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Monoacylated Cellular Prion Protein Modifies Cell Membranes, Inhibits Cell Signaling, and Reduces Prion Formation*[S]

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Prion diseases occur following the conversion of the cellular prion protein (PrP^C) into a disease related, protease-resistant isoform (PrP^{Sc}). In these studies, a cell painting technique was used to introduce PrP^C to prion-infected neuronal cell lines (ScGT1, ScN2a, or SMB cells). The addition of PrP^C resulted in increased PrP^{Sc} formation that was preceded by an increase in the cholesterol content of cell membranes and increased activation of cytoplasmic phospholipase A_2 ($cPLA_2$). In contrast, although PrP^C lacking one of the two acyl chains from its glycosylphosphatidylinositol (GPI) anchor (PrP^C-G-lyso-PI) bound readily to cells, it did not alter the amount of cholesterol in cell membranes, was not found within detergent-resistant membranes (lipid rafts), and did not activate $cPLA_2$. It remained within cells for longer than PrP^C with a conventional GPI anchor and was not converted to PrP^{Sc}. Moreover, the addition of high amounts of PrP^C-G-lyso-PI displaced $cPLA_2$ from PrP^{Sc}-containing lipid rafts, reduced the activation of $cPLA_2$, and reduced PrP^{Sc} formation in all three cell lines. In addition, ScGT1 cells treated with PrP^C-G-lyso-PI did not transmit infection following intracerebral injection to mice. We propose that that the chemical composition of the GPI anchor attached to PrP^C modified the local membrane microenvironments that control cell signaling, the fate of PrP^C, and hence PrP^{Sc} formation. In addition, our observations raise the possibility that pharmacological modification of GPI anchors might constitute a novel therapeutic approach to prion diseases.

The transmissible spongiform encephalopathies, which are also known as prion diseases, are invariably fatal neurodegenerative disorders that include scrapie in sheep, bovine spongiform encephalopathy in cattle, and Creutzfeldt-Jakob disease in human(s). A key event in these diseases is the conversion of a normal host protein, designated PrP^C,[3] into a disease-associated isoform (PrP^{Sc}), which represents the major component of infectious scrapie prions (1). This process involves a portion of PrP^C that is mostly α-helix being refolded into a β-pleated sheet in the PrP^{Sc} molecule (2). PrP^{Sc} acts as a template that facilitates the conversion of PrP^C to PrP^{Sc}. The conversion of PrP^C to PrP^{Sc} is accompanied by changes in biological and biochemical properties, including an increased resistance to proteases (3), resulting in the accumulation of PrP^{Sc} in affected brain areas (4).

Although the presence of PrP^C is essential for prion replication (5, 6), it does not ensure prion replication, and it is thought that the targeting of PrP^C to specific membranes is required for efficient PrP^{Sc} formation. For example, antibody studies suggest that PrP^C conversion occurs at the cell surface (7, 8), and treatments that altered the intracellular trafficking of PrP^C also affected PrP^{Sc} formation (9, 10). In addition, treatment with cholesterol synthesis inhibitors that altered the cellular location of PrP^C also reduced PrP^{Sc} formation (11, 12). Collectively, these observations indicate that the factors that affect the intracellular trafficking of PrP^C affect its conversion to PrP^{Sc}.

The majority of PrP^C molecules are linked to membranes via a glycosylphosphatidylinositol (GPI) anchor (13). The role of the GPI anchor in prion disease is controversial in that although transgenic mice producing anchorless PrP^C produced large amounts of extracellular PrP^{Sc} (14), a recent study showed that cells producing anchorless PrP^C were resistant to scrapie infection (15). Moreover, these studies employed genetic methods that removed the entire GPI anchor rather than specific modifications of GPI anchors. The GPI anchor targets PrP^C to detergent-resistant membrane microdomains that are commonly called lipid rafts and which are necessary for efficient PrP^{Sc} formation (12). Many GPI-anchored molecules transfer between cell membranes, a process called cell painting (16, 17). The transfer of PrP^C between cells *in vitro* (18) showed that cell painting could be used to introduce PrP^C with different GPI anchors to recipient cells. In this study, we report the effects of modification of the GPI anchor attached to PrP^C on PrP^{Sc} formation using a combination of a cell painting technique and PrP^C that had been digested by phospholipase A_2 (PrP^C-G-lyso-PI) or phosphatidylinositol-phospholipase C (PrP^C-IPG) (Fig. 1).

