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Mutant Prion Proteins Are Partially Retained in the Endoplasmic Reticulum*

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Familial prion diseases are linked to point and insertion mutations in the prion protein (PrP) gene that are presumed to favor conversion of the cellular isoform of PrP to the infectious isoform. In this report, we have investigated the subcellular localization of PrP molecules carrying pathogenic mutations using immunofluorescence staining, immunogold labeling, and PrP-green fluorescent protein chimeras. To facilitate visualization of the mutant proteins, we have utilized a novel Sindbis viral replicon engineered to produce high protein levels without cytopathology. We demonstrate that several different pathogenic mutations have a common effect on the trafficking of PrP, impairing delivery of the molecules to the cell surface and causing a portion of them to accumulate in the endoplasmic reticulum. These observations suggest that protein quality control in the endoplasmic reticulum may play an important role in prion diseases, as it does in some other inherited human disorders. Our experiments also show that chimeric PrP molecules with the sequence of green fluorescent protein inserted adjacent to the glycolipidation site are post-translationally modified and localized normally, thus documenting the utility of these constructs in cell biological studies of PrP.

Prion diseases are fatal neurodegenerative disorders of humans and animals that exemplify a novel mechanism of biological information transfer (1, 2). They are thought to be caused by a change in the conformation of PrP\(^{\text{C}}\),\(^*\) a neuronal membrane glycoprotein of unknown function, that results in its conversion to PrP\(^{\text{Sc}}\), a protease-resistant isoform of high \(\beta\)-sheet content that is infectious in the absence of nucleic acid.

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\(^{1}\) The abbreviations used are: PrP, prion protein; PrP\(^{\text{C}}\), scrapie prion protein; CHO, Chinese hamster ovary; GPI, glycosylphosphatidylinositol; WT, wild-type; ER, endoplasmic reticulum; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; BHK, baby hamster kidney; EM, electron microscopic; PDI, protein-disulfide isomerase; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PIPLC, phosphatidylinositol-specific phospholipase C; EYFP, enhanced yellow fluorescent protein.

PrP\(^{\text{Sc}}\) is postulated to physically interact with PrP\(^{\text{C}}\), acting as a nucleus or template in the formation of more PrP\(^{\text{Sc}}\). Self-propagating changes in protein conformation have also been described in lower eukaryotes such as bakers’ yeast and filamentous fungi, underlining the generality of the prion phenomenon in nature (3).

Family prion diseases are due to dominantly inherited mutations in the PrP gene on chromosome 20 that are presumed to favor spontaneous conversion of PrP\(^{\text{C}}\) to PrP\(^{\text{Sc}}\) (4, 5). Point or insertion mutations have been linked to Gerstmann-Sträussler syndrome, fatal familial insomnia, and \(\approx 10\%\) of the cases of Creutzfeldt-Jakob disease. The mechanism by which these mutations promote generation of the PrP\(^{\text{Sc}}\) conformation is still unclear. One possibility is that they structurally destabilize the PrP\(^{\text{C}}\) molecule, lowering the kinetic barrier to PrP\(^{\text{Sc}}\) formation. However, this is not likely to be the mechanism in all cases of familial prion diseases since some pathogenic mutations have little effect on the thermodynamic stability of PrP (6–9). Instead, these mutations may alter the interaction between PrP and other cellular components that influence the PrP\(^{\text{C}}\) \(\rightarrow\) PrP\(^{\text{Sc}}\) conversion reaction.

Cultured cells that express mutant PrP molecules offer an attractive system for analyzing the biochemical mechanisms underlying familial prion diseases. Much of our own work has utilized Chinese hamster ovary (CHO) cells that have been stably transfected to synthesize mouse PrP molecules carrying mutations whose human homologs are associated with familial Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome, or fatal familial insomnia (10–14). We have found that the mutant proteins in these cells display several biochemical properties that are characteristic of PrP\(^{\text{Sc}}\), including protease resistance, insolubility in nondenaturing detergents, and resistance of the C-terminal glycosyl-phosphatidylinositol (GPI) anchor to cleavage by phospholipase. These properties are not manifested by wild-type (WT) PrP or by PrP carrying a mutation that is homologous to a nonpathogenic amino acid polymorphism in human PrP (M129V), thus demonstrating the specificity of the effect. Although there are some differences between the mutant PrP molecules and authentic PrP\(^{\text{Sc}}\), such as their degree of protease resistance, it is nevertheless likely that CHO cells are reproducing several key features of PrP\(^{\text{Sc}}\) formation. We have observed the same three PrP\(^{\text{Sc}}\)-like properties when mutant PrPs are expressed in both undifferentiated and nerve growth factor-differentiated PC12 cells (15), and other investigators have reported similar results in neuroblastoma cells and fibroblasts (16–19). Although the biochemical properties can vary with the cell type and the mutation being expressed, the conclusion from all of these studies is that mutant PrPs can be converted to a PrP\(^{\text{Sc}}\)-like state in cultured cells. The fact that this occurs in non-neuronal as well as neuronal cell types suggests that whatever cellular cofactors play a role in the conversion process are likely to be ubiquitous.
Subcellular Localization of Mutant PrP

Despite extensive work on the biochemical properties of mutant PrPs in cultured cells, less attention has been paid to the precise localization of these proteins at a subcellular level. Several studies have suggested that mutant PrP molecules are inefficiently transported to the cell surface and are trapped in intracellular compartments (17–22). To further investigate these abnormalities, we undertook a detailed investigation of the localization of mutant PrPs in cultured cells using light and electron microscopic techniques. Our results demonstrate that several different pathogenic mutations share the property of impairing delivery of PrP molecules to the cell surface and causing their accumulation within the endoplasmic reticulum (ER). These observations raise the possibility that ER quality control mechanisms play an important role in prion diseases, as they do in some other inherited human disorders. From a technical standpoint, our experiments also document the utility of green fluorescent protein (GFP) chimeras in cell biological studies of PrP.

