A Mutant Prion Protein Displays an Aberrant Membrane Association When Expressed in Cultured Cells*

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Inherited forms of prion disease have been linked to mutations in the gene encoding PrP, a neuronal and glial protein that is attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor. One familial form of Creutzfeldt-Jakob disease is associated with a mutant PrP containing six additional octapeptide repeats. We report here our analysis of cultured Chinese hamster ovary cells expressing a murine homologue of this mutant PrP. We find that, like wild-type PrP, the mutant protein is glycosylated, GPI-anchored, and expressed on the cell surface. Surprisingly, however, cleavage of the GPI anchor using phosphatidylinositol-specific phospholipase C fails to release the mutant PrP from the surface of intact cells, suggesting that it has an additional mode of membrane attachment. The phospholipase-treated protein is hydrophobic, since it partitions into the detergent phase of Triton X-114 lysates; and it is tightly membrane-associated, since it is not extractable in carbonate buffer at pH 11.5. Whether membrane attachment of the mutant PrP involves integration of the polypeptide into the lipid bilayer, self-association, or binding to other membrane proteins remains to be determined. Our results suggest that alterations in the membrane association of PrP may be an important feature of prion diseases.

The spongiform encephalopathies are a group of transmissible neurodegenerative disorders which are characterized by dementia, motor dysfunction, and in some cases by the presence of cerebral amyloid plaques (reviewed by Gajdusek, 1990; Chesebro et al., 1985). Prion replication is hypothesized to result from conversion of endogenous PrP⁰ into infectious PrP⁰⁰, a process that seems to involve species-specific molecular interactions between the two isoforms (Prusiner et al., 1990; Büeler et al., 1993; Kociósko et al., 1994). Recent evidence suggests that the isoforms differ in the conformation of their polypeptide chains, and it has been proposed that conversion of α-helices into β-sheets underlies the formation of PrP⁰⁰ (Caughey et al., 1991; Safar et al., 1993; Pan et al., 1993). The two forms of the protein also exhibit distinct biochemical properties. PrP⁰⁰ aggregates in non-denaturing detergents, and it is more resistant to protease digestion than PrP⁰, yielding an N terminally cleaved core fragment called PrP 27–30 (Oesch et al., 1985; Meyer et al., 1986).

Prion diseases are unique in having both a genetic as well as an infectious origin. Gerstmann-Sträussler syndrome and familial insomnia, and about 10% of the cases of CJD, are inherited in an autosomal dominant fashion (Prusiner and DeArmond, 1994). These inherited forms have been associated with specific mutations in the chromosomal gene that encodes PrP. Genetic linkage or association has been established for several amino acid substitutions in the C-terminal half of the molecule, as well as for insertions of between two and nine additional octapeptide repeats in the N-terminal half (reviewed by Brown et al., 1991; Prusiner and Hsiao, 1994). A genetic form of prion disease can also be produced in mice harboring a PrP transgene with a point mutation (Hsiao et al., 1990); mouse models of insertion mutations have not been reported.

It is presumed that mutant PrPs spontaneously assume the conformation of PrP⁰⁰ in the absence of exogenous prions (Prusiner and DeArmond, 1994). However, there is no experimental evidence for this point, and the cellular mechanisms that might be involved remain unknown. To address these issues, it would be desirable to analyze the processing and metabolism of mutant PrPs in cultured cells. We have previously characterized the trafficking of wild-type PrP⁰ in cultured neuroblastoma cells and have defined the secretory and endocytic pathways followed by the cellular isoform (Harris et al., 1993; Shyng et al., 1993, 1994). We have now extended our studies to a murine form of PrP that carries an insertion of six additional octapeptide repeats. This mutation is homologous to one in human PrP that is strongly linked to CJD in a large family from southeast England. This family comprises seven generations, and its members are all descended.
from a single founder who was born in the late 18th century (Owen et al., 1990; Poulter et al., 1992; Collinge et al., 1992). Recently, two wild-type clones with PrP insertions encoding the same amino acids have been published (Nichol et al., 1995; Oda et al., 1995). We report here that murine PrP containing six additional octapeptide repeats displays an abnormal association with the plasma membrane when expressed in cultured CHO cells.

