Prion Proteins Carrying Pathogenic Mutations Are Resistant to Phospholipase Cleavage of Their Glycolipid Anchors†

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ABSTRACT: Familial prion diseases are linked to mutations in the gene encoding PrP, a protein of unknown function that is attached to the plasma membrane of neurons and several other cell types by a phosphatidylinositol-containing, glycolipid anchor. We have previously found that PrP molecules carrying disease-associated mutations display several biochemical attributes of PrP^{Sc}, the pathogenic isoform of PrP, when expressed in cultured Chinese hamster ovary cells. One of the distinctive properties of these mutant PrPs is their abnormal association with cell membranes, as revealed by their retention on the cell surface after treatment with a bacterial phospholipase that normally cleaves the glycolipid anchor. We demonstrate here that mutant PrP molecules, either expressed on intact cells or solubilized in nondenaturing detergents, are partially resistant to phospholipase cleavage. The anchor becomes fully susceptible to the enzyme when the proteins are denatured in SDS. These results suggest that the mutant PrP conformation, state of aggregation, or association with other molecules renders the glycolipid anchor physically inaccessible to cleavage. This conclusion stands in contrast to our previous suggestion that mutant PrP molecules are poorly released from the cell surface because they possess a secondary mechanism of membrane attachment in addition to the glycolipid anchor. Since PrP^{Sc} from scrapie-infected brain and cultured cells is also inefficiently released from membranes by phospholipase, resistance to this enzyme may be a molecular marker of the scrapie state.

Prion diseases are fatal neurodegenerative disorders that can have an infectious or genetic etiology, or can arise sporadically. This group of diseases includes kuru, Creutzfeldt-Jakob disease (CJD),[1] Gerstmann-Sträussler syndrome (GSS), and fatal familial insomnia (FFI) in human beings, along with scrapie and bovine spongiform encephalopathy in animals. All of these diseases are associated with conversion of a normal, cell-surface glycoprotein designated PrP^{C} to a pathogenic isoform denoted PrP^{Sc} that is hypothesized to be the only component of infectious prion particles (reviewed in refs *1*−*3*). Current evidence suggests that the essential difference between the two forms of PrP lies in their conformation, with PrP^{Sc} having a substantially higher β-sheet content (*4*−*8*).

We have been interested in familial prion diseases, which include 10% of the cases of CJD and all cases of GSS and FFI (*9*−*11*). These cases are all linked to dominantly inherited, germline mutations in the PrP gene on chromosome 20. The mutations are presumed to favor spontaneous conversion of the protein to the PrP^{Sc} state without the necessity for contact with exogenous infectious agent (*12*). Point mutations occur in the C-terminal half of the PrP molecule, and are associated with either CJD, GSS, or FFI. Insertional mutations, which are associated with a variable phenotype that can include features of CJD or GSS, consist of one to nine additional copies of an octapeptide repeat that is normally present in five copies in the N-terminal half of the protein.

We have previously established a cell culture model of familial prion diseases by constructing transfected lines of Chinese hamster ovary (CHO) cells that express mutant mouse PrP (moPrP) molecules whose human homologues are associated with each of the three familial prion diseases of humans (*13*−*18*). We have found that the mutant PrPs acquire several biochemical attributes reminiscent of PrP^{Sc}, including insolubility in nondenaturing detergents and resistance to digestion by low concentrations of proteinase K. We therefore believe that the transfected cells reproduce key events in the conversion of PrP^{C} to the PrP^{Sc} state, and we have used this model system to analyze several mechanistic features of the conversion process.

In earlier studies, we observed that mutant PrP molecules expressed in transfected CHO cells exhibit an abnormal association with cell membranes (*13*, *14*). Both PrP^{C} and PrP^{Sc} are attached to membranes via a glycosylphosphatidylinositol (GPI) anchor, which is added posttranslationally in the endoplasmic reticulum after cleavage of a C-terminal

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[1] Abbreviations: CHO, Chinese hamster ovary; CJD, Creutzfeldt-Jakob disease; ECL, enhanced chemiluminescence; FFI, fatal familial insomnia; GPI, glycosylphosphatidylinositol; GSS, Gerstmann-Sträussler syndrome; HRP, horseradish peroxidase; moPrP, mouse prion protein; 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; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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segment of 23 amino acids (*19*−*21*). Wild-type PrP^C can be efficiently released from cell membranes by treatment with the bacterial enzyme phosphatidylinositol-specific phospholipase C (PIPLC), which cleaves off the glycerolipid portion of the anchor (*13*, *22*, *23*). In contrast, we found that PrP molecules carrying any one of seven different pathogenic mutations were relatively resistant to release from the membranes of CHO cells by PIPLC (*13*, *14*, *16*). Similar results have now been obtained in other cell types for certain of these mutations (*24*; R. Chiesa and D. A. Harris, unpublished data). We previously proposed that the mutant proteins were not released by PIPLC because they possessed a secondary mechanism of membrane attachment in addition to the GPI anchor; this secondary association might include integration of the polypeptide chain into the lipid bilayer, or tight binding of the polypeptide chain to other membrane components (*13*). In support of this explanation, we reported that PIPLC treatment of intact cells removed metabolically incorporated [^{3}H]fatty acid label from the mutant proteins, indicating that the GPI anchor had been cleaved but that some other mechanism was responsible for retaining the proteins on the cell surface.

