Prion diseases are unusual neurodegenerative disorders that can be both infectious and inherited. Both forms are hypothesized to result from a posttranslational structural alteration in the cell surface glycoprotein PrP\(^\text{C}\) (cellular isoform of the prion protein) that converts it into the protease-resistant isoform PrP\(^\text{Sc}\) (scrapie isoform of the prion protein). However, a direct comparison of molecular events underlying these two manifestations of prion diseases has not been possible, because there has been no cell culture model for the familial forms. We report here that when mutant prion proteins associated with three different inherited prion disorders of humans are expressed as their murine homologues in cultured Chinese hamster ovary cells, the proteins are protease-resistant and detergent-insoluble, two biochemical properties characteristic of infectious PrP\(^\text{Sc}\). In addition, each mutant protein remains tightly associated with the plasma membrane after enzymatic cleavage of its glycosylphosphatidylinositol anchor, a property that we now show is also typical of infectious PrP\(^\text{Sc}\). The cell culture system described here is the first in vitro model for familial prion diseases and provides compelling evidence that infectious and genetic cases share common molecular features.

Prion diseases are fatal neurodegenerative disorders of human beings and animals characterized by dementia, ataxia, myoclonus, and spongiform deterioration of the brain and spinal cord (1, 2). A unique feature of these disorders is their manifestation as infectious, genetic, and sporadic forms. Infectious cases, including kuru, experimental scrapie, and iatrogenic Creutzfeldt-Jakob disease (CJD D), \(^1\) are thought to be caused by prions (3). These particles contain little or no nucleic acid and are composed primarily of PrP\(^\text{Sc}\) (4), a posttranslationally modified isoform of a glycolipid-anchored membrane protein of the host called PrP\(^\text{C}\) (5). Familial prion diseases, including Gerstmann-Sträussler syndrome (GSS), familial insomnia (FFI), and about 10% of the cases of CJD, display an autosomal dominant pattern of inheritance with nearly complete penetrance and are linked to insertional and point mutations in the chromosomal gene that encodes PrP\(^\text{C}\) (6).

Both infectious and inherited forms of prion disease are characterized by accumulation in the central nervous system of protease-resistant PrP\(^\text{Sc}\) that is capable of transmitting the disease to laboratory animals (2). It is uncertain, however, whether the cellular mechanisms underlying production of PrP\(^\text{Sc}\) are the same in the two disease states. This uncertainty is due, in part, to the absence of a cell culture model of familial prion diseases, analogous to the ones that have been developed for analysis of scrapie infection (7–10). It is hypothesized that all prion diseases result from a posttranslational change in the conformation of PrP\(^\text{Sc}\) (11–14), but it is possible that distinct cellular compartments and molecular components are involved in inherited and infectious forms. To resolve this issue, it will be necessary to employ cultured cell models in addition to transgenic mice (15) and cell-free systems (16).

To develop such a model, we have constructed stably transfected Chinese hamster ovary (CHO) cells that express murine homologues of mutant PrPs associated with human prion diseases. We previously reported that one of these mutants, a Creutzfeldt-Jakob disease (sCJD) akob homologue containing an insertion of six additional octapeptide repeats, displays an abnormal mode of attachment to the plasma membrane (17). Unlike wild-type PrP, this protein remains tightly bound to the cell surface, even after enzymatic cleavage of its glycosylphosphatidylinositol (GPI) anchor. In the present work, we have analyzed CHO cells expressing mutant PrPs related to all three inherited prion diseases of humans, in order to determine whether these proteins acquire biochemical properties of infectious PrP\(^\text{Sc}\). In addition, we have investigated whether PrP\(^\text{Sc}\) synthesized in scrapie-infected neuroblastoma cells has a membrane attachment similar to that of the mutant PrPs.