MATERIALS AND METHODS

Reagents—PILPC from Bacillus thuringiensis was prepared as described previously (Shyng et al., 1995). Cell culture reagents were from the Tissue Culture Support Center at Washington University. N-Glycosidase F and restriction enzymes were purchased from Boehringer Mannheim, fluorescein-conjugated secondary antibodies from Cappel, and sulfo-biotin-X-NHS from Calbiochem. [1-3H]Ethanolamine (28 Ci/mmol) was obtained from Amersham Corp. [3S]methionine (Trans/3S-Label, 1,000 Ci/mmol) from ICN, and [9,10-3H]leucine (30–50 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were from Sigma.

Antisera—The antibody designated P45–66 was raised by immunization of rabbits with a synthetic peptide (CGGNRYPPQGTGWQPGHGGGWGQ), encompassing moPrP residues 45–66, that was coupled via the additional N-terminal cysteine residue to maleimide-activated keyhole limpet hemocyanin (Pierce). The rabbit antiserum designated 24590 heavy chain of clathrin from bovine brain was a gift of Dr. Frances Kascsak (Kascsak et al., 1995). We note that moPrP contains a methionine residue at position 128, which is homologous to position 129 in human PrP where a valine/methionine polymorphism has been described; the PG11 gene in humans is always found to contain a methionine residue (Poulter et al., 1992).

Cell Lines—Chinese hamster ovary (CHO) cells were grown in MEM containing 7.5% fetal calf serum and penicillin/streptomycin in an atmosphere of 5% CO₂, 95% air. CHO cells were transfected using Lipofectin (Life Technologies Inc.), according to the manufacturer’s directions, with a 1:10 mixture of pRSVneo (Urich and Levy, 1990) and the pBC12/CMV expression plasmid encoding moPrP. Antibiotic-resistant clones were selected in 700 μg/ml geneticin (G418), subcloned once, and maintained in 300 μg/ml geneticin. The experiments described here were carried out on a single cell line expressing each construct.

N2a neuroblastoma cells expressing a chPrP/mannose 6-phosphate receptor (MPR) transmembrane chimera are described elsewhere (Gorodinsky and Harris, 1995).

Immunocytochemistry—Cells grown on glass coverslips were incubated with rabbit polyclonal antibody P45–66 for 1 h at 4°C. After rinsing, cells were fixed with methanol for 30 min at 4°C and then incubated with fluorescein-conjugated secondary antibody for 1 h at room temperature. Coverslips were mounted in glycerol and cells were observed with a Zeiss Axiophot fluorescence microscope equipped with a Bio-Rad MRC1000 laser confocal scanning system.

Metabolic Labeling, Immunoprecipitation, and Biotinylation—Confluent cultures of CHO cells were labeled either in methionine-free MEM containing TranS-Labe (250–500 μCi/ml) in MEM plus 10 mM dialyzed fetal calf serum and non-essential amino acids, containing [3H]ethanolamine (200 μCi/ml), or in DMEM plus 10 mM fatty acid-free bovine serum albumin, containing a mixture of [3H]leucine acid (250 μCi/ml) and [3H]palmitic acid (1,250 μCi/ml). In some experiments, labeled cultures were chased in Opti-MEM (Life Technologies Inc.) for various times before harvesting. Cells were lysed in a buffer that contained 150 mM NaCl, 50 mM Tris, pH 7.5, 0.5% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS supplemented with protease inhibitors (1 μg/ml pepstatin and leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM EDTA). Prior to lysis, cultures in some experiments were incubated with PILPC (1 unit/ml in Opti-MEM) at 4°C for 2 h, or with 0.25% trypsin in PBS at 4°C for 10 min, followed by addition of fetal calf serum to 10% to terminate digestion.

Samples from metabolically labeled cells were immunoprecipitated as described previously (Harris et al., 1993), with the modification that immunocomplexes were collected using protein A-Sepharose. Immunoprecipitated proteins were analyzed by SDS-PAGE, and radioactive gels were quantitated using a Phosphorimager (Molecular Dynamics).

Surface biotinylation was carried out as described previously (Shyng et al., 1995). Rabbit anti-chicken IgG was included when biotinylated proteins were immunoprecipitated with 3F4 antibody.

Deglycosylation of PrP—Where indicated, cell lysates were treated prior to immunoprecipitation with 0.01 units/ml of N-glycosidase F for 16 h at 37°C. For the experiments shown in Fig. 4, immunoprecipitated PrP was eluted from protein A-Sepharose with 20 mM Tris in 4% SDS, 100 μg/ml genetin (G418), subcloned once, and incubated with endoglycosidase H (1.6 milliunits) or neuraminidase (10 milliunits). After addition of 2 × SDS sample buffer, samples were analyzed by SDS-PAGE and autoradiography.