In contrast to these previous results, the data reported here indicate that a substantial proportion of mutant PrP molecules are actually resistant to the action of PIPLC because either their conformation, state of aggregation, or association with other molecules renders their GPI anchors physically inaccessible to cleavage. This conclusion makes it unnecessary to postulate a mechanism of membrane attachment in addition to the GPI anchor. Since PrP^{Sc} from scrapie-infected brain and cultured cells is also inefficiently released from membranes by PIPLC (*14*, *23*, *25*, *26*), resistance to the action of the phospholipase may be a general property of the scrapie state.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies. Cell culture reagents were from the Tissue Culture Support Center at Washington University. PIPLC from *Bacillus thuringiensis* was prepared as described previously (*27*). *N*-Glycosidase F was from Boehringer Mannheim and sulfo-biotin-X-NHS from Calbiochem. [^{35}S]-Methionine (Pro-Mix, 1000 Ci/mmol) was from Amersham and [9,10-^{3}H]palmitic acid (30−60 Ci/mmol) from American Radiolabeled Chemicals. All other reagents were from Sigma.

The antibody designated P45−66, raised by immunization of rabbits with a synthetic peptide encompassing moPrP residues 45−66, has been described previously (*13*). R. Kascsak (Institute for Basic Research, Staten Island, NY) kindly provided 3F4, a mouse monoclonal antibody that was raised against PrP 27−30 from infected hamster brain and that reacts with hamster but not mouse PrP (*28*).

Cell Lines. Chinese hamster ovary (CHO) cells were grown in MEM-α containing 7.5% fetal calf serum and penicillin/streptomycin in an atmosphere of 5% CO_2/95% air. CHO cell lines expressing PG14,[2] D177N (Met-128), and E199K moPrPs have been described previously (*13*, *14*). The wild-type and PG14 constructs contained an epitope tag for the monoclonal antibody 3F4 (L108M, V111M in wild-type numbering). The introduction of this tag does not affect the properties of the protein (*29*).

Metabolic Labeling, Surface Biotinylation, and Immunoprecipitation. Confluent cultures of transfected CHO cells were labeled in methionine-free MEM containing [^{35}S]-methionine (300 μCi/mL), or in DMEM containing [^{3}H]-palmitic acid (5−10 mCi/mL) and 10 mg/mL fatty acid-free bovine serum albumin. Surface biotinylation was carried out with sulfo-biotin-X-NHS as described previously (*27*). Immunoprecipitation was performed as described previously (*13*), using antibody P45−66 to recognize D177N and E199K moPrPs, and antibody 3F4 to recognize wild-type and PG14 moPrPs. Prior to immunoprecipitation, all samples were adjusted to 0.5% SDS, heated at 95 °C for 5 min, and then diluted 5-fold with 0.5% Triton X-100 and 50 mM Tris-HCl (pH 7.5); this procedure improves reactivity of PrP with antibodies. In some experiments, samples were deglycosylated prior to immunoprecipitation with 0.01 unit/mL *N*-glycosidase F for 16 h at 37 °C, to produce a single band of PrP that could be more easily quantitated. SDS−PAGE was carried out on 12% gels.

Phase Partitioning in Triton X-114. Metabolically labeled cells were solubilized at 4 °C in phosphate-buffered saline (PBS) containing 1% Triton X-114 and protease inhibitors (1 μg/mL pepstatin and leupeptin, 0.5 mM PMSF, and 2 mM EDTA). The detergent was diluted from a 12% stock solution that had been precondensed in PBS (*30*). Lysates were treated with PIPLC (1 unit/mL) for 2 h at 4 °C. After incubation at 37 °C for 20 min, aqueous and detergent phases were separated by centrifugation. The aqueous phase was discarded, and the detergent phase was diluted to the initial volume with PBS and incubated with or without PIPLC for 2 h at 4 °C and the phase separation repeated. PrP in the aqueous and detergent phases from the second partitioning was then immunoprecipitated.

Surface-biotinylated cells were treated with PIPLC (1 unit/mL) in Opti-MEM (Life Technologies) for 2 h at 4 °C. Cells were lysed in PBS containing 1% Triton X-114 and protease inhibitors. The medium was clarified by centrifugation for 1 min at 16000*g* and adjusted to 1% Triton X-114 by addition of $^1/_{12}$ volume of the concentrated detergent stock. Phase separation of medium and cell lysates was induced by incubation for 20 min at 37 °C followed by centrifugation. PrP was immunoprecipitated from the aqueous and detergent phases, fractionated by SDS−PAGE, and blotted onto polyvinylidene fluoride membranes. Blots were developed with horseradish peroxidase (HRP)-coupled streptavidin, and visualized using enhanced chemiluminescence (ECL) (Amersham). Films were digitized using an Epson Expression 636 flatbed scanner and images analyzed using SigmaScan Pro (SPSS Science).