EXPERIMENTAL PROCEDURES

Plasmids—DNA fragments encoding 3F4 epitope-tagged WT or PG14 PrP were isolated from previously described plasmids (10) by digestion with HindIII and BamHI and, after blunting with Klenow, were subcloned into the Pml site of pSinRep21, which contains the Sindbis replicon (23, 24). The PG14 mutation was previously designated PGI (25).

To construct PrP-EGFP plasmids, a vector designated pEGFP-NC was first constructed in which the EGFP coding sequence was flanked on both the 5′ and 3′-ends by polylinkers. To create this vector, a 549-base pair fragment from pEGFP-C1 (CLONTECH, Palo Alto, CA) was isolated by digestion with BamHI and DraIII and ligated to a 4248-base pair fragment isolated from pEGFP-N1 (CLONTECH) by digestion with the same two enzymes. A 767-base pair fragment encompassing EGFP was then released from pEGFP-NC by digestion with SmaI and EcoRI; and after blunting with Klenow, it was cloned into the single StuI site that lies within codon 223 (alanine) of mouse PrP. The WT and PG14 mouse PrP sequences, which carried an epitope tag for monoclonal antibody 3F4, had been previously inserted between the HindIII and BamHI sites of the vector pBluescript II KS+ (Stratagene, La Jolla, CA), which does not contain a StuI site. A ClaI/KpnI fragment encompassing PrP-EGFP was then isolated from the Bluescript vector, blunt-ended with Klenow, and subcloned either into the Pml site of pSinRep21 or into the EcoRV site of pcDNA3 (Invitrogen, Carlsbad, CA), which does not contain a StuI site. A ClaI/XhoI fragment encompassing PrP-EGFP was then isolated from the Bluescript vector, blunt-ended with Klenow, and subcloned into either the Pml site of pSinRep21 or into the EcoRV site of pcDNA3 (Invitrogen, Carlsbad, CA), which does not contain a StuI site. A ClaI/XhoI fragment encompassing PrP-EGFP was then isolated from the Bluescript vector, blunt-ended with Klenow, and subcloned into either the Pml site of pSinRep21 or into the EcoRV site of pcDNA3 (Invitrogen, Carlsbad, CA), which does not contain a StuI site. A ClaI/XhoI fragment encompassing PrP-EGFP was then isolated from the Bluescript vector, blunt-ended with Klenow, and subcloned into either the Pml site of pSinRep21 or into the EcoRV site of pcDNA3 (Invitrogen, Carlsbad, CA), which does not contain a StuI site.

Cells—For the creation of stable populations of PrP-expressing cells, BHK-21 cells (American Type Culture Collection) were transfected with pSinRep21 plasmids using LipofectAMINE (Life Technologies, Inc.) and 24–48 h later were transferred to medium containing 5 μg/ml puromycin. After ~1 week (at which time all cells in mock-transfected cultures had been killed), antibiotic-resistant cells were expanded, and frozen stocks were prepared. Cells were maintained as a population without cloning in 5 μg/ml puromycin. All experiments were performed on cells that had been passaged <10 times. BHK cells were grown in α-minimal essential medium supplemented with 10% fetal bovine serum, nonessential amino acids, minimal essential medium vitamins, 100 units/ml penicillin, and 100 μg/ml streptomycin.

For the experiments shown in Fig. 10, PrP-EGFP constructs in pcDNA3 were introduced into BHK cells using LipofectAMINE, and cells were analyzed 24–48 h later. The experiment shown in Fig. 7 utilized stably transfected lines of CHO cells, described previously (11, 26), that express WT, PG14, P101L, D177N/M128, or F197S/M128V PrP.

Antibodies and Organelle Markers—Monoclonal antibody 3F4 was used at dilutions of 1:5000 for Western blotting and 1:500 for immunofluorescence staining. Polyclonal antibody P45-66 was raised against a synthetic peptide encompassing residues 45–66 of mouse PrP (10). The serum was used at a dilution of 1:250 for immunofluorescence staining, and the affinity-purified antibodies were used at a concentration of 4–8 μg/ml for EM immunogold labeling.

We utilized, as an ER marker, antibodies against protein-disulfide isomerase (PDI), either a rabbit polyclonal antisera from Peter Arvan (Albert Einstein College of Medicine) or a mouse monoclonal antibody from Stressgen Biotech Corp. (Victoria, British Columbia, Canada) was used at a dilution of 1:200. We utilized, as a Golgi marker, the plasmid pEYFP (CLONTECH), which was introduced into cells by transient transfection, or a polyclonal antibody against giantin (Covance Inc., Princeton, NJ), which was used at a dilution of 1:1000. To visualize lysosomes, we incubated cells with LysoTracker Red DND-99 (Molecular Probes, Inc. Eugene, OR) at a concentration of 62 nm for 2 h at 37 °C prior to fixation.

Immunofluorescence Staining and Imaging—Cells were seeded on glass coverslips in 35-mm dishes and grown for 24 h to 50% confluence. For surface staining of PrP, cells were washed with ice-cold phosphate-buffered saline (PBS) and incubated for 1 h at 4 °C with antibody 3F4 diluted 2:500 in goat serum and Opti-MEM (Life Technologies, Inc.), followed by washing with PBS and fixation in 4% paraformaldehyde, 5% sucrose, and PBS for 30 min at room temperature. Cells were then washed with PBS, blocked for 1 h with 2% goat serum and PBS, and incubated for 1 h at room temperature with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Inc.) diluted 1:500 in solution. Coverslips were washed with PBS and mounted on microscope slides in 50% glycerol and PBS or Cytoseal-60 (Stephens Scientific, Kankakee, IL, MI).

