Filipin Prevents Pathological Prion Protein Accumulation by Reducing Endocytosis and Inducing Cellular PrP Release*

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Conversion of the normal membrane-bound prion protein (PrP-sen) to its pathological isoform (PrP-res) is a key event in the pathogenesis of transmissible spongiform encephalopathies. Although the subcellular sites of conversion are poorly characterized, several lines of evidence have suggested the involvement of membrane lipid rafts in the conversion process. Here we report that copper stimulates the endocytosis of PrP-sen via a caveolin-dependent pathway in both microglia and neuroblastoma cells. We show that the polynye antibiotic filipin both limits endocytosis of PrP-sen and dramatically reduces the amount of membrane-bound PrP-sen. This reduction results from a rapid and massive release of full matured PrP-sen into the culture medium. Finally, we demonstrate that filipin is a potent inhibitor of PrP-res formation into chronically infected neuroblastoma cells. Our results reinforce the role of rafts in PrP trafficking and raise the possibility that the release of PrP-sen from the plasma membrane decreases the amount of available substrate PrP-sen at the conversion sites.

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Transmissible spongiform encephalopathies (TSE) are infectious neurodegenerative diseases including scrapie of sheep, bovine encephalopathy in cattle, and a variety of human diseases such as Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomnia. TSE are associated with the conversion of the protease-sensitive normal isoform of the prion protein (PrP-sen) to a pathological scrapie isoform named PrPsc or PrP-res based on its protease resistance. No differences in distribution charges and amino-terminal sequences have been detected between the isoforms (1); however, PrP-sen and PrP-res conformations differ markedly (2). PrP-sen is a protease-sensitive monomeric protein, whereas PrP-res forms highly insoluble aggregates characterized by their resistance to proteolytic digestion (3). The conversion may occur via a post-translational process without any chemical modifications of the molecules (4, 5). Direct interactions between PrP-sen and PrP-res aggregates and the conversion process resulting from it are molecular key events in the development of TSE pathogenesis.

PrP-sen is bound to the external surface of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. Like most GPI-anchored proteins, PrP-sen is clustered into cholesterol- and sphingomyelin-rich domains called rafts or caveolae domains, referring to the presence of the protein marker caveolin (6, 7). However, it has been shown that cells poorly expressing caveolin such as neuroblastoma cells (N2a) also contain rafts based on the existence of detergent-resistant membranes enriched for GPI-anchored proteins (8). Caveolae domains are involved in a variety of physiological and pathological situations such as signal transduction (9) and entry of nonenveloped DNA virus SV40, respectively (10, 11). From the plasma membrane, PrP-sen either can be endocytosed, recycled, and degraded or can bind to PrP-res and thus be converted into newly formed PrP-res aggregates (for review see Ref. 12). The subcellular sites for binding of PrP-sen to PrP-res and conversion remain unknown. These events could occur either on the cell surface or into undefined endocytosis organelles during PrP-sen trafficking (13, 14). Recently, Baron et al. (15) have shown that raft-bound PrP-sen can be substrate for conversion only when PrP-res molecules are themselves inserted into contiguous membranes. Once PrP-sen is formed, it appears to accumulate on the surface of cells (6) and/or into lysosomes (13). Both sterol-binding antibiotics amphotericin B and lovastatin, which decrease the membrane cholesterol level, inhibit the formation of PrP-res in scrapie-infected N2a cells (16, 17). Likewise, the replacement of the GPI anchor of PrP by a transmembrane domain targets mutated PrP to clathrin-coated pits, resulting in the decrease of PrP-res formation (18). Collectively, these reports strongly support the idea that rafts could be the sites for conversion and PrP-res accumulation.

The PrP-sen amino terminus contains a series of histidine- and glycine-rich octapeptide repeats that are copper ion-binding sites (19). Interestingly, copper ions stimulate endocytosis of PrP-sen (20), suggesting that PrP could serve as a chelator for uptake and delivery of copper ions to intracellular targets. Alternatively, copper ions could be essential cofactors for some yet unknown physiological functions of PrP-sen.