PIPLC Treatment of Detergent-Solubilized PrP. To treat PrP under nondenaturing conditions, metabolically labeled cells were lysed in a buffer that contained 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% Triton X-100, and 0.5% sodium deoxycholate, supplemented with protease inhibitors. Lysates were centrifuged for 1 min at 16000*g* to remove debris, and PIPLC (0.3−3 units/mL) was added for 2 h at 4 °C. For phospholipase treatment of denatured PrP, metabolically labeled cells were lysed in 50 mM Tris-HCl (pH 7.5) and 0.5% SDS. The lysate was heated at 95 °C for 5 min, diluted

[2] This mutation was originally designated PG11 (*13*−*18*); however, resequencing of the cDNA now reveals that it encodes 14 and not 11 octapeptide repeats (S. Lehmann and D. A. Harris, note in preparation).

5-fold with 0.5% Triton X-100 and 50 mM Tris-HCl (pH 7.5), and then incubated with PIPLC for 16 h at 37 °C.

RESULTS

For these studies, we have used stably transfected lines of CHO cells that express either wild-type moPrP or one of three different moPrP mutants: PG14, D177N, and E199K. The PG14 mutation[2] consists of an insertion of nine octapeptide repeats in addition to the five that are normally present in the N-terminal half of the protein; the homologous mutation in humans is associated with a variant phenotype characterized by dementia, ataxia, and PrP-containing amyloid plaques (*31*−*33*). D177N is homologous to a human mutation (D178N-Met129) that is associated with FFI (*34*). E199K is homologous to a human mutation (E200K) linked to CJD (*35*, *36*).

Mutant PrPs Remain Partially Resistant to PIPLC after Solubilization in Nondenaturing Detergents. We previously reported that mutant moPrPs are poorly released by PIPLC from the surface of intact cells that have been labeled with a membrane-impermeant biotinylation reagent (*13*, *14*). Although we previously suggested that this phenomenon resulted from the existence of a mechanism that retains the protein on the membrane independent of the GPI anchor (*13*), it could also be due to inefficient cleavage of the anchor by the phospholipase. To test the latter possibility, we analyzed the PIPLC sensitivity of PrP that had been solubilized from the membrane under either denaturing or nondenaturing conditions. Solubilization of the PrP allowed us to assay the PIPLC sensitivity of the protein itself, independent of any effects of the lipid bilayer in which it is normally embedded. Moreover, by comparing denaturing and nondenaturing conditions of solubilization, we could determine whether the PIPLC sensitivity of PrP depended on the native protein conformation or intermolecular interactions. To monitor GPI anchor cleavage, we took advantage of the fact that removal of the anchor by phospholipase causes an anomalous decrease in the mobility of proteins on SDS−PAGE, probably as a result of changes in SDS binding after the loss of the glycerolipid moiety (*37*). Although the size of the this mobility shift is small, it could be easily detected if the proteins were enzymatically deglycosylated to eliminate electrophoretic heterogeneity due to the presence of N-linked sugars. Thus, the proportion of PrP molecules that shifted into a higher M_r band could be used as an indicator of the extent of GPI anchor cleavage.

The application of this assay to wild-type and D177N moPrPs is shown in Figure 1. In the absence of PIPLC treatment, both wild-type and D177N proteins migrate as a single, sharp band at 28 kDa (lane 1). After treatment with phospholipase, wild-type PrP is shifted into a single band at 29 kDa, reflecting nearly quantitative cleavage of the GPI anchor (lanes 2−6). This effect was observed regardless of whether the protein was solubilized under denaturing (0.5% SDS) or nondenaturing (0.5% Triton X-100/0.5% deoxycholate) conditions. In contrast, PIPLC treatment of D177N PrP that had been solubilized in a nondenaturing buffer shifted only 40−50% of the molecules into the upper band, even at the highest concentration of phospholipase that was tested (lanes 2−4). This result implied that about half of the PrP molecules were resistant to PIPLC cleavage of their GPI

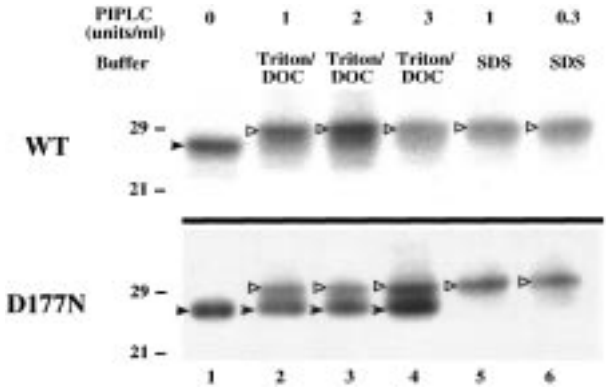

FIGURE 1: Mutant moPrP solubilized in nondenaturing detergents is partially resistant to PIPLC-induced electrophoretic retardation. CHO cells expressing wild-type (WT) or D177N PrP were labeled with [^{35}S]methionine for 4 h and were then lysed in either 0.5% Triton X-100/0.5% deoxycholate (Triton/DOC, lanes 2−4) or 0.5% SDS (lanes 5 and 6). The Triton/DOC samples were incubated with PIPLC at the indicated concentration for 2 h at 4 °C prior to immunoprecipitation of PrP and analysis by SDS−PAGE. The SDS samples were heated at 95 °C for 5 min, diluted 5-fold with 0.5% Triton X-100, and then incubated with PIPLC for 16 h at 37 °C prior to immunoprecipitation and SDS−PAGE. PrP in lane 1 was immunoprecipitated without PIPLC treatment. All samples were enzymatically deglycosylated. PrP lacking a GPI anchor (white arrowhead) migrates slower than PrP containing an anchor (black arrow). Molecular size markers are denoted in kilodaltons. Autoradiograms were exposed for 16 h.