For staining of internal PrP and organelle markers, cells grown on glass coverslips were washed with PBS and fixed for 30 min at room temperature with 4% paraformaldehyde, 5% sucrose, and PBS. Cells were then washed with PBS, permeabilized for 1 min at room temperature with 0.5% Triton X-100 and PBS, and washed again with PBS. Blocking, incubation with primary and secondary antibodies, and mounting were carried out as described above, except that the primary antibody was diluted in blocking solution, and Alexa 594-conjugated secondary antibody was used for some double-labeling experiments.

Cells were viewed on a Zeiss Axioplan fluorescence microscope equipped with a Bio-Rad MRC1024 laser confocal scanning system. Digital images were processed using Adobe Photoshop. Figures show single optical slices through the cells.

For live–cell imaging of PrP-EGFP, cells were grown on 22 × 22-mm No. 1 glass coverslips, which were then mounted with vacuum grease on glass slides to produce a flow chamber and viewed with a 63 × 1.4 NA oil immersion objective in a Zeiss Axioplan 2 upright microscope. Continuous confocal imaging was achieved with a Yokogawa tandem spinning device (Solamere Technologies, Salt Lake City, UT). Live images were captured at magnification ×10 from the Yokogawa device using a highly intensified CCD camera (Solamere Technologies). The analog video signal from the CCD was further intensified, amplified, and smoothed by eight-frame averaging with a Dage-MTI DSP 2000 video image processor. Images were recorded in time-lapse mode on one s on a Panasonic TQ3038 optical memory disc recorder. For Z-series reconstruction, image stacks were obtained at video rates while manually focusing through the cell, and the resultant analog video sequences were compressed to single images with an analog video peak storage device (Model 493, Colorado Video Inc., Boulder, CO). The resultant single-frame C-series projections were transferred to Mac computers as 640 × 480 pixel images with a Targa A-D converter and then manually manipulated and pseudo-colored in green with Adobe Photoshop.

Immunogold Electron Microscopy—Cells were grown to confluence in plastic tissue culture dishes, washed with PBS, detached with 5 mM EDTA and PBS, and fixed for 2 h at room temperature with 2% paraformaldehyde and 0.2% glutaraldehyde. Cells were then rinsed in PBS, embedded in 10% gelatin, and processed for ultramicrotomy as described (27). Ultrathin frozen sections were incubated with blocking buffer containing 10% goat serum in PBS, then with affinity-purified antibody P45-66 for 2 h, and finally with goat anti-rabbit IgG coupled to 12-nm gold (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h. After washing, sections were stained with uranyl acetate and embedded in methyl cellulose. Sections were viewed on a Zeiss 902 electron microscope.

Biochemical Analyses—To assay the detergent insolubility of PrP, cells were solubilized in lysis buffer (0.5% sodium deoxycholate, 0.5% Triton X-100, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.5) supplemented with protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mM EDTA). The lysate was clarified by centrifugation at 16,000 × g for 3 min, followed by ultracentrifugation at 186,000 × g for 40 min. The pellet from the second
centrifugation, containing detergent-insoluble PrP, was resuspended in SDS-PAGE sample buffer. Detergent-soluble PrP was precipitated from the supernatant by addition of 0.05 volumes of 10% SDS and 4 volumes of methanol at 20°C. PrP was visualized by Western blotting with antibody 3F4. All lanes were from the same film. Molecular mass markers are given in kilodaltons. B, PG14 PrP in BHK cells is detergent-insoluble. Lysates of cells expressing WT or PG14 PrP were subjected to centrifugation at 186,000 × g, and PrP in the supernatants (S lanes) and pellets (P lanes) was detected by Western blotting. C, PG14 PrP in BHK cells is proteinase K-resistant. Lysates of cells expressing WT or PG14 PrP (300 μg of total protein) were treated with the indicated concentrations of proteinase K (PK) for 30 min at 37°C, and then PrP was detected by Western blotting. The bracket marks the position of the PrP27–30 fragment. D, PG14 PrP in BHK cells is PIPLC-resistant. Cells expressing WT or PG14 PrP were incubated with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) PIPLC for 2 h at 4°C, and then PrP in the incubation medium (M lanes) and cell lysates (C lanes) was revealed by Western blotting.

FIG. 1. Biochemical properties of PrP expressed in BHK cells using a Sindbis replicon. A, comparison of the expression levels of WT PrP (lanes 1 and 3) and PG14 PrP (lanes 2 and 4) in stably transfected lines of CHO cells (lanes 1 and 2) and in populations of BHK cells transfected with the Sindbis replicon (lanes 3 and 4). Each lane represents 100 μg of total cellular protein. PrP was visualized by Western blotting with antibody 3F4. All lanes were from the same film. Molecular mass markers are given in kilodaltons. B, PG14 PrP in BHK cells is detergent-insoluble. Lysates of cells expressing WT or PG14 PrP or were subjected to centrifugation at 186,000 × g, and PrP in the supernatants (S lanes) and pellets (P lanes) was detected by Western blotting. C, PG14 PrP in BHK cells is proteinase K-resistant. Lysates of cells expressing WT or PG14 PrP (300 μg of total protein) were treated with the indicated concentrations of proteinase K (PK) for 30 min at 37°C, and then PrP was detected by Western blotting. D, PG14 PrP in BHK cells is PIPLC-resistant. Cells expressing WT or PG14 PrP were incubated with (lanes 3, 4, 7, and 8) or without (lanes 1, 2, 5, and 6) PIPLC for 2 h at 4°C, and then PrP in the incubation medium (M lanes) and cell lysates (C lanes) was revealed by Western blotting.