EXPERIMENTAL PROCEDURES

Cell Lines—Prion-infected ScN2a, ScGT1, and SMB cells were grown in Ham's F12 medium containing 2 mM glutamine,

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[3] The abbreviations used are: PrP^C, cellular prion protein; $cPLA_2$, cytoplasmic phospholipase A_2; PI, phosphatidylinositol; GPI, glycosylphosphatidylinositol; IPG, inositolphosphoglycan.

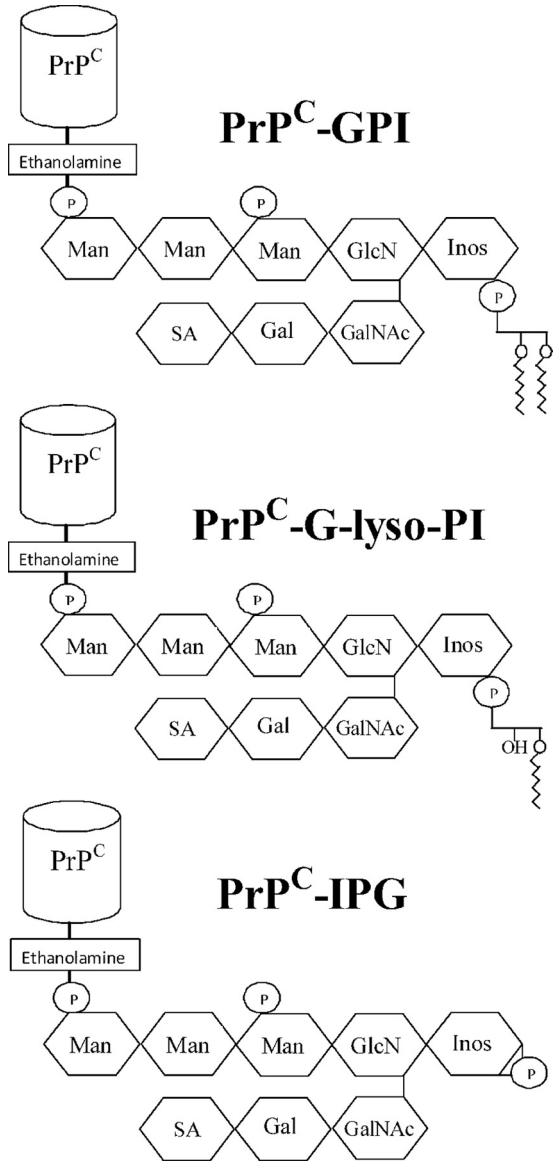

FIGURE 1. **Phospholipase digestion of PrP^C affects the GPI anchors.** Shown is a cartoon displaying the putative GPI anchor attached to PrP^C. Glycan residues shown include inositol (*Inos*), mannose (*Man*), sialic acid (*SA*), galactose (*Gal*), *N*-acetyl galactosamine (*GalNAc*), and glucosamine (*GlcN*) as well as phosphate (*P*). Also shown are the products of PrP^C after digestion with either PLA_2 (PrP^C-G-lyso-PI) or phosphatidylinositol-phospholipase C (PrP^C-IPG).

2% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. To determine the effect of PrP^C preparations on PrP^{Sc} formation, ScN2a, SMB, or ScGT1 cells were plated in six-well plates at 10^5 cells/well and allowed to adhere overnight. Cells were then cultured in the presence or absence of test preparations, with daily changes of media, for 7 days. Cells were washed twice in PBS, and extracts were collected. Spent medium was collected and concentrated by centrifugation with a 10-kDa filter (Vivaspin, Sartorius) to examine whether PrP^{Sc} was released into culture supernatants. The survival of cells was determined by the addition of 25 μM thiazolyl blue tetrazolium for 3 h and reported as a percentage of untreated cells.

Evaluation of Infectivity—Treated ScGT1 cells were detached and counted, washed twice with PBS, frozen, and thawed. Membranes were centrifuged at 16,000 $\times$ *g* for 30 min, washed twice, and homogenized in sterile 0.9% (w/v) saline at 2.5×10^6 cell equivalents/ml. C57/BL mice under halothane anesthesia were injected intracerebrally with 30 μl (7.5×10^4 cell equivalents) of this homogenate. Mice were monitored for clinical signs of scrapie until reaching a predefined clinical end point. All animal work was conducted according to local and national guidelines.