Triton X-114 Phase Separation—Surface-biotinylated cells were solubilized at 4°C in 1% Triton X-114 in PBS containing protease inhibitors; the detergent was diluted with 1:12 Opti-MEM stock solution that had been preincubated according to Bordier (1981). After centrifugation at 37°C for 20 min, aqueous and detergent phases were separated by centrifugation. The detergent phase was diluted to the initial volume with PBS, incubated with or without PILPC for 2 h at 4°C, and the phase separation repeated. PrP in each phase was immunoprecipitated and biotinylated proteins visualized as described (Shyng et al., 1995).

Alkaline Carbonate Extraction—Confluent CHO cells expressing PG11 moPrP protein were surface biotinylated as described above and incubated with PILPC for 2 h at 4°C. Cells were then collected in PBS and passed nine times through 27-gauge needles connected by plastic tubing. The homogenate was centrifuged for 3 min at 300 × g to remove nuclei and debris and then centrifuged for 5 min at 16,000 × g to collect membranes. Membranes and pelleted pellets were resuspended in either PBS or carbonate buffer (0.1 M, pH 11.5), and kept on ice for 10 min. After a further centrifugation for 5 min at 16,000 × g, PrP was immunoprecipitated from pellets and supernatants; each fraction was also immunoblotted for clathrin heavy chain using antibody TD.1. It should be noted that the 5-min centrifugation at 16,000 × g used to collect membranes is not sufficient to pellet PG11 moPrP protein from detergent lysates (not shown).

RESULTS

CHO Cells Expressing Murine PrP—Fig. 1 shows that wild-type mouse PrP (moPrP) contains five octapeptide repeats, each of which displays one of three related amino acid sequences: PQQGTTWQ (R1), PHGGGWWQ (R2), or PHGG-SWWQ (R3). The repeats are arranged in the order R1-R2-R3-
rate lines, behaved similarly (see Fig. 6). For a particular clone of cells, however, since both the untagged and PG11 transfected lines of CHO cells, which synthesize undetectable levels of endogenous hamster PrP (data not shown). A single transfected line was used when the amounts of wild-type and mutant proteins were compared during prolonged passage. In most experiments, cells were kept in culture medium containing 10 μg/ml of insulin (blocks labeled +1 through +6 in Fig. 1). To detect moPrP synthesized by transfected cells, we used a rabbit anti-peptide antibody designated P45–66, which is directed against moPrP residues 45–66 and that does not cross-react with hamster PrP.

In some experiments, we used epitopically tagged versions of wild-type and PG11 moPrP, in which methionine residues were substituted for a leucine and a valine residue at two nearby positions (Fig. 1). Methionine residues are found at the homologous positions in hamster and human PrP, and their introduction into moPrP allows the protein to be recognized by the species-specific monoclonal antibody 3F4 (Bolton et al., 1991). MoPrPs tagged with the 3F4 epitope have been used extensively in cell biological and transgenic studies (Rogers et al., 1993; Scott et al., 1993).

We have expressed each of these moPrP molecules in stably transfected CHO cells, which synthesize undetectable levels of endogenous hamster PrP (data not shown). A single cell line was analyzed for each construct. The unusual properties of PG11 moPrP that we describe here are not peculiar to a particular clone of cells, however, since both the untagged and 3F4-tagged versions of the mutant protein, expressed in separate lines, behave similarly (see Fig. 5A). We noted that even though they had been subcloned, the lines expressing PG11 moPrP showed some decrease in their levels of PrP expression during prolonged passage. In most experiments, cells were used when the amounts of wild-type and mutant proteins were roughly equivalent (Figs. 2, 3, 4, 6, and 7). In two experiments (Figs. 5 and 8A), the amount of PG11 PrP was approximately 3-fold less than the amount of wild-type PrP. In none of our experiments, however, have we found that differences in expression levels had any effect on the results obtained.