PBS and incubated with PIPLC (Glyko, Novato, CA) at a concentration 1 unit/ml in Opti-MEM for 2 h at 4°C. The medium was collected and centrifuged at 16,000 × g for 5 min to remove loose cells, and proteins in the supernatant were precipitated with methanol. Precipitated proteins were collected by centrifugation at 16,000 × g for 30 min and resuspended in SDS-PAGE sample buffer. Cells were lysed directly in sample buffer. PrP was visualized by SDS-PAGE and Western blotting with antibody 3F4.

RESULTS

Expression of PrP Using a Sindbis Viral Replicon—To maximize our ability to visualize the subcellular localization of PrP in cultured cells, we utilized a high level expression system driven by a replicon derived from Sindbis, a cytoplasmic RNA virus belonging to the Alphavirus group (23, 24). The replicon contains a promoter that controls replication of full-length viral RNA and two subgenomic promoters, one of which drives expression of the protein of interest, and the other expression of the enzyme puromycin N-acetyltransferase, which confers resistance to the antibiotic puromycin. This system does not result in the production of infectious viral particles, an important biohazard consideration, since genes for viral structural

FIG. 2. PG14 PrP is present at low levels on the cell surface. BHK cells (expressing WT or PG14 PrP or untransfected) were stained with antibody 3F4 prior to fixation and application of Alexa 488-conjugated secondary antibody. The scale bar, applicable to all panels, is 50 μm.
proteins are absent. Since replication of Sindbis virus in most cell types results in substantial cellular damage within 24–48 h, we employed a replicon that carries a mutation in the nsp2 gene encoding one of the nonstructural proteins. This mutation renders the replicon nontoxic in BHK cells and allows the cells to be propagated indefinitely without apparent cytopathology (23, 24). BHK cells were transfected with a DNA version of the replicon and, after selection with puromycin, were maintained (23, 24). BHK cells were transfected with a DNA version of the replicon and, after selection with puromycin, were maintained as an uncloned population to avoid clonal artifacts. To avoid the appearance of mutations that can occur during RNA replication, all analyses were performed on cells that had been carried to be propagated indefinitely without apparent cytopathology (23, 24). BHK cells were transfected with a DNA version of the replicon and, after selection with puromycin, were maintained as an uncloned population to avoid clonal artifacts. To avoid the appearance of mutations that can occur during RNA replication, all analyses were performed on cells that had been carried for <10 passages.

We initially focused on cells expressing either WT PrP or PG14 PrP, a mutant containing a nine-octapeptide insertion associated with Creutzfeldt-Jakob disease that we have previously characterized in CHO cells, PC12 cells, and transgenic mice (12–15, 28). We first confirmed that both PrP molecules were correctly processed despite high levels of expression. As shown in Fig. 1A, WT and PG14 PrPs were expressed at similar levels in BHK cells using the Sindbis replicon. These levels were ∼10-fold higher than those in the lines of stably transfected CHO cells that we have used in previous studies in which PrP synthesis was driven by a cytomegalovirus promoter. Both proteins were efficiently processed in BHK cells to yield monoglycosylated (33 kDa) and diglycosylated (35–40 kDa) species, with a relatively small proportion of undiglycosylated polypeptide (28 kDa). The glycosylated species were quantitatively converted to the 28-kDa form by treatment with peptide N-glycosidase F (data not shown), confirming the presence of N-linked oligosaccharide chains.

We next tested whether PG14 PrP synthesized in BHK cells acquired the distinctive biochemical characteristics we have previously documented for mutant PrPs expressed at lower levels in other cell types. These properties, which are reminiscent of PrPSc, include detergent insolubility; proteinase K resistance; and resistance to the bacterial enzyme PIPLC, which cleaves the C-terminal GPI anchor (11, 14). In contrast to WT PrP, which remained entirely in the supernatant after ultracentrifugation of detergent lysates, PG14 PrP was partially insoluble, with about half found in the pellet (Fig. 1B). PG14 PrP was also resistant to digestion with low concentrations of proteinase K, yielding a protease-resistant fragment (PrP27–30) after treatment with 0.5–1 μg/ml proteinase K, a concentration range in which WT PrP was completely digested (Fig. 1C). Finally, treatment of intact cells with PIPLC released about half of the WT protein, but none of the mutant protein (Fig. 1D). Thus, WT and PG14 PrPs expressed at high levels in BHK cells using the Sindbis replicon are correctly processed, and the mutant protein displays typical PrPSc-like properties.

PG14 PrP Is Weakly Expressed on the Cell Surface—To visualize PrP on the cell surface, living cells were stained with anti-PrP antibody 3F4 and then fixed and reacted with Alexa 488 (green) secondary antibody without permeabilization. Despite equivalent expression levels by Western blotting (Fig. 1A), virtually all cells synthesizing PG14 PrP showed much weaker surface staining than those expressing WT PrP (Fig. 2). As a negative control, untransfected BHK cells showed no detectable staining. The difference in surface staining between WT and mutant PrPs is likely to be caused by a decreased amount of the mutant protein on the plasma membrane, rather than by inaccessibility of the 3F4 epitope, for the following reasons. First, permeabilization of fixed cells with Triton X-100 prior to immunostaining revealed intracellular deposits of PG14 PrP (see below), implying that the mutant protein is not intrinsically unreactive with the primary antibody. Second, treatment of fixed cells with guanidine HCl to enhance immunoreactivity of PrP (29) did not increase surface or internal staining of the mutant protein (data not shown). Third, PG14

**FIG. 3.** PG14 PrP has a more widespread intracellular distribution compared with WT PrP. Cells expressing WT PrP (A–F) or PG14 PrP (G–L) were fixed, permeabilized, and stained with antibody 3F4 followed by Alexa 488-conjugated secondary antibody. The panels show representative examples of each cell type. WT PrP was confined to the perinuclear area in all cells. PG14 PrP was largely perinuclear in some cells (G and H), but had a much more extensive distribution in other cells (I–L). PrP on the cell surface was not well visualized in these optical slices. The scale bar, applicable to all panels, is 50 μm.
PrP-EGFP fusion proteins displayed the same lack of surface fluorescence (see below), even though these molecules do not require antibody staining for visualization.