Here we have assessed the copper-induced endocytosis of PrP-sen using two complementary approaches. First, by immunolocalization and confocal laser microscopy observation, we show that copper stimulates PrP-sen endocytosis through a caveolin-dependent pathway in both human microglia and mouse neuroblastoma cells. Second, we demonstrate that the binding of a radiolabeled PrP-recognizing monoclonal antibody can be used to assess quantitatively internalization of mem-
brane-bound PrP-sen. Moreover, we show that the polyclonal antibody filipin that binds to membrane sterols both inhibits PrP endocytosis and provokes the release of the PrP molecules from the plasma membrane. As a result, filipin is found to be a potent inhibitor of PrP-res formation in scrapie-infected N2a cells.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Proteinase K and Pefabloc® were purchased from Roche Molecular Biochemicals. Dulbecco’s modified Eagle’s medium (DMEM), Opti-MEM, trypsin, and Geneticin G418 were from Life Technologies, and bovine serum albumin (BSA) was from Biowhittaker. Final solutions of chlorpromazine, nystatin, and filipin (Sigma) were prepared every day. Mouse monoclonal antibodies SAF32 and SAF70 were raised in knock-out mice (21) by immunizing with a scrapie-associated fibril (SAF) preparation obtained from infected hamster brain (263K). SAF32 was directed against the entire fibrils (SAF) preparation obtained from infected hamster brain (263K) raised in knock-out mice (21) by immunizing with a scrapie-associated fibril (SAF) preparation obtained from infected hamster brain (263K) strain. SAFs were denatured before immunization by treatment with formic acid. Monoclonal antibody SAF32 was directed against the entire octapeptide region, whereas SAF70 recognized the 142–162 sequence (22). Both antibodies cross-react with PrP from most mammalian species (bovine, ovine, mouse, hamster, and human). Monoclonal antibodies Pri-308 was raised in Biozi’s mice by immunizing with a synthetic peptide representative of the human 106–126 sequence (22) and binds only PrP from infected hamster. The incubations were performed at 37 °C for the indicated times for kinetics or 90 min for equilibrium studies. At steady state (i.e., 90 min at 37 °C), the incubation medium was rapidly removed, and the endocytosis of 125I-Pri308 was stimulated by incubating cells with 300 μl of Earle’s buffer containing 500 μM CuSO4. At the end of the incubation time, the medium was removed, and the cells were rapidly washed twice with 1 ml of Earle’s buffer. To estimate the amount of cell-associated 125I-Pri308, the surface-bound radioactivity was removed by washing cells for 4 min at 37 °C with 500 μl of Earle’s buffer pH 4 containing 0.5 mM NaCl (acid-NaCl buffer). In control conditions, the acid wash consistently removed ~95% of the cell surface radioactivity. Finally, the cells were lysed with 500 μl of 0.2 N NaOH, and the radioactivity was counted in a γ counter (Packard). The protein concentration was assayed by measuring the ratio of optical densities at 260 nm/280 nm. Nonspecific binding was determined in the presence of a large excess of unlabeled Pri308 (50 μg/ml; 1 μg/well) or of synthetic human prion peptide 106–128 (10 μM). In both cases, the nonspecific binding represented less than 8% of the total binding. Specific binding was calculated by subtracting nonspecific binding from total binding.

**Assay for Release of PrP-sen from Cells**—Confluent cultures of C13-NJ cells were washed three times at 4 °C with PBS containing 1% glucose, 10 μM bestatin, and a complete mixture of protease inhibitors EDTA-free (Roche Molecular Biochemicals) prior incubation in the presence or in the absence of filipin (5 μg/ml) at 37 °C. At the end of the incubation time, the culture media were collected and trichloroacetic acid-precipitated. The pellets were resuspended in 50 μl of denaturing loading buffer (65 mM Tris-HCl, pH 6.8, containing 2% SDS, 5% glycerol, 0.05% bromphenol blue), boiled for 5 min, and loaded onto a 12% polyacrylamide gel. The proteins were separated by SDS-PAGE and then electroblotted onto a nitrocellulose membrane (Protran BA83; Schleicher & Schuell). The membranes were treated with 5% nonfat dry milk in TBST (20 mM Tris-HCl, pH 8, 100 mM NaCl, 0.1% Tween 20) and incubated overnight with the appropriate primary antibody at 4 °C. The blots were developed by using an enhanced chemoluminescence system (Roche Molecular Biochemicals) and exposed on x-ray film (X-Omat AR; Eastman Kodak Co.).