Both Wild-type and PG11 moPrPs Are Glycosylated—We noted in the experiment shown in Fig. 3A that there was a marked shift in the molecular weight distribution of the labeled PrP molecules during the chase period. At the end of the pulse, PrP migrated as a series of three bands, ranging in size from 28 to 33 kDa for the wild-type protein and from 30 to 40 kDa for the PG11 protein. By 40 min of chase, the majority of the protein had shifted to 40 kDa for the wild-type form and to 40–45 kDa for PG11. These time-dependent alterations in the molecular mass of each protein reflect changes in N-linked glycosylation, since N-glycosidase F digestion at each time point produces a single band at 29 kDa for wild-type moPrP and 33 kDa for PG11 (see Fig. 5A). These results indicate that the PG11 mutation does not block glycosylation of PrP. However, the pattern of glycosylation appears to be affected, since the mutant protein displays a greater proportion of lower molecular mass glycoforms than the wild-type protein (see Fig. 4A) and sensitive to digestion by neuraminidase (Fig. 4B). These results indicate that both proteins traverse the mid-Golgi stack (site of acquisition of the glycolipid anchor). One of the consequences of this is that both proteins become resistant to digestion by neuraminidase and trypsin by 20 min of chase, and by 40 min approximately 60% was susceptible to digestion by the enzyme. Similar kinetics have been reported for delivery of pulse-labeled molecules of chicken PrP (Shyng et al., 1993) and moPrP (Caughey et al., 1989, Borchelt et al., 1992) to the surface of mouse neuroblastoma cells.

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sition of endoglycosidase H resistance) and the trans-Golgi network (site of sialic acid addition) during their passage to the surface of CHO cells. Maturation of the oligosaccharide chains of moPrP to an endoglycosidase H-resistant form has also been observed in neuroblastoma cells (Caughey et al., 1989; Taraboulos et al., 1992). Taken together, our results indicate that the presence of additional octapeptide repeats does not markedly affect the kinetics or route of biosynthetic processing of PrP, but does affect the specific pattern of glycosylation.

MoPrP PG11 is Not Released from the Cell Surface by PIPLC—Because of its GPI anchor, PrP2 can be released from the surface of intact cells by digestion with the bacterial enzyme PIPLC, which removes the diacylglycerol portion of the anchor (Englund, 1993). Since our previous results indicated that PG11 moPrP was present on the cell surface, we asked whether it was PIPLC-releasable like wild-type moPrP.

We first examined this question by performing a pulse-chase labeling experiment similar to that shown in Fig. 3, but using PIPLC rather than trypsin (Fig. 5). Cells were incubated with PIPLC at each time point and then lysed; the amount of PrP in cell lysates and PIPLC incubation media was determined by immunoprecipitation. During the chase period, wild-type moPrP gradually became accessible to digestion by PIPLC, so that by 30 min almost 40% of the protein was recovered in the PIPLC incubation medium (Fig. 5B). This increase reflects delivery of newly synthesized molecules to the cell surface, where they were susceptible to release by the phospholipase. The kinetics of surface delivery of wild-type moPrP measured in this way was quite similar to that measured using external trypsin (compare Figs. 3B and 5B).

Surprisingly, a very different result was obtained with CHO cells expressing untagged wild-type (WT) or PG11 moPrP were labeled with [35S]methionine for 20 min and then chased in medium containing unlabeled methionine for 0, 20, or 40 min. At the end of the chase period, cells were either treated with trypsin for 10 min at 4°C (lanes) or remained untreated (lanes) before lysis. MoPrP in cell lysates was then analyzed by immunoprecipitation with antibody P45-66, either in the presence (lanes) or absence (lanes) of the peptide immunogen. PrP-specific bands are those that are absent when the peptide is included. Exposure time was 48 h for both gels. Panel B, PrP-specific bands in panel A were quantitated by PhosphorImager analysis, and the amount of PrP digested by trypsin at each time point was plotted as a percentage of the PrP present in the absence of trypsin treatment.

**Fig. 3.** Pulse-labeled molecules of both wild-type and PG11 moPrP become accessible to external trypsin during the chase period. Panel A, CHO cells expressing untagged wild-type (WT) or PG11 moPrP were labeled with [35S]methionine for 20 min and then chased in medium containing unlabeled methionine for 0, 20, or 40 min. At the end of the chase period, cells were either treated with trypsin for 10 min at 4°C (lanes) or remained untreated (lanes) before lysis. MoPrP in cell lysates was then analyzed by immunoprecipitation with antibody P45-66, either in the presence (lanes) or absence (lanes) of the peptide immunogen. PrP-specific bands are those that are absent when the peptide is included. Exposure time was 48 h for both gels. Panel B, PrP-specific bands in panel A were quantitated by PhosphorImager analysis, and the amount of PrP digested by trypsin at each time point was plotted as a percentage of the PrP present in the absence of trypsin treatment.

