × g centrifugation (Fig. 3). Immediately after labeling, 33% of the mutant PrP pellet (lanes 1 and 3), and this amount increased to 65% after a 3-h chase (lanes 5 and 7). Of note, the insoluble PrP present immediately after labeling was protease-sensitive, with <1% of the pellet protein remaining after proteinase K
digestion (lane 4). In contrast, 15% of the insoluble protein was protease-resistant following a 3-h chase (lane 8). We conclude from this result that following synthesis mutant PrP is initially converted into a form that is detergent-insoluble and protease-sensitive and only later acquires protease resistance.

Brefeldin A and 18 °C Incubation Inhibit Acquisition of Detergent Insolubility and Protease Resistance—To define the cellular compartments in which mutant PrPs become detergent-insoluble and protease-resistant, we utilized two treatments that inhibit the trafficking of proteins along the secretory pathway. First, we incubated cells expressing PG11 moPrP with the fungal metabolite brefeldin A (BFA) which causes fusion of the ER with the cis/medial-Golgi and thereby inhibits movement of proteins out of these compartments (34). Second, we incubated cells at 18 °C, a treatment that blocks movement of proteins from the trans-Golgi network to the cell surface (35, 36). We found that both of these manipulations caused a marked inhibition of the acquisition of both detergent insolubility (Fig. 4) and protease resistance (Fig. 5). The efficacy of BFA and 18 °C incubation in blocking movement of protein along the biosynthetic pathway was confirmed by the fact that these treatments inhibited maturation of PrP oligosaccharide chains (note the lower molecular weight of the two glycosylated PrP species in Figs. 5, B-C, compared with Fig. 5A, lanes 1, 3, and 5); in addition, these treatments prevented delivery of wild-type moPrP to the cell surface as assayed by PIPLC accessibility (data not shown). We conclude from our results that transformation of PG11 moPrP to a detergent-insoluble and protease-resistant form occurs primarily in a post-Golgi compartment.

We observed that in the presence of BFA a small amount of protease-resistant PrP was detectable after 4 h of chase (Fig. 5, C–D). This result was not likely to be a consequence of escape from the BFA block, since the protease-resistant protein was underglycosylated (23–27 kDa), and was therefore probably derived from molecules that were still trapped in the mixed ER-Golgi compartment (Fig. 5C, lane 6). Similarly, a small percentage of PG11 moPrP became detergent-insoluble after a 4-h chase in the presence of BFA (data not shown). Taken together, these results suggest that detergent insolubility and protease resistance can develop in the ER or Golgi in the presence of BFA, albeit more slowly than under control conditions. Interestingly, no detergent-insoluble or protease-resistant PrP is observed even after prolonged incubation at 18 °C (Fig. 5B, lane 6; and data not shown), possibly because of blockade of a second temperature-sensitive step that is separate from trans-Golgi network-cell surface transport (19).
Identification of an Early Intermediate in the Transformation of Mutant PrP—We sought to identify a step in the conversion of mutant PrP to a PrPSc-like state that preceded the acquisition of detergent insolubility and therefore occurred within 30 min after synthesis of the protein. A clue that such a step might exist was our previous observation that newly synthesized PG11 moPrP molecules are resistant to PIPLC release by the time they reach the cell surface (26), suggesting that an alteration in membrane topology might be an early event in the metabolism of the mutant protein, and one that may occur along the secretory pathway. Since it was technically difficult to test the PIPLC releasability of molecules prior to their arrival at the plasma membrane, we chose instead to assay the hydrophobicity of the protein after treatment with PIPLC, a characteristic that was likely to correlate with aberrant membrane attachment. We had previously shown that, after treatment with PIPLC, surface-biotinylated PG11 moPrP is partially retained in the detergent phase following Triton X-114 phase partitioning, whereas wild-type PrP is shifted almost entirely into the aqueous phase (26).