Primary Cortical Neurons—Cortical neurons were prepared from the brains of mouse embryos (day 15.5) derived from PrP null mice as described (11) and plated at 10^6 cells/well in six-well plates precoated with poly-L-lysine. Neurons were grown in neurobasal medium containing B27 components (PAA) for 10 days. Neurons were incubated with PrP^C preparations for different time periods and washed three times with PBS, and extracts were prepared. In some assays, cells were pulsed with PrP^C for 2 h, washed three times with PBS, and incubated in fresh culture medium for between 24 and 96 h. The amount of PrP^C expressed at the cell surface was determined by treating cells with 0.2 units of phosphatidylinositol-phospholipase C/10^6 cells for 1 h at 37 °C, and the amount of PrP^C released into the supernatant was measured by ELISA.

Cell Membrane Extracts—Treated cells were homogenized in an extraction buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.2% SDS) at 10^6 cells/ml, and nuclei and large fragments were removed by centrifugation (300 $\times$ *g* for 5 min). Mixed protease inhibitors (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, aprotinin, leupeptin, bestatin, pepstatin A, and E-46) (Sigma) and a phosphatase inhibitor mixture including PP1, PP2A, microcystin LR, cantharidin, and *p*-bromotetramisole (Sigma) were added to extracts where appropriate. To determine the amount of PrP^{Sc} produced, cell extracts/supernatants were digested with 1 μg/ml proteinase K for 1 h at 37 °C. Samples were heated to 95 °C for 5 min and tested in a PrP ELISA.

Isolation of Detergent-resistant Membranes—Cells were homogenized in an ice-cold buffer containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 10 mM EDTA, and mixed protease inhibitors at 10^6 cells/ml. Nuclei and large fragments were removed by centrifugation (500 $\times$ *g* for 5 min). The postnuclear supernatant was incubated on ice for 60 min and centrifuged (16,000 $\times$ *g* for 30 min at 4 °C). The soluble material contained the normal cell membrane (detergent-resistant membranes). Pellets were homogenized in 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.2% SDS and mixed protease inhibitors, centrifuged again (16,000 $\times$ *g* for 10 min), and the supernatant containing the lipid raft constituents was collected.

Sucrose Density Gradients—Cultured neurons were harvested with a Teflon scraper and homogenized in 250 mM sucrose, 10 mM Tris-HCl, pH 7.2, 1 mM EDTA, and 1 mM dithiothreitol at 10^6 cells/ml. Nuclei and membrane fragments were removed by centrifugation (1000 $\times$ *g* for 5 min). Membranes were washed by centrifugation at 16,000 $\times$ *g* for 10 min at 4 °C and suspended in an ice-cold buffer containing 1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, and protease and phosphatase inhibitors (as above). 5–40% sucrose solutions were prepared and layered to produce a gra-

dient. Membranes were added and centrifuged (50,000 × *g* for 4 h at 4 °C). Fractions were collected from the bottom of gradients.

Isolation of PrP^C—PrP^C was isolated from GT1 neuronal cell membranes that had been homogenized in a buffer containing 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and mixed protease inhibitors using an affinity column loaded with mAb ICSM35 (d.gen, Inc.). Bound PrP^C was digested with 0.2 units/ml phosphatidylinositol-phospholipase C extracted from *Bacillus cereus* (Sigma) to generate PrP^C-IPG, or 100 units/ml bee venom phospholipase A_2 (PLA_2) (Sigma) to generate PrP^C-G-lyso-PI at 37 °C for 1 h. Digested PrP^C preparations were eluted using glycine-HCl, neutralized with 1 M Tris, and isolated via reverse phase chromatography on C18 columns (Waters). Proteins were eluted using a gradient of acetonitrile in water and 0.1% TFA as shown in supplemental Fig. 1*A*. PrP containing fractions were pooled, desalted, and concentrated. For bioassays, samples were diluted in culture medium and solubilized by sonication.

Isolation of GPI Anchors—GPIs were isolated from PrP^C by digestion with 100 μg/ml proteinase K at 37 °C for 24 h. The released GPIs were extracted with water-saturated butan-1-ol, washed with water, lyophilized, and stored in ethanol at 20 μM. GPIs were examined by high performance thin-layer chromatography on silica gel 60 plates and probed with mAb 5AB3-11 that binds to phosphatidylinositol as described (19).

PrP ELISA—The amount of PrP in samples was measured by ELISA as described (20). Maxisorb Immunoplates (Nunc) were coated with mAb ICSM18 (d.gen, Inc.) and blocked with 5% milk powder. Samples were applied and detected with biotinylated mAb ICSM35 (d.gen, Inc.), followed by extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate (Sigma). Absorbance was measured on a microplate reader at 405 nm, and the amount of PrP in samples was calculated by reference to a standard curve of recombinant murine PrP (Prionics).