**Fig. 4.** Pulse-labeled molecules of both wild-type and PG11 moPrP become resistant to endoglycosidase H and sensitive to neuraminidase during the chase period. Panel A, CHO cells expressing untagged wild-type (WT) or PG11 moPrP were labeled with [35S]methionine for 20 min and then chased in medium containing unlabeled methionine for either 0 or 40 min. MoPrP was immunoprecipitated from cell lysates using antibody P45-66 and was then either left untreated (lanes) or was digested with endoglycosidase H (+lanes) prior to SDS-PAGE. After 0 min of chase, both proteins show an increase in electrophoretic mobility following endoglycosidase H treatment; this shift is absent after 40 min of chase, indicating loss of high-mannose-type glycans. Panel B, CHO cells were labeled as in panel A. MoPrP was immunoprecipitated from cell lysates using antibody P45-66, and was then either left untreated (lanes) or was digested with neuraminidase (+lanes) prior to SDS-PAGE. After 40 min of chase, both wild-type and PG11 moPrPs show an increase in electrophoretic mobility following treatment with neuraminidase, indicating acquisition of sialic acid residues.
cells expressing the PG11 form of moPrP. Even after 30 min of chase, very little of the mutant protein was released by PIPLC, and most of it remained associated with the cell lysates (Fig. 5B). A similar result was obtained when cells were labeled continuously for 4 h with \([^{35}S]\)methionine, eliminating the possibility that PG11 molecules become releasable by PIPLC at later times (Fig. 5C). The fact that PG11 moPrP cannot be released by the PIPLC contrasts with our observation that almost half of the mutant protein is susceptible to digestion by external trypsin 30 min after pulse labeling (Fig. 3B). Taken together, these data indicate that the PG11 protein is not releasable by PIPLC, even though substantial amounts of it are present on the cell surface.

To confirm this conclusion, we analyzed the effect of PIPLC on cell surface PrP that had been labeled by biotinylation of intact cells with the membrane-impermeant reagent sulfo-biotin-X-NHS. We found that \(\sim 90\%\) of wild-type moPrP was released by PIPLC, in contrast to \(\sim 5\%\) of PG11 moPrP (Fig. 6A, lanes 1, 2, 5, and 6). As a control to confirm that only PrP molecules on the cell surface had been biotinylated, we found that both forms of PrP were completely digested by externally applied trypsin (Fig. 6A, lanes 3, 4, 7, and 8). Lack of release of PG11 did not result from a slower action of PIPLC on the mutant protein, since extending incubation with the phospholipase to as long as 6 h failed to liberate any additional PrP (Fig. 6B). We obtained similar results after surface iodination of cells, indicating that the failure of PIPLC to release PG11 is not an artifact of biotinylation (data not shown).

To confirm that the unusual behavior of the mutant protein was not a peculiarity of the particular clone of CHO cells we were analyzing, we performed an identical biotinylation experiment on a separate pair of cell lines that expressed moPrPs tagged with the 3F4 antibody epitope (Fig. 6C). In agreement with our previous results, we found that \(>90\%\) of wild-type moPrP was released by PIPLC, in contrast to \(\sim 5\%\) of the PG11 mutant. We noted in this and other experiments that the small amount of the mutant protein released was usually the most heavily glycosylated isoform (Fig. 6C, lane 3). In preliminary
Our data thus far indicated that PG11 moPrP molecules are GPI-anchored, even though they cannot be released from the cell surface by digestion with PIPLC. A possible explanation for this fact might be that the mutant protein contains a modified GPI anchor that is intrinsically resistant to PIPLC cleavage, as has been reported for anchor structures in which the inositol ring is acylated (Rosenberry, 1991). Alternatively, aggregation of the protein on the cell surface, or interaction with other membrane components, might render the anchor physically inaccessible to the phospholipase. To investigate these possibilities, we incubated CHO cells with a mixture of \([\text{H}]\)palmitate and \([\text{H}]\)stearate in order to label the fatty acyl residues of the GPI anchor and then treated the intact cells with PIPLC. Since PIPLC cleaves the diacylglycerol moiety from the GPI anchor, the activity of this enzyme would be revealed by a loss of incorporated \(^{3}\text{H}\) label after PIPLC treatment. We also labeled parallel cultures with \(^{35}\text{S}\)methionine to track the polypeptide chain. We found that both wild-type and PG11 moPrP molecules were labeled with \(^{3}\text{H}\)-fatty acids, consistent with the presence of a GPI anchor on both proteins (Fig. 7B, lanes 5 and 7). Importantly, PIPLC treatment of the cells removed the \(^{3}\text{H}\) label from the PG11 protein almost completely (Fig. 7B, lane 8). Analysis of the \(^{35}\text{S}\)methionine-labeled dishes confirmed that after removal of the diacylglycerol moiety by PIPLC, PG11 molecules were retained on the cell surface (Fig. 7B, lane 4), while the wild-type molecules were released into the medium (Fig. 7B, lane 2). This result demonstrates that the GPI anchor of the PG11 mutant is cleavable by PIPLC, but that cleaved molecules are not released from the cell surface. Consistent with this conclusion, we also observed a small (1–2 kDa) decrease in SDS-PAGE mobility of the PG11 protein after PIPLC treatment (Fig. 7B, lanes 3 and 4). This shift in mobility is characteristic of many GPI-anchored proteins after digestion with PIPLC and is probably due to changes in SDS binding after removal of diacylglycerol (Ferguson and Williams, 1988; Rosenberry, 1991).