**PG14 PrP Is Localized in Both the Golgi and the ER**—To visualize intracellular PrP, fixed cells were permeabilized with Triton X-100 prior to application of primary and secondary antibodies. We found that, in virtually all cells expressing WT PrP, staining was tightly restricted to the region immediately surrounding the nucleus (Fig. 3, A–F). In contrast, many PG14 PrP-expressing cells showed a much more widespread staining pattern that included large areas of the cytoplasm, often extending into the long filopodia elaborated by the cells (Fig. 3, G–L). This pattern was seen in 20–30% of all cells expressing mutant PrP, with the remainder showing the perinuclear distribution typical of WT PrP-expressing cells (Fig. 3, G and H). Untransfected cells showed no detectable PrP staining (data not shown).

To determine the identity of the intracellular compartments where PrP resided, we colocalized PrP with a series of marker proteins. We used, as a Golgi marker, EYFP-Golgi (30), which consists of enhanced yellow fluorescent protein fused to the Golgi localization signal of /H9252, an enzyme that resides in the trans- and medial-Golgi stacks. BHK cells expressing PrP from the Sindbis viral replicon were transiently transfected with the plasmid encoding EYFP-Golgi and, after staining with anti-PrP antibody and an Alexa 594 (red) secondary antibody, were viewed with either green excitation/emission settings to detect EYFP-Golgi (A, D, G, and J) and with red excitation/emission settings to detect PrP (B, E, H, and K). Merged green and red images are shown in C, F, I, and L. In the merged images, there is red staining for WT PrP on the cell surface, but all of the intracellular staining is yellow, corresponding to complete colocalization with EYFP-Golgi. In contrast, there is no red staining for PG14 PrP on the cell surface, and the intracellular staining is yellow only in certain regions, indicating that the mutant protein is present in compartments in addition to the Golgi. The scale bar, applicable to all panels, is 50 μm.

**Fig. 4.** Intracellular WT PrP precisely colocalizes with a Golgi marker, but intracellular PG14 PrP only partially colocalizes with this marker. Cells expressing WT PrP (A–F) or PG14 PrP (G–L) were transiently transfected with a plasmid encoding Golgi-targeted EYFP and then fixed, permeabilized, and stained with antibody 3F4 followed by Alexa 594-conjugated secondary antibody. Cells were viewed with green excitation/emission settings to detect EYFP-Golgi (A, D, G, and J) and with red excitation/emission settings to detect PrP (B, E, H, and K). Merged green and red images are shown in C, F, I, and L. In the merged images, there is red staining for WT PrP on the cell surface, but all of the intracellular staining is yellow, corresponding to complete colocalization with EYFP-Golgi. In contrast, there is no red staining for PG14 PrP on the cell surface, and the intracellular staining is yellow only in certain regions, indicating that the mutant protein is present in compartments in addition to the Golgi. The scale bar, applicable to all panels, is 50 μm.
marker, a fluorescent version of wheat germ agglutinin, a lectin that binds to oligosaccharide structures present in the Golgi (31) (data not shown). We conclude from these results that both WT and PG14 PrPs are concentrated in the Golgi region, but that in a subset of the cells, the mutant protein resides in other intracellular structures as well.

We next utilized PDI as an ER marker (Fig. 5). In BHK cells, which display a flat, fibroblast-like morphology, PDI is distributed in a fine reticular pattern that fills much of the cytoplasm, including filopodial extensions. We found that WT PrP showed no colocalization with PDI, with the merged red and green images showing no yellow color (Fig. 5, A–F). Even in the perinuclear area, where both proteins are present, there was no overlap of the PrP and PDI staining patterns, demonstrating our ability to resolve the ER and Golgi compartments even when they are present in the same region of the cell. In contrast, PG14 PrP showed remarkably precise colocalization with PDI, except in the perinuclear area (Fig. 5, G–L). These results indicate that much of the PG14 PrP that lies outside of the Golgi apparatus resides in the ER. Similar results were obtained using calnexin as an ER marker (data not shown).

We investigated whether any PrP was also present in lysosomes and late endosomes using, as a marker, LysoTracker Red, a weakly basic, fluorescent amine that accumulates in these acidic compartments (32). Cells were incubated with LysoTracker Red for 2 h prior to fixation, permeabilization, and staining with antibody 3F4. Although some of the LysoTracker Red was washed out during fixation and permeabilization, it was nevertheless possible to observe punctate structures throughout the cytoplasm that correspond to lysosomes or large endosomes (Fig. 6). None of these structures colocalized with either WT or PG14 PrP (Fig. 6).