anchors. Denaturing the mutant protein by heating it at 95 °C in SDS prior to PIPLC treatment rendered the molecules fully susceptible to anchor cleavage, as indicated by a complete upward shift in electrophoretic mobility, even at the lowest enzyme concentration that was tested (lanes 5 and 6). These results indicate that a substantial proportion of mutant PrP molecules are resistant to the action of PIPLC, even when solubilized from the membrane, and this property is abolished by a denaturing detergent.

Cleavage of the GPI anchor can also be scored by partitioning proteins in the nondenaturing detergent Triton X-114 to separate hydrophobic from hydrophilic molecules. Solutions of Triton X-114 separate into detergent-rich and aqueous phases at 37 °C (*30*). The presence of a hydrophobic GPI anchor causes proteins to partition into the detergent phase, while removal of the anchor shifts them into the aqueous phase. Figure 2 shows the application of the method to wild-type moPrP and three different mutants. Prior to PIPLC treatment, both wild-type and mutant PrPs partitioned into the detergent phase, as expected for proteins bearing a GPI anchor. After PIPLC treatment of the solubilized protein, wild-type PrP was almost completely shifted into the aqueous phase, consistent with removal of the anchor structure. In contrast, and as reported previously (*13*, *18*), about half of the PG14 moPrP remained in the detergent phase; the same was true for the D177N moPrP, consistent with the results of the mobility shift assay in Figure 1. Interestingly, E199K moPrP, like the wild-type protein, was shifted almost entirely into the aqueous phase. We have previously observed that E199K moPrP is more readily releasable from the surface of biotinylated cells than other mutant moPrPs (50% released, compared to <5% for other mutants; *14*).

The two assays utilized thus far measure the PIPLC sensitivity of PrP molecules indirectly, on the basis of changes in the biochemical properties of the proteins after

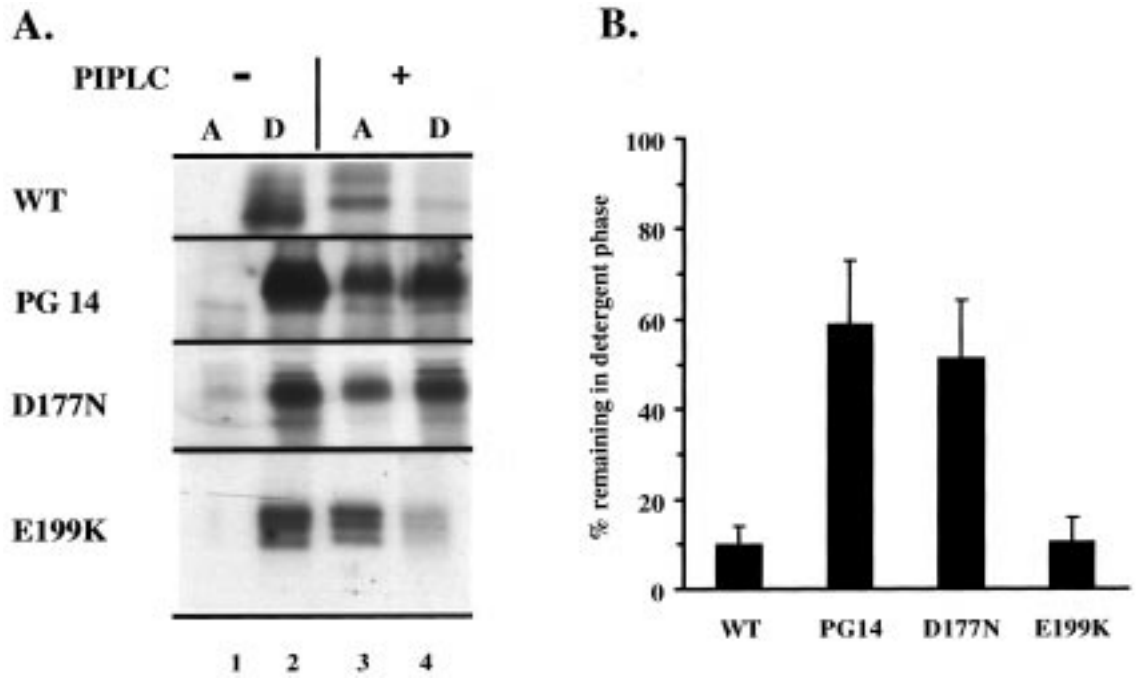

FIGURE 2: PG14 and D177N moPrPs remain partially hydrophobic after PIPLC treatment and phase partitioning in Triton X-114. (A) CHO cells expressing wild-type (WT) or mutant PrPs were labeled with [^{35}S]methionine for 4 h and were then lysed in 1% Triton X-114. Lysates were then incubated at 4 °C for 2 h either in the absence (lanes 1 and 2) or in the presence (lanes 3 and 4) of PIPLC. After phase partitioning, moPrP in the aqueous phase (A, lanes 1 and 3) and detergent phase (D, lanes 2 and 4) was immunoprecipitated and run on SDS−PAGE. Autoradiograms were exposed for 16 h. (B) PrP bands were quantitated by film densitometry, and the amount of PrP remaining in the detergent phase after PIPLC treatment was expressed as a percentage of the total amount of PrP (detergent and aqueous phases). Each bar represents the mean ± standard deviation of values from at least three separate experiments.