**Assay for PrP-res Accumulation in N2aS12cells—**N2aS12 cells were seeded at 10–15% confluent density in a 24-well culture plate in Opti-MEM supplemented with 10% FBS. Four hours after plating at 37 °C, the cells were treated with the indicated concentrations of filipin, nystatin, or chlorpromazine for 4 days. Confluent cultures were lysed for 10 min at 4 °C in lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA) and then centrifuged for 5 min at 3,000 × g. For detection of PrP-sen, one-tenth of the post-nuclear supernatants were directly mixed with the same volume of 2× denaturing loading buffer. For detection of PrP-res, the radiolabeled anti-caveolin-1 antibody, Pri308 (50 μg/ml) in Earle’s buffer was incubated with 10 μg of total protein for 30 min at 37 °C. The digestion was stopped by adding Pefabloc® (1 mM) for 5 min before centrifugation at 20,000 × g for 90 min at room temperature. The pellets were resuspended in 30 μl of denaturing loading buffer, sonicated, boiled for 5 min, and loaded onto a 12% polyacrylamide gel. PrP-sen and partially digested PrP- res were detected with SAF70 monoclonal antibody diluted 1:1000 in TBST.
At this point of our study, we cannot rule out the copper mechanism. To further characterize this mechanism, microglia and macrophages were first incubated with Pri308 for 90 min at 37 °C. After paraformaldehyde fixation, Pri308 monoclonal antibody was detected with a secondary conjugated antibody on permeabilized cells as described under “Experimental Procedures.” Scale bar, 15 μm.

The results were developed and exposed as described above. Densitometry was performed with National Institutes of Health Image software, computing at least four independent experiments, and the results are expressed as percentages of the control condition.

RESULTS

Copper-induced Subcellular Relocalization of Cell Surface PrP−sen—A previous report showed that copper stimulates the endocytosis of PrP−sen in neuroblastoma cells. Using confocal laser microscopy, we further investigated the relocalization of cell surface PrP−sen induced by copper. Human microglia cells were incubated in the absence (Fig. 1A) or in the presence of 500 μM CuSO4 (Fig. 1B) for 30 min at 37 °C. After paraformaldehyde fixation, the cell surface PrP was assayed by incubation with Pri308 antibody and revealed by a Texas Red-conjugated secondary antibody under nonpermeabilized conditions (Fig. 1, A and B). Whereas the fluorescence was found associated to the cell surface of the microglia cells in control conditions, the copper incubation induced a significant disappearance of the cell-associated fluorescence (Fig. 1, A and B), suggesting the existence of a copper-induced PrP endocytosis mechanism. To further characterize this mechanism, microglia cells were first incubated with Pri308 for 90 min at 37 °C to specifically label the surface-bound PrP−sen and then submitted to copper stimulation. The confocal microscopy observations were performed on permeabilized cells. Copper-stimulated cells displayed an intense perinuclear fluorescent pattern, confirming that copper induced the relocation of PrP−sen from the cell membrane to intracellular compartments (Fig. 1C). At this point of our study, we cannot rule out the possibility that PrP−sen trafficking is disturb by the binding of Pri308. However, several lines of evidence will be provided later in the manuscript showing that the binding of Pri308 does not modify the copper-induced internalization of PrP.