**Several Mutant PrPs Display an Altered Distribution in CHO Cells**—To determine whether other mutations affected the cel-
lular distribution of PrP and whether these changes could be observed in other cell types at lower expression levels, we analyzed the localization of WT PrP and four different disease-associated mutants in stably transfected lines of CHO cells. We have previously carried out an extensive biochemical characterization of PrP in these cell lines (11, 12, 14, 26), where expression levels are 10-fold lower than in BHK cells carrying the Sindbis replicon (Fig. 1A). By Western blotting, PrP expression levels were equivalent in all five of the CHO cell lines examined here and were similar to those in mouse brain when normalized for protein content (data not shown).

By performing labeling of unpermeabilized cells, we found that all the mutant PrPs (PG14, P101L, D177N/M128, and F197S/M128V) showed less intense surface fluorescence compared with WT PrP (Fig. 7, left-hand panels). There were noticeable differences among the mutants in the extent of this effect, however, with PG14 PrP showing the least intense surface staining. When permeabilized cells were examined (Fig. 7, right-hand panels), WT PrP was concentrated in a small perinuclear patch that colocalized with Golgi markers (data not shown). In contrast, 20–30% of the cells expressing each of the mutant PrPs showed a more widespread cytoplasmic staining pattern that largely colocalized with ER markers (data not shown). There were also differences among the mutants in the pattern of internal staining; for example, P101L PrP appeared to be most concentrated in the parts of the ER closest to the nucleus.

**PG14 PrP Is Present in the ER at the Ultrastructural Level**—We carried out EM immunogold labeling of ultrathin cryostat sections of BHK cells to localize PrP at the ultrastructural level. Optimal ultrastructural preservation required inclusion of 0.2% glutaraldehyde in the fixative; but even at this low concentration, glutaraldehyde was found to abolish the reactivity of PrP with antibody 3F4. Therefore, EM localization studies were carried out with an affinity-purified antibody (P45-66) raised against the octapeptide repeat region of PrP. The reactivity of this antibody was not affected by glutaraldehyde fixation, and it gave the same pattern of immunofluorescence staining as antibody 3F4 (data not shown). Consistent with the light microscope results, immunogold labeling of the

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**Fig. 6.** Neither WT nor PG14 PrP colocalizes with a lysosomal marker. Cells expressing WT PrP (A–F) or PG14 PrP (G–L) were incubated for 2 h with LysoTracker Red and then fixed, permeabilized, and stained with antibody 3F4 followed by Alexa 488-conjugated secondary antibody. Cells were viewed with green excitation/emission settings to detect PrP (A, D, G, and J) and with red excitation/emission settings to detect LysoTracker Red (B, E, H, and K). Merged green and red images are shown in C, F, I, and L. There is no overlap of green staining for PrP (WT or PG14) with the punctate red structures labeled by LysoTracker Red. The scale bar, applicable to all panels, is 25 μm.
plasma membrane was abundant for WT PrP, but was virtually absent for PG14 PrP (Fig. 8, A and B). Inside the cell, labeling of WT PrP was seen over the Golgi stacks (Fig. 8C), whereas labeling of PG14 PrP was observed over tubular elements of the ER (D and E) as well as over the Golgi complex (data not shown). Vector-transfected cells showed only a few scattered gold particles (data not shown), confirming the specificity of the staining.

PrP-EGFP Fusion Proteins Are Correctly Processed and Localized—To rule out the possibility that the altered cellular staining pattern observed for mutant PrPs was an artifact of differences in the reactivity of these molecules to the primary antibodies used for immunolocalization, we analyzed the distribution of PrP-EGFP fusion proteins. EGFP is an altered version of jellyfish GFP carrying two mutations in the chromophore region of the polypeptide that augment quantum yield and red-shift the excitation spectrum (33); codon usage is also optimized for expression in mammalian cells. To preserve GPI anchoring of the fusion proteins, we inserted the EGFP sequence at codon 223, just N-terminal to the presumptive GPI attachment site of PrP at serine 230 (Fig. 9A). We reasoned that inserting the EGFP sequence at this location, rather than near the signal peptide cleavage site at residues 22 and 23, was more likely to produce a functional protein since the PrP segment could fold normally during translocation into the ER before the EGFP segment was even translated.

We first assessed the effect of the large EGFP moiety on the biochemical properties of PrP. WT PrP-EGFP and PG14 PrP-EGFP expressed in BHK cells using the Sindbis replicon migrated on SDS-polyacrylamide gel as broad bands at 60 and 65 kDa, respectively, ~8 kDa higher than the predicted sizes of the polypeptide chains themselves (Fig. 9, B and C). These results suggest that the fusion proteins are glycosylated, an inference confirmed by sensitivity of the proteins to digestion with peptide N-glycosidase F (data not shown). In addition, WT PrP-EGFP was releasable from the cell surface by PIPLC, indicating that the protein has been properly GPI-anchored (Fig. 9C). Thus, introduction of the EGFP moiety does not appear to interfere with biosynthetic processing of PrP.

We next determined whether the presence of the EGFP segment influenced acquisition of PrPSc-like properties by mutant PrP. We found that, like its non-fluorescent counterpart, PG14 PrP-EGFP was partially detergent-insoluble (Fig. 9B), suggesting that the EGFP tag does not prevent conversion of mutant PrP to an aggregated state. Interestingly, although the non-fluorescent version of PG14 PrP was completely resistant to PIPLC release (Fig. 1D), the EGFP version was partially releasable (Fig. 9C). We have previously postulated that the

Fig. 7. Several mutant PrPs display an altered distribution in CHO cells. To visualize surface PrP, stably transfected CHO cells expressing the indicated PrP molecules were stained with antibody P45-66 prior to fixation and application of Alexa 488-conjugated secondary antibody (unpermeabilized; left-hand panels). To visualize internal PrP, cells were fixed, permeabilized, and then stained with antibody 3F4 followed by Alexa 488-conjugated secondary antibody (permeabilized; right-hand panels). The scale bar, applicable to all panels, is 50 μm. The human homologs of the PrP mutations are associated with the following diseases: Creutzfeldt-Jakob disease (PG14), Gerstmann-Sträussler syndrome (P101L and F197S/M128V), and fatal familial insomnia (D177N/M128).
abnormal conformation of mutant PrP blocks access of PIPLC to the GPI anchor (14). It is likely that the presence of the EGFP moiety at the C terminus of the fusion protein diminishes this effect, thus allowing PG14 PrP-EGFP to be released by the phospholipase.