treatment with phospholipase. To directly determine whether PIPLC treatment cleaves the GPI anchor of mutant PrPs, we metabolically labeled cells with [^{3}H]palmitate, a fatty acid which becomes incorporated into the glycerolipid portion of the anchor. Proteins were then solubilized in either Triton/deoxycholate or SDS and treated with PIPLC. We found, as expected, that PIPLC fully removes the [^{3}H]palmitate label from wild-type moPrP when the protein is solubilized in either the denaturing or nondenaturing buffers (Figure 3). In contrast, approximately 70% of the label remains on PG14 moPrP and about 60% on D177N moPrP when PIPLC treatment is carried out in Triton/deoxycholate. After denaturation in SDS, both proteins become fully susceptible to removal of [^{3}H]palmitate. This result directly demonstrates that a substantial proportion of PG14 and D177N molecules are resistant to GPI anchor cleavage in the native state. Again, E199K moPrP behaves in a manner different from those of the other two mutants, with all of the [^{3}H]palmitate being removed by PIPLC, similar to the result for the wild-type protein.

Mutant PrPs Are Partially Resistant to PIPLC when Expressed on the Cell Surface. The previous results establish that mutant PrPs are partially resistant to PIPLC-induced anchor cleavage when solubilized from cell membranes under native but not denaturing conditions. To investigate whether the proteins are phospholipase-resistant when attached to the plasma membrane of intact cells, we labeled cells by surface biotinylation, treated them with PIPLC, and then carried out phase partitioning of Triton X-114 extracts of the cells and media (Figure 4). Prior to PIPLC treatment, all the moPrPs partitioned into the detergent phase because of the presence of the GPI anchor. As expected, wild-type moPrP was quantitatively released into the medium by PIPLC, and was recovered in the aqueous phase, consistent with removal of the GPI anchor. In contrast, the PG14 and D177N proteins were completely retained on the cell surface, with 85 and 50%, respectively, partitioning into the detergent phase. About half of the E199K molecules were released by PIPLC in a hydrophilic form that presumably lacks the GPI anchor, but those that remained on the cell surface were largely (80%) recovered in the detergent phase. These results indicate that all three mutant PrP molecules are partially resistant to anchor removal in the context of an intact membrane. In fact, the PG14 and E199K proteins appear to be even more PIPLC-resistant when attached to the cell membrane (Figure 4) than when solubilized in nondenaturing detergents (Figures 2 and 3), raising the possibility that detergent extraction can modify the properties of the molecules.

DISCUSSION

Mutant moPrP molecules whose human homologues are associated with inherited prion diseases display several biochemical attributes of PrP^{Sc} when expressed in cultured CHO cells (*13*−*18*). One of the distinctive features of these mutant PrPs is their abnormal association with cell membranes, as manifested by retention on the cell surface after treatment with PIPLC, a bacterial enzyme that normally cleaves the GPI membrane anchor (*13*, *14*). We demonstrate here that this property is largely a consequence of the resistance of mutant PrPs to PIPLC-induced anchor cleavage, and is dependent on the preservation of the native structure or intermolecular associations of the protein. Our conclusions are based upon the fact that a substantial proportion of the mutant PrP molecules retain their GPI anchors after PIPLC treatment, either on intact cells or after solubilization in