The PG11 moPrP Molecule Is Hydrophobic, and Carbonate Inextractable, Even after Cleavage of Its GPI Anchor—The fact that the PG11 protein is retained on the cell surface even after removal of the GPI anchor by PIPLC suggests that the polypeptide chain has an additional mode of attachment to the plasma membrane. One possibility is that the polypeptide chain interacts directly with the lipid bilayer. Alternatively, the protein may aggregate with itself or bind tightly to another membrane protein. To investigate these possibilities, we performed two tests that are commonly used to distinguish integral from peripheral membrane proteins.

First, we subjected moPrP to phase partitioning in the detergent Triton X-114. In this method, integral membrane and other hydrophobic proteins are found in the detergent phase, while peripheral membrane and soluble proteins are found in the aqueous phase (Bordier, 1981). We found that before treatment with PIPLC, both wild-type and PG11 moPrPs partitioned almost exclusively into the detergent phase, as would be expected for molecules carrying an intact GPI anchor (Fig. 8A, lanes 1 and 2 and 5 and 6). PIPLC treatment shifted the wild-type protein almost completely into the aqueous phase (Fig. 8A, lanes 3 and 4 and Fig. 8B), a phenomenon that is observed for other GPI-anchored proteins (Englund, 1993), and which results from loss of the hydrophobic diacylglycerol por-
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**Fig. 8.** PIPLC-treated PG11 PrP is retained in the detergent phase after Triton X-114 phase partitioning and is not extractable with alkaline carbonate buffer. Panel A, CHO cells expressing untagged wild-type (WT) or PG11 moPrP, and N2a cells expressing a chPrP/M6PR transmembrane chimera, were surface-biotinylated, and lysed at 4°C in a buffer containing 1% Triton X-114. After the temperature was raised to 37°C, the detergent phase was recovered, diluted to the original volume, and split in half. Half was incubated with PIPLC at 4°C for 2 h (+ lanes), and the other half was left untreated (− lanes). Phase separation was then repeated, and moPrP in the detergent (D) and aqueous (A) phases was immunoprecipitated with antibody P45–66 (lanes 1–8); chPrP/M6PR was immunoprecipitated with antibody F35–96 (lanes 9–12). SDS-polyacrylamide gels of the immunoprecipitates were electoblotted, and the blots developed with horseradish peroxidase-streptavidin and ECL to visualize biotinylated proteins. Panel B, FPR bands from the experiment shown in panel A, and from two additional experiments, were quantitated by densitometry, and the amount of PrP in the detergent phase after PIPLC treatment (lanes 3, 7, and 11) was plotted as a percentage of the total amount of PrP present (detergent + aqueous phases). Each bar represents the mean ± S.D. Panel C, CHO cells expressing PG11 moPrP were surface-biotinylated and incubated with PIPLC for 2 h at 4°C. Cells were then homogenized in PBS, and a postnuclear membrane fraction prepared. Membranes were resuspended either in PBS or in carbonate buffer and were then collected by centrifugation. MoPrP in the pellet and supernatant fractions was then analyzed by immunoprecipitation, followed by SDS-PAGE, electrobblotting, and visualization with HRP-streptavidin and ECL (lanes 1–4; bracket indicates PrP-specific bands). Pellet and supernatant fractions were also immunoblotted to detect clathrin heavy chain (arrow), a marker peripheral membrane protein (lanes 5–8).