We initially imaged PrP-EGFP fusion proteins in fixed and permeabilized BHK cells by confocal microscopy to analyze their localization with respect to subcellular markers. We observed that, whereas WT PrP-EGFP was easily visualized as a rim of fluorescence corresponding to the cell surface, both PG14 PrP-EGFP and D177N/M128 PrP-EGFP did not display this peripheral localization (Fig. 10, A, D, and G). Absence of the mutant fusion proteins from the cell surface was confirmed by staining unpermeabilized cells with antibody 3F4 (data not shown). Whereas the wild-type fusion protein was concentrated almost exclusively in the Golgi region, where it colocalized with the marker protein giantin (34) (Fig. 10, A–C), the two mutant fusion proteins had a more widespread cytoplasmic distribution that was partly coincident with that for the ER marker PDI (D–I). We also noticed that the mutants were sometimes concentrated in highly fluorescent puncta in the cytoplasm that did not colocalize with either the Golgi or ER markers (Fig. 10, D and G, arrows). The identity of these structures, which were occasionally observed when the non-EGFP versions were analyzed, remains to be determined. Thus, the localization of the PrP-EGFP fusion proteins mirrored the localization of the non-fluorescent versions, indicating that the EGFP moiety does not interfere with cellular trafficking mechanisms for PrP.

**FIG. 8.** Localization of PrP at the ultrastructural level. BHK cells expressing WT PrP (A and C) or PG14 PrP (B, D, and E) were processed for EM immunogold labeling with anti-PrP antibody P45-66. A and B show gold labeling at the cell surface; C shows labeling over stacks of Golgi cisternae; and D and E show labeling associated with ER tubules and tubulovesicular elements. The scale bars in all panels are 150 nm.
One of the advantages of tagging a protein with GFP is that it permits visualization of the protein inside living cells. We have therefore carried out a preliminary analysis of the distribution and movements of PrP-EGFP in living BHK cells using time-lapse confocal video microscopy. We found that WT PrP-EGFP was present at much higher levels on the cell surface than PG14 PrP-EGFP (Fig. 11), analogous to what was observed in fixed and permeabilized cells. The fluorescence of both proteins was uniform over the entire surface of the cell, with no obvious foci or other substructure visible. This diffuse pattern is consistent with evidence that, although GPI-anchored proteins are clustered into raft domains on the plasma membrane, the size of these domains is below the resolution limit of the light microscope (35, 36). Both WT PrP-EGFP and PG14 PrP-EGFP were also concentrated in the central region of the cell in a large, stationary, brightly fluorescent structure near the nucleus that corresponds to the Golgi apparatus (Fig. 11, C and G, asterisks). Smaller spherical fluorescent vesicles with a diameter of 0.3–0.5 μm could also be observed moving in a saltatory and bidirectional fashion in the peripheral cytoplasm of cells expressing either protein (Fig. 11, B and H, arrows). The features of these vesicles are characteristic of endosomes and lysosomes, consistent with our previous observation that PrP can be endocytosed (37). Our failure to observe these structures after immunofluorescence staining of cells incubated with LysoTracker Red (Fig. 6) may be due to their small size or the fact that some of them may not have retained the dye after fixation and permeabilization.

**DISCUSSION**

Using immunofluorescence and immunogold staining techniques as well as GFP fusion proteins, we have demonstrated that pathogenic mutations alter the subcellular localization of PrP, shifting the protein from the cell surface to intracellular compartments that include the ER. We have seen this effect with four different disease-associated mutations in two different types of transfected cells and at both low and high expression levels. Using immunofluorescence staining, we have also recently observed a reduced amount of PrP on the surface of cerebellar granule neurons cultured from transgenic mice that express PG14 PrP compared with those from mice that express WT PrP (28). Thus, the altered cellular distribution of PrP molecules carrying pathogenic mutations appears to be a general phenomenon with respect to mutation, cell type, and expression level.

We found that, compared with WT PrP, all of the mutant PrPs were present at reduced levels on the surface of unpermeabilized BHK and CHO cells. Since the WT and mutant proteins were expressed at equivalent levels as judged by Western blotting, one would expect that a greater proportion of mutant PrP would be found in intracellular compartments that are not accessible to surface staining. Consistent with this prediction, when we stained Triton X-100-permeabilized cells, we observed that mutant PrPs were distributed in a pattern that overlapped with markers for the ER in 20–30% of the BHK and CHO cells in each culture. In contrast, ER localization was never seen for the WT protein in either cell type. Both WT and mutant PrPs were also found to be concentrated in the Golgi apparatus, a phenomenon seen for many other plasma membrane proteins; this probably reflects relatively slow transit of secretory proteins through this compartment on their way to the cell surface (38). Although much of the mutant PrP that accumulated intracellularly outside of the Golgi region colocalized with ER markers, we cannot rule out the possibility that some of the protein was present in other cellular locations as well. In fact, we observed that the PG14 PrP-EGFP and D177N/M128 PrP-EGFP fusion proteins were sometimes concentrated in small puncta that did not colocalize with either the ER or Golgi markers.