We asked when after pulse labeling the PG11 molecule first displays this aberrant behavior in Triton X-114 phase partitioning (Fig. 6, lanes 1–8). We found that both wild-type and PG11 moPrP partitioned into the detergent phase prior to PIPLC treatment, a behavior that is expected because of the fatty acyl or alkyl chains present on the glycosylphosphatidylinositol (GPI) anchors of both proteins. After PIPLC treatment, wild-type moPrP was shifted entirely into the aqueous phase because of cleavage of the anchor. In contrast, approximately half of the PG11 moPrP protein was retained in the detergent phase following phospholipase treatment. This result may be attributable to failure of PIPLC to remove the GPI anchor from the PG11 molecule or to an intrinsic hydrophobicity of the polypeptide chain after the anchor has been removed (26). Significantly, retention in the detergent phase was seen after a 20-min pulse labeling period with no chase, arguing that whatever molecular change was responsible for this aberrant behavior in Triton X-114 occurred very early after synthesis of PG11 PrP molecules.

We used binding to phenyl-Sepharose as a second method to assay the hydrophobicity of PG11 molecules after PIPLC treatment (Fig. 7, lanes 1–4). This resin binds hydrophobic molecules, and the distribution of a protein between bound and unbound fractions is a measure of its hydrophobic character (37, 38). As expected because of the presence of the GPI anchor, both wild-type and PG11 moPrP were concentrated in the bound fraction prior to PIPLC treatment. Incubation with PIPLC shifted all of the wild-type protein into the unbound fraction, whereas only half of the PG11 protein was shifted. Retention of PG11 moPrP in the bound fraction was observed immediately following the 30-min labeling period in the absence of a chase. These results are analogous to those obtained using partitioning in Triton X-114 to study hydrophobicity.

We obtained similar results when cells expressing PG11 moPrP were pulse-labeled for as little as 10 min prior to Triton X-114 phase partitioning or phenyl-Sepharose fractionation (data not shown).

Brefeldin A Does Not Affect Triton X-114 Phase Partitioning or Phenyl-Sepharose Binding—Our results suggested that the partial hydrophobicity of PG11 PrP following PIPLC treatment was a feature that developed during or very soon after synthesis of the polypeptide chain, at a time when the protein is likely to reside in the ER or Golgi. Consistent with this hypothesis, we found that BFA had no effect on the behavior of PG11 moPrP as assessed by either Triton X-114 phase partitioning (Fig. 6, lanes 9–16) or binding to phenyl-Sepharose (Fig. 7, lanes 9–16).
lanes 5–8); in both assays, about half of the molecules remained hydrophobic after PIPLC treatment whether or not BFA was present. These results indicate that whatever molecular change alters the response of the PG11 molecule to PIPLC in the two assays is likely to develop within the ER or cis/medial-Golgi.

**DISCUSSION**

We have shown previously that moPrP molecules carrying disease-related mutations display a number of biochemical markers characteristic of PrPSc when expressed in CHO cells (26–28). Our objective in this study was to examine the time course over which each of these operational properties is acquired after synthesis of the protein, with a view to defining intermediate stages in the acquisition of the PrPSc-like state. We have assayed three different biochemical hallmarks of the scrapie isoform and found that although all of them are present
in mutant PrP molecules at steady state, each property develops with different kinetics in pulse-chase experiments. In addition, we have used inhibitors of protein trafficking to help pinpoint the cellular compartments where these biochemical transformations take place. Our results suggest the model shown in Fig. 8. We propose that mutant PrPs are initially synthesized in the PrPSc state and then acquire the characteristics of PrPSc in a stepwise fashion during passage through various cellular compartments.

The earliest biochemical change we could detect in mutant PrP was a partial resistance to the effect of PIPLC in rendering the protein hydrophilic. About half of the mutant protein remained hydrophobic after treatment with the phospholipase, as assessed by partitioning in Triton X-114 or binding to phe- nyl-Sepharose. In contrast, essentially all of the wild-type protein was a partial resistance to the effect of PIPLC in rendering the protein hydrophilic. Whatever the relative importance of the two mechanisms, however, the unusual behavior of mutant PrP following PIPLC treatment was already observable in molecules that had been pulse-labeled for only 10 min and was not affected by treatment of cells with BFA. These observations suggest that an initial alteration in the mutant PrP molecule occurs in the ER, during or very soon after translation of the polypeptide chain. We cannot, however, rule out the possibility that this change takes place in the cis or medial compartments of the Golgi, which become fused with the ER after BFA treatment (34). We think it is probable that abnormal behavior in the Triton X-114 and phenyl-Sepharose assays correlates with inefficient release of mutant PrPs from the cell membrane by PIPLC, since both properties develop prior to arrival of the protein at the cell surface, and since both are likely to reflect an alteration in membrane association (26).