The data reported here demonstrate that a mutant form of moPrP containing 11 octapeptide repeats displays an aberrant mechanism of membrane association when expressed in cultured CHO cells. These results are directly relevant to human prion diseases, since an insertional mutation in the PrP gene that results in 11 octapeptide repeats has been strongly linked to familial CJD in a large English family (Poulter et al., 1992; Collinge et al., 1992) and has also been described in two smaller families (Nicholl et al., 1995; Oda et al., 1995). This report presents the first description of an abnormality in the biochemical properties of a mutant PrP molecule, and it raises the possibility that an alteration in the membrane attachment of PrP is a critical feature of prion diseases.

We have presented several lines of evidence indicating that, while both wild-type and PG11 forms of PrP are glycosylated and delivered to the cell surface, they differ dramatically in their mode of membrane association. The surface localization of both proteins in intact cells has been shown by immunofluorescence staining, as well as by accessibility to externally applied biotinylation and iodination reagents, and to trypsin. Both wild-type and PG11 PrPs also contain a GPI anchor, as demonstrated by metabolic incorporation of [3H]ethanolamine and [3H]-fatty acids, which are anchor constituents. The critical difference in membrane attachment between wild-type and PG11 PrP is revealed by digestion of intact cells with PIPLC, which is a bacterial enzyme that specifically cleaves the GPI anchor, removing the diacylglycerol portion that is embedded in the lipid bilayer. While the wild-type protein is released near quantitatively by PIPLC, the PG11 protein is almost completely retained on the cell surface after digestion. We have shown that the inability of PIPLC to release PG11 PrP is not due to a failure to cleave the GPI anchor, since the phospholipase almost completely removes [3H]-fatty acid label from the protein when applied to intact cells, and produces a character-
istic decrease in its electrophoretic mobility. These observations make it unlikely that aggregation of the mutant protein on the cell surface, or interaction with other membrane components, renders the GPI anchor inaccessible to enzymatic cleavage, at least for the majority of the molecules; however, our data do not rule out the possibility that the anchors of a small proportion of the molecules are resistant to cleavage.

The results indicate that PG11 PrP must have a second mechanism of membrane attachment in addition to the GPI anchor. Several possible models can be envisioned. One is that the PG11 polypeptide chain is directly integrated into the lipid bilayer. In support of this proposal, we find that after cleavage of the GPI anchor with PIPLC, 65% of the mutant PrP still partitions into the detergent phase after Triton X-114 phase separation (Bordier, 1981), in comparison to 15% for wild-type PrP (Fig. 8B). These results indicate that the PG11 molecule is quite hydrophobic, even after removal of diacylglycerol from the GPI anchor, suggesting that a portion of the protein has the potential to interact with lipids. The fact that 85% of a bona fide transmembrane protein was retained in the detergent phase after partitioning in Triton X-114 (Fig. 8B) might indicate that PG11 possesses a shorter or less hydrophobic lipid-associated domain than the transmembrane protein. Direct association between the polypeptide chain and the lipid bilayer is also suggested by failure to release PIPLC-digested PG11 from isolated membranes using alkaline carbonate buffer, which disrupts many protein-protein interactions. We note that transmembrane forms of PrP have been observed when PrP mRNA is translated in cell-free systems (Hay et al., 1987; Yost et al., 1990; Lopez et al., 1990; DeFea et al., 1994) and that several other proteins are proposed to have both a GPI anchor and a membrane-integrated polypeptide chain (Köster and Strand, 1994; Hitt et al., 1994; Howell et al., 1994). Nevertheless, it will be necessary to employ additional techniques, such as labeling with lipid-soluble reagents and mapping the accessibility of sequence epitopes, to decide whether or not the PG11 polypeptide chain loops into or traverses the lipid bilayer.

A second possible model is that PG11 moPrP binds tightly to other membrane-associated molecules, thereby preventing release of mutant PrP after cleavage of its PIPLC anchor. This hypothetical interaction would have to be stable in carbonate buffer at pH 11.5. One potentially relevant class of surface binding sites is proteoglycan molecules. PrPSc binds to heparinagarose beads (Caughney et al., 1994), and we have found that PrP-containing bacterial fusion proteins bind to glycosaminoglycan sites on the surface of cultured cells.3 In this model, one would have to postulate that the conformation of the PG11 molecule is altered in such a way that it binds more tightly than the wild type molecule to membrane-associated sites.