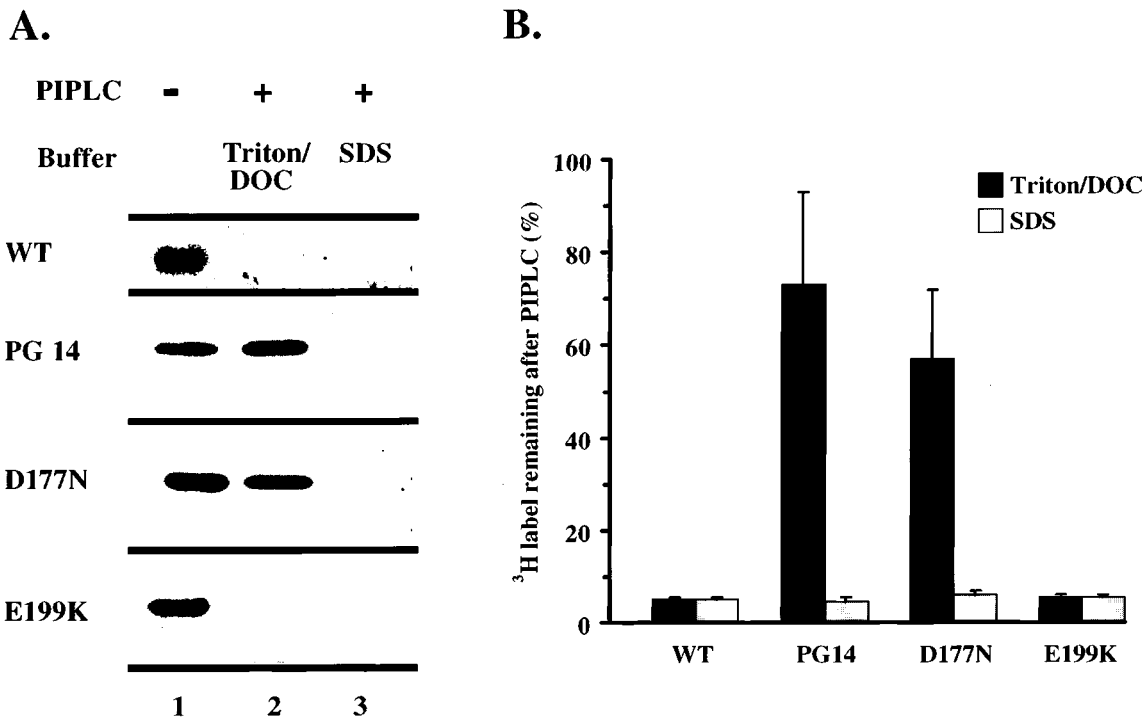

FIGURE 3: PG14 and D177N moPrPs solubilized in nondenaturing detergents are resistant to PIPLC cleavage of metabolically incorporated [^{3}H]palmitate. (A) CHO cells expressing wild-type (WT) or mutant PrPs were labeled for 16 h with [^{3}H]palmitate and were then lysed in either 0.5% Triton X-100/0.5% deoxycholate (Triton/DOC, lane 2) or 0.5% SDS (lane 3). Samples in lanes 2 and 3 were treated with PIPLC as described in the legend of Figure 1, prior to immunoprecipitation and SDS−PAGE. PrP in lane 1 was immunoprecipitated without PIPLC treatment. All samples were enzymatically deglycosylated. Autoradiograms were exposed for 10−20 days. (B) PrP bands were quantitated by film densitometry, and the amount of ^{3}H label remaining after PIPLC treatment in either Triton/DOC or SDS was expressed as a percentage of the amount of ^{3}H label present in samples not treated with PIPLC. Each bar represents the mean ± standard deviation of values from at least three separate experiments.