MATERIALS AND METHODS

PrP Constructs and Cell Lines—CHO cell lines expressing PG11 moPrP have been described previously (17). Point mutations in moPrP were introduced using recombinant polymerase chain reaction (18); the second stage of amplification employed a 5' primer containing a HindIII site and a 3' primer containing a BamHI site (primers 1 and 2 in Ref. 17). All cDNAs were cloned into the expression vector pBC12/CMV and were stably transfected into CHO cells, as described (17). The data shown here were obtained from a single subcloned line expressing each construct, although similar results have been obtained from additional clones and from pools of transiently transfected cells (data not shown).

Scrapie-infected N2a mouse neuroblastoma cells were kindly provided by Dr. Byron Caughey (Rocky Mountain Laboratories, Hamilton, MT) and were grown as described (19, 20).
MoPrP contains Met at position 128 (30). However, the homologous codon 129 in human PrP is polymorphic in the normal population, encoding either Met or Val. The D178N mutation is linked to FFI if amino acid 129 is Met and Cj D if it is Val. The six-octapeptide insertion and the P102L and E200K point mutations are in linkage with Met at position 129 in all patients examined to date (31).

Table I, along with their human homologues and the phenotypes that account for lack of PIPLC release are also likely to confer protease resistance on mutant PrP molecules, since we noted that E199K moPrP was more detergent-soluble than the other mutants (Figs. 1 A and B). E199K moPrP was released, which correlates with the higher solubility of this protein compared to the other disease-related mutants (Fig. 1B). Confirming that the GPI anchor on each protein had been cleaved, we observed a characteristic decrease in the electrophoretic mobility of the protein after PIPLC treatment (17, 34) (Fig. 2C). The structural features that account for lack of PIPLC release are also likely to confer protease resistance on mutant PrP molecules, since we found that the pool of E199K protein that was retained on the cell surface after PIPLC treatment was protease-resistant (Fig. 2D, lanes 4 and 8), while the released pool was completely digested (Fig. 2D, lanes 3 and 7). Several possible explanations may account for retention of mutant PrPs on the cell surface following GPI anchor cleavage, including integration of the PrP polypeptide chain into the lipid bilayer, tight association with other membrane components, or aggregation (17).

Although all of the moPrPs carrying disease-related mutations were more detergent-insoluble and protease-resistant, and less PIPLC-releasable than wild-type moPrP, we noted several biochemical features that distinguished the mutant PrPs from each other. First, the protease-resistant fragments of the D177N and E199K proteins consistently migrated 1–2 kDa more slowly than the fragments of the other mutant proteins (Fig. 1C), suggesting that the cleavage site may not be identical for all of the mutants. Second, the glycosylation patterns of the mutants were different from each other, although all of the disease-related mutants displayed a higher proportion of lower molecular mass glycoforms than wild-type PrP (Fig. 2A). Third, E199K moPrP was more detergent-soluble and PIPLC-releasable than the other mutants (Figs. 1B and 2B). These observations raise the possibility that mutant PrPs possess “strain-specific” molecular properties that account for the

RESULTS AND DISCUSSION

The mutant moPrP constructs employed here are listed in Table I, along with their human homologues and the phenotypes with which they are associated. M128V moPrP was analyzed as a negative control, because a Met → Val substitution at codon 129 is a nonpathogenic polymorphism in the human population (26).

To test the detergent solubility of the moPrPs, lysates of metabolically labeled CHO cells expressing each protein were centrifuged at 265,000 × g for 40 min, a protocol that sediments PrPSc but not PrP (19). We found that significantly more PG11, P101L, D177N, and E199K moPrPs than wild-type moPrP sedimented (Fig. 1, A and B). We noted that E199K moPrP was more soluble than the other mutants (60% versus 80–90% in the pellet). Importantly, very little M128V moPrP sedimented, indicating that the effect on detergent solubility was specific for disease-related mutations.