The second step we have identified is acquisition of detergent insolubility. Some detergent insolubility is detectable at the end of a 30-min labeling period (Fig. 3), but this property is not maximal until 1 h of chase (28), arguing that it occurs after the alteration in hydrophilicity properties. Consistent with this proposal, development of detergent insolubility is inhibited by BFA and incubation at 18 °C, suggesting that it occurs in a post-Golgi location. The relevant sites remain to be identified, but they could include the plasma membrane, caveolae-like microdomains (39, 40), or endosomes. Detergent insolubility presumably reflects aggregation of PrP molecules, and by sucrose gradient fractionation we were able to detect aggregates ranging in size from 4 S (monomeric) to >16.6 S (>10 PrP molecules). Although we have not directly proven that smaller aggregates are converted to larger aggregates, we speculate that PrP oligomers exist in intact cells and grow in size over time. Whether this process has the properties of a nucleated polymerization, as proposed by some investigators (12, 14–16), remains to be proven.

The third step in the transformation of mutant PrP is acquisition of protease resistance. This property is not maximal until several hours after labeling (28), and its development is blocked by BFA treatment and 18 °C incubation (Fig. 5), consistent with it happening in a post-Golgi location. We have shown that mutant PrP is detergent-insoluble and protease-sensitive at early times after pulse labeling but then becomes protease-resistant with subsequent chase. This result directly demonstrates that acquisition of detergent insolubility and protease resistance are temporally distinct steps connected by an intermediate state. It is easy to imagine, however, that the two steps might be related if, for example, PrP aggregates became protease-resistant upon reaching a certain minimum size. Gabizon et al. (41) have detected a form of wild-type PrP that is detergent-insoluble but protease-sensitive in the brains of heterozygous patients carrying an E200K mutation, although it was not possible in their experiments to determine by metabolic labeling whether this was a true intermediate that could be converted into a protease-resistant form. Priola et al. (42) have described a 60-kDa covalently linked dimer of PrP that is aggregated but protease-sensitive, but we have not observed this species in our cells.

Although we have shown that mutant PrPs synthesized in CHO cells display all the biochemical hallmarks of PrPSc, we are still in the process of testing whether the proteins are infectious in animal bioassays. If the proteins should turn out to be infectious, it will be important to determine at what point in the pathway shown in Fig. 8 this critical attribute is acquired. Infectivity could develop simultaneously with the earliest biochemical changes that we hypothesize take place in the ER, or it may represent an additional step that depends on PrP aggregates growing beyond a certain size, or attaining a minimum level of protease resistance. Even if the mutant PrPs from CHO cells should turn out to lack infectivity, it seems likely that the steps presented in Fig. 8 represent necessary prerequisites for acquisition of this property.

Our data provide the first evidence that conversion to the PrPSc state may begin at an early point in the biosynthetic pathway, perhaps even simultaneously with translation of the PrP polypeptide chain in the ER. Previous studies have emphasized events occurring subsequent to arrival of PrP molecules at the cell surface (18, 19). A role for the ER in transformation of mutant PrPs is appealing from a theoretical standpoint because of the well known role of this organelle in protein folding and glycosylation. Recent evidence suggests that prion strains are distinguished by differences in utilization of the two consensus sites for N-linked glycosylation (43–45), and it is possible that these differences arise in the ER as a result of variations in the efficiency with which oligosaccharyl transferase transfers the dolichol-linked precursor to nascent PrP chains. It is also tempting to speculate that chaperone molecules in the ER, which catalyze the folding of newly synthesized polypeptide chains (22), play an important role in the conformational changes that are thought to underlie generation of PrPSc. Mutant glycoproteins associated with several other human genetic diseases are known to associate abnor- mally with ER chaperones (46), and it will be interesting to see if the same is true for mutant PrPs. Several cytoplasmic heat shock proteins that function as molecular chaperones have been implicated in prion phenomena in scrapie-infected cells (47) and in yeast (48), although there has not been any experimental investigation of the role of ER chaperones. Recent genetic experiments have also been interpreted to suggest the involvement of accessory proteins, some of which may be mo-