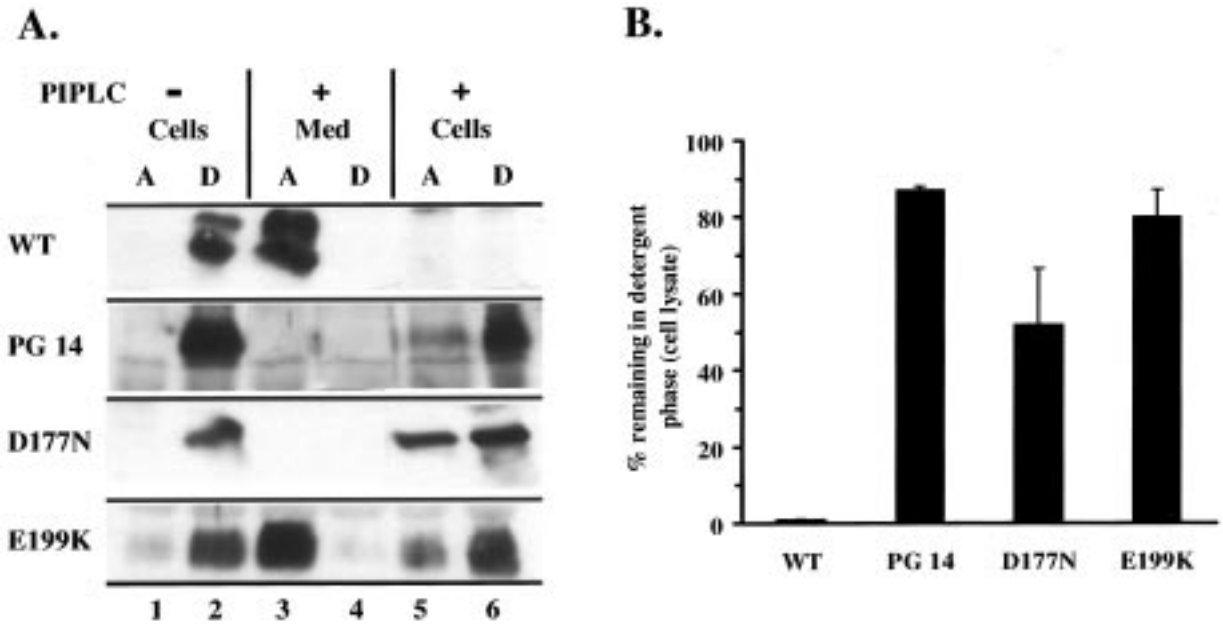

FIGURE 4: Mutant moPrPs remain partially hydrophobic after PIPLC treatment of intact cells followed by phase partitioning in Triton X-114. (A) CHO cells expressing wild-type (WT) or mutant PrPs were biotinylated at 4 °C with a membrane-impermeant reagent and were then incubated for 2 h at 4 °C either in the absence (lanes 1 and 2) or in the presence (lanes 3−6) of PIPLC. Cells were lysed in 1% Triton X-114 (Cells, lanes 1, 2, 5, and 6), and the PIPLC incubation medium was adjusted to 1% Triton X-114 (Med, lanes 3 and 4). After phase partitioning, moPrP in the aqueous phase (A, lanes 1, 3, and 5) and detergent phase (D, lanes 2, 4, and 6) was immunoprecipitated, separated by SDS−PAGE, and visualized by developing blots of the gel with HRP−streptavidin and ECL. The medium from cells incubated without PIPLC is not shown, since it contained no PrP. (B) PrP bands were quantitated by film densitometry, and the amount of PrP remaining in the detergent phase of the cell lysate after PIPLC treatment was expressed as a percentage of the total amount of PrP in the lysate (detergent and aqueous phases). Each bar represents the mean ± standard deviation of values from at least three separate experiments.

nondenaturing detergents, as assayed by electrophoretic migration, Triton X-114 phase partitioning, and [^{3}H]palmitate labeling. Denaturation by heating in SDS renders the anchors of the mutant PrPs fully susceptible to cleavage by phospholipase.

In a previous publication, we proposed several hypothetical mechanisms to explain why PIPLC fails to release mutant PrPs from cell membranes (*13*). One possible model was that the mutant proteins possessed a second mode of attachment to the lipid bilayer in addition to the GPI anchor. For example, the polypeptide chain of the mutant PrP might integrate into the lipid bilayer, or bind strongly to other membrane-associated molecules. If this were the case, then the protein would be retained on the cell surface even if its GPI anchor had been cleaved by PIPLC. To test this prediction, we carried out [^{3}H]fatty acid labeling experiments, and found that PIPLC treatment of cells substantially reduced the amount of ^{3}H label recovered in PG14 PrP that remained on the cell surface (*13*). This result, which supports the existence of a secondary membrane anchor, contrasts with the data reported here (Figure 3). However, in the earlier experiments, in which a mixture of [^{3}H]palmitate and [^{3}H]stearate was used, the PrP band was weak, and it was difficult to accurately gauge how much ^{3}H label was actually removed from the protein by treatment with the phospholipase; consequently, we could not rule out the possibility that some of the molecules were resistant to removal of their anchors. In the work presented here, we have been able to substantially increase the signal by labeling with large amounts of [^{3}H]palmitate. It is now clear from multiple repetitions that the majority of the radioactive label remains associated with PG14 and D177N PrP molecules after PIPLC treatment, even when digestion is carried out with proteins solubilized in Triton/deoxycholate.

For those mutant molecules that are resistant to GPI anchor cleavage, it is therefore unnecessary to postulate a secondary mechanism of membrane attachment to explain the lack of PIPLC release. Nevertheless, our data do not rule out the existence of additional modes of association between PrP and cell membranes. For example, Lingappa and colleagues have postulated the existence of two transmembrane species of PrP, each with the same membrane-spanning segment (residues 112−135), but with opposite orientations of the polypeptide chain (*38*, *39*). It was suggested that an increase in the proportion of one of these species was induced by the presence of a disease-causing mutation in PrP (A117V), and that this transmembrane form was a cause of neurodegeneration. However, a difficulty with these studies is that the percentage of the transmembrane forms is usually quite low, and in addition it was not determined whether these molecules contained a GPI anchor. It is also not known if mutations other than A117V have a similar effect.

Although the majority of PG14 and D177N PrP molecules are resistant to anchor cleavage, we have detected a population (30−50% of the total) that is PIPLC-sensitive in each of three different assays (Figures 1−3). We do not think that these molecules represent a distinct population with a different mode of membrane association, but rather that they are generated by the conditions used to solubilize the protein. In support of this idea, we have found that alteration of the detergent lysis conditions can either increase or decrease the proportion of cleavable molecules (data not shown). Moreover, we have noted that the proportion of mutant molecules that escapes cleavage is higher when the phospholipase is applied to intact cells than when it is added to detergent lysates; this is especially noticeable for E199K PrP, but can be seen for PG14 also (compare Figure 4 to Figures 2 and 3). This observation suggests that solubilization, even in nondenaturing detergents, can increase the accessibility of the GPI anchor. However, we cannot rule out the possibility that some mutant PrP molecules are PIPLC-sensitive prior to their extraction from the cell membrane. Such molecules are not likely to possess a transmembrane domain that retains them on the cell surface after anchor cleavage, since they are hydrophilic in Triton X-114 partitioning experiments (Figure 4, lane 5). A more likely possibility is that they reside on the exterior of PrP aggregates, and remain membrane-bound after anchor cleavage by virtue of their association with PIPLC-resistant molecules in the core of the aggregates (*13*).