Differentiation of Copper-induced PrP−sen Endocytosis through Coated and Noncoated Pathways—To further investigate the pathway of copper-induced PrP−sen internalization, we performed immunocytochemical colocalization experiments both with anti-PrP monoclonal antibody and with a specific marker of the clathrin-dependent endocytosis pathway (transferrin-Alexa 488) and/or anti-caveolin-1 antibody to label the caveole structures. As shown in Fig. 2, when endocytosis of PrP is induced by copper ions, PrP is relocalized into caveolin-1-positive intracellular compartments in human microglia cells. Moreover, the superimposed images clearly revealed the absence of colabeling fluorescence between the anti-PrP antibody and the transferrin receptor marker in both microglia and neuroblastoma cells. These results strongly suggest that copper-induced PrP endocytosis occurred via a caveole-dependent pathway. Although it is well established that microglia cells contain caveolin and caveolin (27), no caveolin labeling was detected in mouse N2a by immunofluorescence confocal microscopy and Western blotting analysis (results not shown), in agreement with previous report (28).

Effects of Filipin, Nystatin, and Chlorpromazine on the Relocalization of PrP−sen Induced by Copper—In the absence of marker of the caveole-dependent endocytosis pathway in neuroblastoma cells, we examined the effect of two classes of drugs on PrP−sen endocytosis. Sterol-binding agents such as filipin and nystatin bind to cholesterol, a major component of glycolipid microdomains and caveole, and disrupt caveole structure and function (27, 29). On the other hand, chlorpromazine inhibits the clathrin-dependent pathway (30, 31). Human microglia and 3F4-PrP expressing neuroblastoma cells were incubated in the absence or in the presence of filipin, nystatin, or chlorpromazine prior to copper stimulation for 30 min and paraformaldehyde fixation. The cell surface PrP−sen molecules were monitored by staining with Pri308 antibody. The observations were performed by confocal laser microscopy on nonpermeabilized cells. In control conditions, the cells exhibited a loss of fluorescence at the plasma membrane after copper stimulation, confirming the endocytosis of PrP−sen from the surface to the inside of the cell (Fig. 3A). In contrast, fluorescence was found only at the cell surface of filipin- or nystatin-treated cells, indicating that these cells were unable to significantly internalize PrP−sen after copper stimulation. In contrast, chlorpromazine-treated cells displayed a loss of cell surface fluorescence similar to that of untreated cells. Thus, the results with filipin and nystatin indicated that copper-induced PrP endocytosis occurred through cholesterol-rich microdomains including the possible involvement of caveole in both human microglia.
and neuroblastoma cells. The interaction of filipin with cholesterol and its inhibitory effects on the copper-induced PrP-sen endocytosis can be demonstrated in both caveolin-expressing or caveolin-devoid cells.

Knowing that transferrin receptors are internalized via clathrin-coated pits, we further compared the distribution of Alexa 488-transferrin into neuroblastoma cells in control, filipin-treated, and chlorpromazine-treated cells. As shown in Fig. 3B, control and filipin-treated cells exhibited a largely perinuclear fluorescence pattern with a concomitant loss of plasma membrane labeling. In contrast, Alexa 488-transferrin fluorescence was found predominantly at the cell surface of chlorpromazine-treated cells, indicating that these cells did not internalize transferrin receptors. Other fields from chlorpromazine-treated neuroblastoma cells occasionally showed some internalized Alexa 488-transferrin fluorescence;