Although we have focused on the common features that characterize the staining patterns of all four mutant PrPs, it is

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2 L. Ivanova, B. Drisaldi, and D. A. Harris, unpublished data.
likely that there are also differences among the mutants. For example, we have noticed that PG14 PrP tended to have weaker surface expression than the other mutants and that P101L PrP tended to be more concentrated in the portion of the ER immediately surrounding the nucleus, in contrast to the more widespread ER distribution of the other mutants. We have also observed that some mutant PrP molecules, for example those carrying an E199K substitution, are expressed on the cell surface more efficiently than any of the four mutants examined here. Interestingly, these mutants are also less “PrPSc-like” in their biochemical properties (11), suggesting a correlation between the effects of a mutation on the cellular localization and biochemical properties of PrP.

Our results provide a picture of the steady-state distribution of mutant PrP molecules that can be combined with data from recent pulse-chase labeling experiments to suggest a major route for the metabolism of these molecules. We have found that all four of the mutant PrPs examined here have a significantly shorter metabolic half-life compared with WT PrP, with much of the mutant protein being degraded immediately after synthesis before it has a chance to transit beyond the ER.3 We therefore propose that a substantial proportion of mutant PrP molecules fail to reach the cell surface after their synthesis.

3 B. Drisaldi, R. S. Stewart, and D. A. Harris, manuscript in preparation.
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Because they are partially retained in the ER and are then degraded. This interpretation is consistent with the reduced surface levels of mutant PrPs and with the ER localization of the proteins in a percentage of the cells. Our failure to observe mutant PrP in the ER of all cells in a culture may be due to the fact that the newly synthesized molecules are usually degraded before they can accumulate to detectable levels. The subpopulation of cells in which mutant PrP can be visualized may represent those cells in which the rate of PrP synthesis has temporarily exceeded the rate of ER degradation, possibly due to fluctuations in the metabolic state of individual cells as they grow and divide.

Reduced delivery of mutant PrPs to the cell surface and their accumulation in the ER suggest the possibility that ER quality control processes are involved in the metabolism of these proteins. Secretory and membrane proteins are subject to a stringent surveillance mechanism that causes retention in the ER of misfolded or nonfunctional forms, such as those carrying mutations, and this prevents them from reaching the cell surface (39, 40). Proteins retained in the ER are then eliminated by a pathway that is now known to involve reverse translocation of the polypeptide chain into the cytoplasm, followed by proteasomal degradation (41). Consistent with the involvement of such a mechanism in the metabolism of mutant PrPs, we have recently found that proteasome inhibitors cause a marked accumulation of these molecules in cultured CHO cells and a significant prolongation of their metabolic half-lives. Presumably, ER retention and degradation depend on certain molecular features that are common to PrPs carrying disease-causing mutations. It is noteworthy that one of the PrPSc-like biochemical properties of these proteins, PIPLC resistance, is acquired at the time the polypeptides are synthesized in the ER (13). It is attractive to hypothesize that this early molecular alteration is recognized by the cell and triggers retention of the mutant protein along the secretory pathway.

Taken together, our morphological and biochemical results link prion diseases for the first time to other inherited human disorders that are thought to involve the action of protein quality control mechanisms. These include other neurodegenerative diseases such as Huntington’s and Parkinson’s diseases as well as disorders that affect peripheral organ systems such as cystic fibrosis, congenital hypothyroidism, and α1-antitrypsin deficiency (42, 43). The common theme in all of these diseases is thought to be misfolding of a mutant polypeptide that is recognized by the cell as abnormal and is then subject to retention in the ER (for secreted or membrane proteins) or sequestration in cytoplasmic inclusions (for soluble proteins), followed by proteasomal attack. It is noteworthy that quality control of the PrP mutants examined here is incomplete since a fraction of each protein clearly escapes retention in the ER and reaches the cell surface. Whether the PrP molecules responsible for neurodegeneration are those that remain trapped inside the cell or those that escape will be an interesting subject for future investigation. Retention of abnormal proteins in the ER is known to trigger stress response pathways (44), some of which could be involved in the pathogenic effects of mutant PrPs. We have recently demonstrated that CmvPrP, a transmembrane form of PrP that is postulated to be a key intermediate in prion-induced neurodegeneration, is completely retained in the ER, suggesting that it may act via such a mechanism (45).

Although several other studies have examined the subcellular distribution of mutant PrP molecules in transfected cells, the generality of the patterns observed has not been emphasized, and it has not been recognized that ER retention is likely to be a common theme in familial prion diseases. Using immunofluorescence techniques, Singh and colleagues (17, 20) found that human PrP carrying a Q217R mutation, linked to Gerstmann-Sträussler syndrome, was expressed at reduced levels on
the cell surface and also accumulated in the ER, Golgi, and endosomes/lysosomes. This group also reported that human PrP with another Gerstmann-Sträussler mutation (Y145stop) could be visualized only at low levels in control cells, where it was localized to the Golgi; but after treatment with a proteasome inhibitor, the amount of the mutant protein increased, and it was found in the ER and the nucleus (19). Finally, biochemical analyses have suggested that human E200K and D178N PrPs are not transported efficiently to the cell surface (18, 21), and localization studies using GFP chimeras indicate the functionality of PrP-GFP chimeras. We have demonstrated here that attachment of a GFP reporter adjacent to the C-terminal GPI addition site of either WT or mutant PrP does not affect a number of key attributes of these molecules, including their biosynthetic maturation, subcellular localization, and biochemical properties. These results lay the foundation for the use of PrP-EFGP chimeras in a wide range of experiments on the distribution, trafficking, and molecular transformations of PrP in living cells and in animals.

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