To assess the protease resistance of the moPrPs, lysates of metabolically labeled cells were treated for 10 min with 3.3 μg/ml of proteinase K, conditions similar to those that have been used for digestion of PrPSc derived from some scrapie strains (32, 33). We observed that PG11, P101L, D177N, and E199K moPrPs were cleaved by the protease to yield fragments that migrated between 27 and 30 kDa, the same size as the protease-resistant core of PrPSc (4, 5) (Fig. 1C). In contrast, wild-type moPrP, as well as M128V moPrP, were completely degraded under these conditions (Fig. 1, C and D). In a separate study, we have shown that protease K truncates the PG11 protein within a segment of ~20 amino acids following the octapeptide repeats, the same region within which cleavage of infectious PrPSc occurs.2

More than half of each of the mutant PrPs is present on the cell surface at steady state, as determined by susceptibility to externally applied trypsin (data not shown). To test whether the proteins can be released from the surface by cleavage of their GPI anchors, cells were biotinylated with a membrane-impermeant reagent and incubated with the bacterial enzyme PIPLC, which cleaves the diacylglycerol portion of the anchor. We found that ~5% of PG11, P101L, and D177N moPrPs were released by PIPLC, compared with ~90% for wild-type and M128V moPrPs (Fig. 2, A and B). An intermediate amount (~50%) of E199K moPrP was released, which correlates with the higher solubility of this protein compared to the other disease-related mutants (Fig. 1B). Confirming that the GPI anchor on each protein had been cleaved, we observed a characteristic decrease in the electrophoretic mobility of the protein after PIPLC treatment (17, 34) (Fig. 2C). The structural features that account for lack of PIPLC release are also likely to confer protease resistance on mutant PrP molecules, since we found that the pool of E199K protein that was retained on the cell surface after PIPLC treatment was protease-resistant (Fig. 2D, lanes 4 and 8), while the released pool was completely digested (Fig. 2D, lanes 3 and 7). Several possible explanations may account for retention of mutant PrPs on the cell surface following GPI anchor cleavage, including integration of the PrP polypeptide chain into the lipid bilayer, tight association with other membrane components, or aggregation (17).
different clinical and neuropathological phenotypes with which they are associated (32, 33, 35–39).

Although numerous reports have emphasized the hydrophobicity of infectious PrPSc (40–42), there has been uncertainty about how this isomer is associated with cell membranes. Scrapie-infected mouse neuroblastoma cells have been used extensively to analyze the biochemical mechanisms underlying prion generation, since these cells produce PrPSc that is both protease-resistant and infectious (Fig. 3A; Refs. 7, 8, 19, and 23). To determine whether infectious PrPSc, like mutant PrPSc, remains membrane-bound after PIPLC treatment, we surface-biotinylated scrapie-infected and uninfected neuroblastoma cells and incubated them with the phospholipase. In agreement with previous studies (43, 44), the majority of the protease-sensitive moPrP in both types of cells was released by PIPLC (Fig. 3B, lanes 1–4). In contrast, protease-resistant PrPSc was retained on the surface of infected cells after PIPLC treatment (Fig. 3B, lanes 7 and 8). This behavior is similar to that of the mutant PrPs and suggests an important similarity between the membrane topologies of mutant and infectious molecules. The fact that PrPSc can be biotinylated in these cells indicates that at least some must be present on the plasma membrane and must therefore be accessible to externally applied PIPLC, a result consistent with previous reports (20, 45, 46).

Several results argue strongly that the cell culture system employed here is a faithful in vitro model of familial prion diseases. First, the mutant PrPs synthesized in this system display all of the major biochemical properties of authentic PrPSc, including detergent insolubility, protease resistance, abnormal attachment to the plasma membrane, and, as shown in a separate study, metabolic stability. Second, these PrPSc-like biochemical properties are detected in four different moPrP mutants whose human homologues are associated with each of the known forms of inherited prion disorder (familial CJD, GSS, and FFI). Third, a mutation in moPrP (M128V) whose human homologue represents a nonpathogenic polymorphism fails to induce PrPSc properties. Fourth, there are variations among the disease-related mutants in their biochemical properties, consistent with the concept that structurally distinct PrP “strains” are associated with different disease phenotypes (32, 33, 35–39).