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**FIG. 3.** Effects of filipin, nystatin, and chlorpromazine on the relocalization of cell surface-bound PrP and transferrin receptors. A, C13-NJ and 3F4-N2a cells were treated with 5 µg/ml filipin or 50 µg/ml nystatin for 15 min or with 5 µg/ml chlorpromazine for 30 min at 37 °C. CuSO₄ (500 µM) was then added to the incubation medium for 30 min. After a rapid wash with PBS, surface-bound PrP was revealed on unpermeabilized cells with Pri308 and Texas Red-conjugated secondary antibody. Identical photomultiplier values and parameters of the laser scanning confocal microscope were used for all of the panels. B, 3F4-N2a cells were preincubated with DMEM containing 1% bovine serum albumin for 90 min at 37 °C. The cells were then incubated in the absence (panels a and a’) or in the presence of 5 µg/ml filipin (panels b and b’) or 5 µg/ml chlorpromazine for 30 min (panels c and c’). The incubation medium was removed, and the cells were incubated in the presence of a solution of Alexa 488-transferrin for 30 min at 37 °C. The cells were then rinsed, fixed with 3% paraformaldehyde, and observed with a laser scanning confocal microscope. Panels a, b, and c, confocal microscopy images. Panels a’, b’, and c’, overlay representations of confocal and transmission microscopy observations.
The goal of these studies was to characterize the cell surface binding of radiolabeled Pri308. Confluent C13-NJ cells plated into 24-well tissue culture plates were incubated at 37 °C in Earle’s buffer containing 125I-Pri308 monoclonal antibody (800,000 cpm/300 μl) in the absence (black circles) or in the presence (open circles) of a large excess of unlabeled Pri308 or 10 μm synthetic peptide P106-128. At the indicated times, the cells were rapidly washed twice with 1 ml of Earle’s buffer and lysed with 500 μl of 0.2 M NaOH, and the radioactivity bound to the cells was counted. Inset, C13-NJ cells were incubated in Earle’s buffer containing radiolabeled Pri308 antibody for 90 min at 37 °C. Then the incubation medium was rapidly removed, and 300 μl of Earle’s buffer was added in the absence (open squares) or in the presence (black squares) of 500 μM CuSO4. At the indicated times, the cells were treated with Earle’s buffer, pH 4, containing 0.5 M NaCl for 4 min at 37 °C. The results are expressed as the percentages of acid wash-resistant binding compared with the associated cell-specific binding. The results are the means of six independent experiments where each point is the average of duplicate determinations.

However, the pattern was diffuse and remained in the region underlying the plasma membrane. In summary, we confirmed that transferrin receptor, a typical clathrin-dependent endocytosis pathway, was insensitive to filipin and also to nystatin treatment (data not shown), whereas it could be efficiently inhibited by chlorpromazine.

**Binding of 125I-Pri308 Antibody to Human Microglia Cells (C13-NJ)**—The goal of these studies was to characterize the kinetic parameters of PrP-sen internalization induced by copper using the approach of binding of radiolabeled monoclonal antibody Pri308 to the cell surface. This antibody was chosen from a panel of antibodies developed against PrP essentially because Pri308 is able to recognize the human PrP-sen in native conditions. The binding of the 125I-Pri308 antibody to the cell surface was specific because the radiolabeled IgG bound can be displaced by 10 μM of synthetic peptide sequence 106–128 or by an excess of unlabeled Pri308 (Fig. 4) but not by a 100-fold excess of unlabeled nonimmune mouse IgG (data not shown). Moreover, the binding of 125I-Pri308 on confluent mouse PrP-overexpressing N2a cells was similar to nonspecific binding (data not shown). The binding equilibrium of 125I-Pri308 was reached by 90 min at 37 °C. In subsequent experiments, the cells were incubated with 125I-Pri308 for 90 min at 37 °C, then rapidly rinsed, and incubated in the presence or in the absence of 500 μM of CuSO4 for different times. At the end of the incubation time, the cells were submitted to a hypertonic acid wash for 4 min at 37 °C. Then the incubation medium was rapidly removed, and 300 μl of Earle’s buffer containing radiolabeled Pri308 (800,000 cpm/300 μl) for 90 min. Filipin or chlorpromazine (chlorpro) were added to the incubation medium 5, 15, and 30 min prior to the end of the radiolabeled IgG incubation time at a final concentration of 5 μg/ml. The medium was then removed and replaced by 500 μl of Earle’s buffer containing 500 μM CuSO4 and 5 μg/ml of filipin or chlorpromazine as indicated for an additional incubation time of 30 min at 37 °C. Finally, the cells were treated with Earle’s buffer, pH 4, containing 0.5 M NaCl for 4 min at 37 °C (gray histograms) or by two washes with 1 ml of Earle’s buffer (white histograms). The radioactivity was recovered by scraping the cells with 500 μl of 0.2 M NaOH and counted. A, representation of the effects of drugs on cell-associated radiolabeled Pri308 binding as a function of time. The results are expressed as percentages of specific binding (i.e. total binding – nonspecific binding) of the control experiment performed in the absence of drug (left panel). For endocytosis experiments (right panel), the results are expressed as follows: acid wash-resistant binding × 100/cell-associated specific binding. B, dose-response of filipin on the radiolabeled IgG binding. The results are the means of five independent experiments where each point is the average of duplicate determinations.