---

3 S. Lehmann and D. A. Harris, unpublished data.
lœcal chaperones, in the generation of the PrPSc (23).

Our results invite speculation about the relationship between the operational biochemical parameters we have assayed and the fundamental conformational change that is thought to underlie conversion of PrPC into PrPSc. Spectroscopic data indicate that PrPSc has a higher content of β-sheets and a lower content of α-helices than PrPC, and it has been proposed that conversion of α-helices into β-sheets is responsible for generation of the scrapie isoform (5–8). We favor the possibility that this conformational transition happens either during or soon after synthesis of mutant PrP molecules in the ER and that a direct manifestation of this change is the aberrant response of the protein to PIPLC in the Triton X-114 and phenyl-Sepharose assays. In this view, detergent insolubility and protease resistance are secondary properties that develop only some time after the initial molecular conversion. On the other hand, we cannot rule out the possibility that the altered behavior of the protein in the two hydrophobicity assays is unrelated to the fundamental change in PrP conformation, which might occur later and be directly responsible for development of insolubility and protease resistance. It is also conceivable that the conformational change occurs in a gradual rather than an all-or-none fashion and that each of the three biochemical transitions we have observed is related to an incremental change in folding of the PrP molecule.

We have assumed that each of the biochemical transformations shown in Fig. 8 depends on the mutant PrP molecule through a specific cellular compartment. A particular subcellular location may provide a favorable environment for a given transition because of the presence of cofactor molecules, for example chaperones in the ER, or because of physical conditions, like the acidic milieu of endocytic organelles. However, it is also likely that mutant PrPs have some intrinsic tendency for detergent insolubility and protease resistance are secondary properties that develop only some time after the initial molecular conversion. On the other hand, we cannot rule out the possibility that the altered behavior of the protein in the two hydrophobicity assays is unrelated to the fundamental change in PrP conformation, which might occur later and be directly responsible for development of insolubility and protease resistance. It is also conceivable that the conformational change occurs in a gradual rather than an all-or-none fashion and that each of the three biochemical transitions we have observed is related to an incremental change in folding of the PrP molecule.

but that there may be an earlier step that takes place in infected cells prior to arrival of PrP at the cell surface. By analogy to mutant PrP, this step might take place in the ER and be detectable experimentally by testing the ability of PIPLC to convert the PrPSc precursor to a hydrophilic form and to completely release it from the cell membrane. In fact, we have already shown that at steady state PrPSc in infected neuroblastoma cells is retained on the cell surface after PIPLC treatment (27). It will be important now to carefully compare the intermediate steps underlying PrPSc production in infected cells with those operative in cells expressing mutant PrPs to define differences and similarities between the infectious and inherited manifestations of prion diseases.

Acknowledgments—We thank Rick Kacskos for antibodies as well as Cy Pauly and Maurine Linder for critical evaluation of the manuscript.

REFERENCES

1. Prusiner, S. B. (1996) in Virology (Fields, B. N., Knipe, D. M., and Howley, P. M., eds) 3rd Ed., pp. 2901–2950, Lippincott-Raven Press, Philadelphia.

2. Gajdusek, D. C. (1996) in Virology (Fields, B. N., Knipe, D. M., and Howley, P. M., eds) 3rd Ed., pp. 1633–1637, Lippincott-Raven Press, Philadelphia.

3. Will, R. G., Ironside, J. W., Zeidler, M., Cousens, S. N., Estibeiro, K., Alperovitch, A., Pusey, S., Pocchiari, M., Hofman, A., and Smith, P. G. (1999) Lancet 347, 921–925.