It might be argued that there is a difference between our cell culture system and the brains of patients afflicted with inherited prion diseases in the time course and extent of PrPSc production. The human disorders evolve slowly, not manifesting themselves clinically until adulthood, and even in the terminal stages only a small proportion of the available PrPSc is converted to PrPSc (6). In contrast, we find that CHO cells convert a substantial fraction of mutant PrP they express into PrPSc within a matter of hours. Possible explanations for this discrepancy are that cell- and tissue-specific factors influence the efficiency of the conversion reaction and that PrPSc accumulates in the brain for a period of time before pathological damage and clinical symptoms ensue.

We now plan to test whether mutant moPrPs expressed in CHO cells are infectious, as are PrP molecules from the brains of some patients with familial prion diseases. It has been observed, however, that inherited prion diseases as a group dis-
Properties of Mutant and Infectious Prion Proteins

MoPrPs carrying disease-related mutations are retained on the cell surface after cleavage of their GPI anchors. A, surface-biotinylated CHO cells expressing each protein were treated with PIPLC (1 unit/ml) for 2 h at 4 °C prior to lysis. MoPrP in the PIPLC incubation media (M lanes) and cell lysates (C lanes) was immunoprecipitated, separated by SDS-PAGE, and visualized by developing blots of the gel with horseradish peroxidase-streptavidin and ECL. B, PrP bands from the ECL film shown in A, and from two additional experiments, were quantitated by densitometry, and the amount of PrP released by PIPLC was plotted as a percentage of the total amount of PrP (medium + cell lysate). Each bar represents the mean ± S.D. Values that are significantly different from wild-type moPrP by t test are indicated by single (p < 0.001) and double (p < 0.001) asterisks. C, lysates of metabolically labeled cells expressing each protein were incubated with (+ lanes) or without (− lanes) PIPLC. MoPrP was then immunoprecipitated and analyzed by SDS-PAGE. PrP-specific bands (arrowheads) are reduced in electrophoretic mobility after PIPLC treatment. D, CHO cells expressing wild-type and E199K moPrPs were surface-biotinylated and treated with PIPLC as described in the legend of A. Proteins in the PIPLC incubation media (M lanes) and cell lysates (C lanes) were then either digested at 37 °C for 10 min with 3.3 μg/ml of proteinase K (PK) (+ lanes) or were left untreated (− lanes), prior to recovery of moPrP by immunoprecipitation. Biotinylated moPrP was then visualized as described in the legend of A. Five times as many cell equivalents were loaded in the + lanes as in the − lanes. Only the fraction of E199K moPrP not released by PIPLC (lane 4) generates a protease-resistant fragment (bracket, lane 8). WT, wild-type moPrP.

PrPSc molecules have remarkably similar biochemical properties, including an unusually tight attachment to the plasma membrane that had not been previously appreciated. In addition, acquisition of at least some of these properties occurs posttranslationally, as demonstrated by pulse-chase labeling studies of mutant2 and infectious PrPs (20, 23) in cultured cells. Taken together, these results strongly support the hypothesis (11–14) that all prion diseases are initiated by a conformational transition of the PrP polypeptide chain that profoundly alters its biochemical properties following synthesis, formational transition of the PrP polypeptide chain that profoundly alters its biochemical properties following synthesis, and at least in most cases, endows it with infectivity. It remains to be determined whether this transition occurs along the same cellular pathways in all forms of the disease and whether the same set of molecular intermediates and accessory proteins is involved. Further analysis of PrPSc production in infected cell lines and in cultured cells expressing mutant PrPs will make it possible to resolve these issues and for the first time to directly compare the cellular and biochemical mechanisms underlying two major etiologies of prion disease.

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