Effects of Filipin and Chlorpromazine on Binding and Copper-induced Internalization of 125I-Pri308 Antibody to Human Microglia Cells—We next examined the effects of filipin and chlorpromazine on both the 125I-Pri308 specific binding and the copper-induced endocytosis assays to human microglia cells. As expected, the cells treated with filipin exhibited a significant decrease in the amount of acid wash-resistant binding of 125I-Pri308 antibody after copper stimulation (Fig. 5A, right panel). Surprisingly, the filipin treatment also induced a decrease of the specific binding of 125I-Pri308 antibody in a time-dependent manner (Fig. 5A, left panel). One hour of filipin treatment resulted in a 60% decrease of the 125I-Pri308-specific binding (Fig. 5A). The inhibition of the cell surface binding of radiolabeled IgG by filipin was concentration-dependent (Fig. 5B). In the course of these experiments, there was no evidence for cytotoxicity or reduced protein synthesis in human microglia cultures exposed to filipin. Chlorpromazine had no effect on the
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125I-Pri308 antibody-specific binding to C13-NJ cells nor on copper-induced endocytosis mechanism (Fig. 5A). In summary, we show that filipin decreases the cell surface-specific binding of 125I-Pri308 antibody and inhibits the copper-induced endocytosis of the remaining PrP-sen molecules.

Filipin Induces Release of PrP-sen from Cell Surface of Human Microglia and Mouse Neuroblastoma Cells—The decrease in cell surface-specific binding of 125I-Pri308 antibody induced by filipin could be due to a release of PrP-sen from the cell surface into the culture medium. To investigate this possibility, C13-NJ and 3F4-N2a cells were treated with filipin, and the culture media were assayed for the presence of PrP-sen by Western blot. As shown in Fig. 6, treatment of both type of cells by filipin induces the release of fully matured PrP-sen into the medium in a time-dependent manner. In these experiments, if the mixture of protease inhibitors was omitted, the aminoterminal epitope of SAF32 antibody was rapidly destroyed (data not shown), whereas the epitopes of SAF70 and Pri308 remained intact. In summary, filipin induces a continuous release of membrane-bound PrP-sen into the medium.

Inhibition of PrP-res Formation in N2a S12sc− Cells by Filipin—Because filipin treatment results in a cell surface PrP-sen, we asked whether filipin could also inhibit the PrP-res accumulation in chronically infected N2a S12sc− cells. N2a S12sc− cells were incubated continuously for 4 days with various concentrations of filipin, and then cell lysates were assayed for the presence of protease K-resistant isoforms of PrP (Fig. 7A). Alternatively, discontinuous treatment of cells was performed by treating cells in the absence (0) or in the presence of the indicated concentrations of filipin for 4 days. PrP-res was detected with the monoclonal antibody SAF70. B, dose-response curve of the inhibition of PrP-res accumulation induced by filipin. The data represent the means of four independent experiments ± S.D. (bars) and were expressed as relative quantities of PrP-res in the presence of the drug compared with the control culture. MW, molecular mass.

FIG. 6. Kinetics of release of surface-bound PrP induced by filipin treatment. Shown is a representative experiment performed by treating C13-NJ or N2aS12 cells in the absence (control) or in the presence of 5 µg/ml of filipin at 37 °C in PBS containing 1% glucose and a mixture of inhibitors. At the indicated times, the incubation medium was collected and trichloroacetic acid-precipitated. The pellets were resuspended in denaturing loading buffer and analyzed by Western blot. PrP was revealed with the indicated anti-PrP antibodies and developed with the enhanced chemiluminescence system. MW, molecular mass.

FIG. 7. Effect of filipin on the accumulation of PrP-res in infected N2aS12. A, immunoblot of a representative experiment performed by treating cells in the absence (0) or in the presence of the indicated concentrations of filipin for 4 days. PrP-res was detected with the monoclonal antibody SAF70. B, dose-response curve of the inhibition of PrP-res accumulation induced by filipin. The data represent the means of four independent experiments ± S.D. (bars) and were expressed as relative quantities of PrP-res in the presence of the drug compared with the control culture. MW, molecular mass.