4. Parchi, P., and Gambetti, P. (1995) Curr. Opin. Neurobiol. 5, 286–293.

5. Cohen, F. E., and Peretz, D., Pan, K. M., Prusiner, S. B. (1993) Science 264, 530–531.

6. Caughey, B., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, B. (1994) Nature 370, 471–474.

7. Kaneko, K., Peretz, D., Pan, K. M., Ernst, D., Hayes, S. F., and Caughey, B. (1994) Nature 370, 471–474.

8. Prusiner, S. B., Scott, M., Foster, D., Pan, K. M., Groth, D., Mirenda, C., Torcia, M., Yang, S. L., Serban, D., Carlson, G. J., Hoppe, P. C., Westaway, D., and DeArmond, S. J. (1995) Cell 63, 673–686.

9. Caughey, B., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, B. (1994) J. Biol. Chem. 270, 7672–7680.

10. Lehmann, S., and Harris, D. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1633–1637.

11. Priola, S. A., and Chesebro, B. (1995) J. Virol. 69, 7754–7758.

12. Caughey, B., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, B. (1994) J. Biol. Chem. 270, 471–474.

13. Caughey, B., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, B. (1994) J. Biol. Chem. 270, 471–474.

14. Caughey, B., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, B. (1994) J. Biol. Chem. 270, 471–474.

15. Caughey, B., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, B. (1994) J. Biol. Chem. 270, 471–474.

16. Caughey, B., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, B. (1994) J. Biol. Chem. 270, 471–474.

17. Caughey, B., Dong, A., Bhat, K. S., Ernst, D., Hayes, S. F., and Caughey, B. (1994) J. Biol. Chem. 270, 471–474.
Intermediate Steps in the Formation of Scrapie Prion Protein

(1986) Biochemistry 25, 6740–6747
38. Davitz, M. A. (1988) in Post-translational Modification of Proteins by Lipids
(Brodbeck, U., and Bordier, C., eds) pp. 40–42, Springer-Verlag, Berlin
39. Anderson, R. G. W. (1993) Curr. Opin. Cell Biol. 5, 647–652
40. Lisanti, M. P., Scherer, P. E., Tang, Z.-L., and Sargiacomo, M. (1994) Trends
Cell Biol. 4, 231–235
41. Gabizon, R., Telling, G., Meiner, Z., Halimi, M., Kahana, I., and Prusiner, S. B.
(1996) Nat. Med. 2, 59–64
42. Priola, S. A., Caughey, B., Wehrly, K., and Chesebro, B. (1995) J. Biol. Chem.
270, 3299–3305
43. Collinge, J., Sidle, K. C. L., Meads, J., Ironside, J., and Hill, A. F. (1996) Nature
383, 685–690
44. Telling, G. C., Parchi, P., DeArmond, S. J., Cortelli, P., Montagna, P., Gabizon,
R., Mastriani, J., Lugaresi, E., Gambetti, P., and Prusiner, S. B. (1996)
Science 274, 2079–2082
45. Parchi, P., Castellani, R., Capellari, S., Ghetti, B., Young, K., Chen, S. G.,
Farlow, M., Dickson, D. W., Sima, A. A. F., Trojanowski, J. Q., Petersen, R.
B., and Gambetti, P. (1996) Ann. Neurol. 39, 767–778
46. Amara, J. F., Cheng, S. H., and Smith, A. E. (1992) Trends Cell Biol. 2, 145–149
47. Tatziel, J., Zhao, J., Voellmy, R., Scott, M., Hartl, U., Prusiner, S. B., and Welch,
W. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2944–2948
48. Chernoff, Y. O., Linquist, S. L., Ono, B., Inge-Vechtomov, S. G., and Liebman,
S. W. (1995) Science 268, 880–884
49. Taraboulos, A., Raebber, A. J., Borchelt, D. R., Serban, D., and Prusiner, S. B.
(1992) Mol. Biol. Cell 3, 851–863