A final possibility is that PG11 moPrP forms aggregates on the cell surface and that these remain attached to the membranes after PIPLC treatment by virtue of a small number of molecules whose GPI anchors have escaped, or are resistant to cleavage. In fact, we have found the PG11 protein expressed in CHO cells forms detergent-insoluble aggregates that can be recovered by ultracentrifugation.4 Arguing against an effect of aggregation, however, is the high efficiency of PIPLC cleavage (~90%, as judged by loss of $^{3}$H-fatty acid label; Fig. 7B, lanes 7 and 8); and the observation that no additional PG11 protein is released by prolonging the period of PIPLC digestion to 6 h (Fig. 6B).

We have noted that the glycosylation pattern of PG11 PrP differs from that of wild-type PrP, with the mutant protein displaying a larger proportion of less glycosylated forms (Fig. 6A). We have also observed that the small amount (1–5%) of PG11 PrP that can be released by PIPLC is usually the most heavily glycosylated form (Fig. 6C). These results suggest that there may be a relationship between the altered glycosylation state of mutant PrP and its aberrant membrane association, a proposal consistent with the observation that PrPSc synthesized in infected neuroblastoma cells also displays a predominance of less glycosylated forms (Caughney and Raymond, 1991)

We have recently found that PG11 moPrP produced in CHO cells displays two of the characteristic biochemical properties of PrPSc, aggregation in non-ionic detergent, and resistance to protease digestion.4 We have not yet tested whether the protein is infectious. It will be important now to determine how these scrapie-like properties are related to the abnormal membrane association of PG11, and whether the conformational changes that underlie generation of PrPSc involve alterations in membrane attachment. Although there are no data available on the protease sensitivity of PG11 PrP from human patients, these individuals do not develop symptoms until adulthood (Collinge et al., 1992), suggesting that accumulation of PrPSc is a slow process that may require many years. Since we find that PG11 moPrP molecules in CHO cells acquire an aberrant membrane association, and become protease-resistant and detergent-insoluble,4 within a matter of hours, the generation of PrPSc may be more efficient in CHO cells than in neurons in vivo. There is one report that CHO cells cannot be infected by scrapie prions (Butler et al., 1988), but the cellular factors that influence prion replication are poorly understood and may be different from those that affect PrPSc production from mutant molecules.

Although our study has focused on a mutant form of PrP, our results lead us to hypothesize that the PrPSc produced during prion infection possesses an aberrant membrane association similar to that of PG11. This proposal is generally consistent with a large body of literature, some of it dating back more than 30 years, suggesting that infectious PrPSc is tightly associated with cellular membranes, and is very hydrophobic (Hunter et al., 1964; Gibbons and Hunter, 1967; Prusiner et al., 1978). Of particular relevance, it has been reported that, although PrPSc possesses a GPI anchor, it is not readily releasable from brain membranes by PIPLC (Stahl et al., 1990; Safran et al., 1991). Lack of release by PIPLC does not result from sequestration in a membrane-bound compartment, since PrPSc is susceptible to biotinylation with membrane-impermeant reagents, and to cleavage by proteases; moreover, the GPI anchor of purified PrPSc is PIPLC-sensitive, arguing that the anchor is not intrinsically uncleavable. These properties of PrPSc from infected brain are strikingly reminiscent of those we have described for PG11 PrP in CHO cells. It is uncertain from the published studies whether the failure of PIPLC to release membrane-bound PrPSc results from inaccessibility of the anchor structure, or rather, signifies a second, GPI-independent form of membrane attachment. Our results with PG11 PrP argue for the second possibility. It will be useful now to reexamine the membrane association of PrPSc from scrapie-infected cells in culture, using experimental approaches similar to those we have described here.

Most of the known pathogenic mutations in human PrP are single amino acid substitutions in the C-terminal half of the molecule, in contrast to the 48 amino acid insertion in the N-terminal half that we have modeled here (Prusiner and Hsiao, 1994). We find that several moPrPs carrying point mutations homologous to those found in human PrP are not released from the surface of transfected CHO cells by PIPLC.
implying an aberrant form of membrane association like that described for PG11. These data support the idea that alterations in membrane attachment may be a general feature of both genetic and infectious forms of prion disease.

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