E199K moPrP clearly displays biochemical properties that are distinct from those of other mutants. E199K is considerably more PIPLC-releasable, with 50% remaining on the surface after phospholipase treatment compared to >95% for other mutants that have been tested (*14*). Moreover, its GPI anchor is significantly more susceptible to cleavage by PIPLC after solubilization, and indeed, it is indistinguishable from wild-type PrP when cleavage is assayed by either Triton X-114 phase partitioning (Figure 2) or [^{3}H]palmitate labeling (Figure 3). Interestingly, the E199K molecules that are not released from cells by PIPLC appear to have intact GPI anchors, as assessed by Triton X-114 partitioning (Figure 4 and ref *18*). In previous studies, we have shown that only these surface-retained molecules, but not those released into the medium, possess PrP^{Sc}-like properties, including detergent insolubility and protease resistance (*14*, *18*). Taken together, these results suggest that the E199K mutation alters the structure of the PrP molecule in a way that is different from that of some other pathogenic mutations. Consistent with this proposal, recent thermodynamic studies have suggested that some mutations (including D177N) significantly destabilize the structure of the molecule, while others (including E199K) do not (*40*−*42*). Interestingly, it has been reported that mutants in the first group aggregate as inclusion bodies when synthesized in the periplasm of bacteria, while mutants in the second group are recovered in a soluble form (*41*, *42*). Similarly, E199K moPrP is more detergent-soluble than other mutants when expressed in CHO cells (*14*, *15*). These results suggest the possibility that E199K is less PIPLC-resistant because it is less aggregated.

PIPLC resistance appears to depend on the cellular context in which PrP is expressed. Thus, the same set of octapeptide insertion mutants that are inefficiently released by PIPLC from 3T3 cells are released normally when expressed in N2a neuroblastoma cells (*24*). In addition, human D178N PrP appears to be fully PIPLC-releasable when synthesized in human neuroblastoma cells (*43*). Despite these differences among transformed cell lines, however, PIPLC resistance appears to be a bona fide property of mutant PrPs expressed in neurons in vivo, since we observe it for PG14 moPrP molecules expressed in the brains of transgenic mice (*44*). There are no reports of the PIPLC sensitivity of mutant PrPs from the brains of human patients.

What is the structural basis for the partial PIPLC resistance of mutant PrPs? The fact that the proteins are fully sensitive to cleavage after denaturation in SDS rules out the possibility that the GPI anchors are intrinsically resistant to cleavage because of a chemical modification such as acylation of the inositol ring (*45*). The fact that the mutant proteins remain largely PIPLC-resistant after solubilization in nondenaturing detergents suggests that this property does not depend on interaction with an intact lipid bilayer. However, it remains possible that detergent-stable associations with lipids or other membrane molecules could influence access to the enzyme. Indeed, if such interactions were cell type-specific, they could explain why PIPLC resistance varies among cell lines.

The fact that the mutant proteins are resistant to phospholipase digestion under mild but not denaturing detergents suggests that some aspect of secondary or higher-order structure may be involved in conferring PIPLC resistance. One possibility is that the mutant molecules are aggregated in such a way that access to the phospholipase is physically blocked. In support of this hypothesis, mutant PrPs are aggregated when expressed in CHO cells, as evidenced by detergent insolubility, and this property correlates with their degree of PIPLC resistance (*14*, *15*). Dense packing of GPI-anchored VSG molecules on the surface of trypanosomes is thought to prevent their cleavage by PIPLC (*46*, *47*). It is also possible that conformational alterations in individual PrP molecules could reduce the accessibility of the GPI anchor. Recent studies in which monoclonal antibodies were used indicate that the C-terminus of the polypeptide chain does not undergo major conformational change during the transition from PrP^{C} to PrP^{Sc} (*48*, *49*), but these studies do not directly address the disposition of the GPI anchor itself.

It is attractive to hypothesize that the biochemical alteration that inhibits PIPLC cleavage, whatever its underlying mechanism, reflects specific steps along the pathway from PrP^{C} to PrP^{Sc}. On the basis of their biochemical properties, we have postulated that mutant PrPs undergo stepwise conversion to a PrP^{Sc}-like state in CHO cells (*18*). The earliest step we have detected is the acquisition of PIPLC resistance, which we have shown takes place in the endoplasmic reticulum within minutes of polypeptide chain synthesis. We thus view PIPLC resistance as an operational property analogous to protease resistance, and suggest that it reflects an early and fundamental change in the structure or associations of mutant PrP attendant upon its conversion to the PrP^{Sc} state.

Like mutant PrPs synthesized in cultured cells, infectious PrP^{Sc} from scrapie-infected brain tissue and neuroblastoma cells is not efficiently released from membranes by PIPLC (*14*, *23*, *25*, *26*). The lack of release by PIPLC is not due to sequestration of the protein in the lumen of a vesicular compartment, since membrane-bound PrP^{Sc} is accessible to biotinylation and protease digestion (*14*, *25*, *26*). The GPI anchor of purified and denatured PrP^{Sc} can be cleaved by PIPLC (*21*, *26*), suggesting that the anchor is not chemically modified so it could be made intrinsically PIPLC-resistant. Taken together with the work reported here, these results suggest that PIPLC resistance may be a general property or marker of the PrP^{Sc} state. Further investigation of the structural and cellular mechanisms that account for this property are likely to provide new insights into the generation of prions.

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