**DISCUSSION**

In the present report, we investigate the mechanism of PrP endocytosis induced by copper ions in both overexpressing mouse PrP neuroblastoma and endogenous PrP expressed human microglia cells. Having quantitatively assayed the surface-bound and the caveolae-mediated trafficking pathway of PrP, we showed that disruption of cholesterol-rich domains could prevent PrP-res accumulation.

PrP trafficking has been previously investigated by indirect procedures using cell surface iodination or biotinylation (20, 33). In the radiolabeling IgG binding assay used in this study, several technical differences should be pointed out. First, the use of monoclonal antibody Pri308, which recognizes the 106–126 epitope of the human PrP sequence, allows us to selectively measure the unprocessed PrP molecules. Indeed, it has been shown that PrP-sen is submitted to a physiological proteolytic process occurring around amino acid residues 110–111 (34, 35), giving rise to disruption of the Pri308-recognized epitope. A significant part of PrP-sen molecules present at the surface of cells are amino terminus-shortened and thus are not taken into account in our radiolabeled IgG assay. Second, in light of studies showing that bivalent antibodies can alter the normal endocytic pathway of receptor (36, 37), we could not be sure a priori that this approach accurately reflected PrP trafficking. Several lines of evidence show that binding of whole IgG to PrP does not induce internalization. In the absence of copper stimulation, monoclonal wash-resistant (i.e. internalized) 125I-Pri308 was measured in the course of the incubations. Our results were consistent with those obtained using the cell surface iodination/biotinylation assay, in terms of endocytosis kinetics, divalent ions efficiencies, and concentrations of copper required for induction of PrP endocytosis (20, 33). Moreover, a similar approach has been successfully used to characterize the trafficking of cell surface amyloid β-protein precursor (38). Together, these observations strongly suggested that the binding
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PrP molecules. To our knowledge, this is the first report showing that filipin is capable of releasing a GPI-anchored protein. Acute or chronic filipin treatments of scrapie-infected N2a efficiently inhibit PrP-res accumulation. It was proposed that the PrP-sen/PrP-res conversion process could be compared with a autocatalytic polymerization reaction (48) occurring somewhere between the cell surface and the organelles of the endocytic pathway. Thus, decreasing the level of PrP-sen substrate leads to an inhibition of PrP-res formation. Both inhibition of endocytosis of PrP and release of membrane-bound PrP induced by filipin could produce additional effects by removing the substrate available for conversion. Note that phosphatidylinositol-specific phospholipase C treatment inhibits PrP-res formation into infected N2a cells likely by cleavage of the GPI anchor and release of PrP from the cell surface (4). There is a lot of evidence showing that filipin, amphotericin B, and nystatin, which belong to the same polycyclic antibiotic family, interact with cholesterol (for review see Ref. 49). Although amphotericin B is one of the most potent anti-scrapie agents, its mechanism of action remains unclear (16, 50). In our radiolabeled IgG assays, no effect of amphotericin B was found on PrP endocytosis and on the release of cell surface PrP, suggesting that its mechanism of action differs from that of filipin. The same statement has been made concerning chlorpromazine, which inhibits PrP-res formation in infected N2a (32) remains without effect on surface-bound PrP relocation. The antiscrapie mechanisms of action of both chlorpromazine and amphotericin B remain to be characterized.

It has been shown that caveolae-like domains isolated from infected N2a contain PrP-res (6). However, we never detected PrP-res in the culture medium of infected N2a treated by filipin, suggesting that the anchorage of PrP-res at the cell surface may be insensitive to disruption of lipid rafts.

In conclusion, our results further document the crucial role played by caveola-like domains in the cellular events leading to PrP-res formation. The identification of drugs such as filipin that disturb the trafficking of PrP underscores the role of lipid rafts in the pathogenesis of TSE and may provide new therapeutic strategies.

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