Activated $cPLA_2$ ELISA—The activation of $cPLA_2$ is accompanied by phosphorylation of the serine 505 residue, which creates a unique epitope, and the amount of activated $cPLA_2$ in samples was measured by ELISA as described (20). Maxisorb immunoplates were coated with 0.5 μg/ml of mouse mAb anti-$cPLA_2$ (clone CH-7 (Upstate)). Samples were incubated for 1 h, and the amount of activated $cPLA_2$ was detected using a rabbit polyclonal antiphospho-$cPLA_2$ (Cell Signaling Technology) followed by a biotinylated anti-rabbit IgG (Dako), extravidin-alkaline phosphatase, and 1 mg/ml 4-nitrophenyl phosphate. Samples were expressed as "units of activated $cPLA_2$," where 100 units was the amount of activated $cPLA_2$ in extracts from 10^6 untreated cells. To measure $cPLA_2$ protein immunoplates were coated with mAb CH-7, and samples were applied as above. Bound $cPLA_2$ was detected using a goat polyclonal anti-$cPLA_2$ (Santa Cruz Biotechnology) followed by biotinylated anti-goat IgG, extravidin-alkaline phosphatase, and 1 mg/ml 4-nitrophenyl phosphate.

Immunoprecipitations—Treated cells were solubilized in ice cold 1% Triton X-100, 10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 10 mM EDTA 10^6 cells/ml for 1 h at 4 °C. Cell debris was removed by centrifugation (500 × *g* for 5 min), and the supernatant was incubated with mAbs to PrP (ICSM35), $cPLA_2$ (CH-7), or isotype controls for 30 min at 4 °C on rollers. Magnetic microbeads containing protein G were added (10 μl/ml) (Miltenyi Biotech) for 30 min, and protein G bound antibody complexes were isolated using a μMACS magnetic system (Miltenyi Biotech.) at 4 °C. To determine PrP^{Sc} levels, isolated beads were predigested with 1 μg/ml proteinase K.

Western Analysis—Samples were mixed with Laemmli buffer containing β-mercaptoethanol, boiled, and separated by PAGE on a 15% gel. Proteins were transferred onto a Hybond-P PVDF membrane (Amersham Biosciences) by semi-dry blotting. Membranes were blocked using 10% milk powder. PrP was detected by incubation with mAb ICSM18 (d.gen, Inc.), β-actin by mAb clone AC-74 (Sigma), and $cPLA_2$ by mAb CH-7, followed by a secondary anti-mouse IgG conjugated to peroxidase. Bound antibody was visualized using enhanced chemiluminescence.

Cholesterol and Protein Content—Protein concentrations were measured using a micro-BCA protein assay kit (Pierce), and the amount of cholesterol was measured using the Amplex Red cholesterol assay kit (Invitrogen). The assay was performed after digestion with cholesterol esterase to determine the amount of esterified cholesterol within samples.

Statistical Analysis—Comparison of treatment effects was carried out using one-way and two-way analysis of variance. For all statistical tests, significance was set at the 1% level.

RESULTS

Addition of PrP^C Increased PrP^{Sc} Formation in Prion-infected Cells—Control and phosphatidylinositol-phospholipase C- or PLA_2-digested PrP^C preparations were isolated by reverse phase chromatography on C18 columns. Mock-treated PrP^C containing an intact GPI anchor (PrP^C-GPI) was eluted between 68 and 74% acetonitrile, PrP^C-G-lyso-PI was eluted between 30 and 36% acetonitrile, whereas PrP^C-IPG did not bind (supplemental Fig. 1*A*). Immunoblots showed that digestion with PLA_2 or phosphatidylinositol-phospholipase C did not affect the protein or glycan components of PrP^C (supplemental Fig. 1*B*). However, the GPI anchor isolated from PrP^C-G-lyso-PI and PrP^C-IPG had differed to that isolated from PrP^C-GPI (supplemental Fig. 1*C*).

The daily addition of PrP^C-GPI increased the amount of PrP^{Sc} in ScN2a cells in a dose-dependent manner (Fig. 2*A*). The increased PrP^{Sc} formation did not affect the survival of ScN2a cells, for example the addition of 10 ng PrP^C-GPI increased PrP^{Sc} formation without affecting cell survival (97% cell survival ± 9 compared with 100% ± 7, $n = 12$, $p = 0.7$). Immunoblots confirmed ELISA data and showed that the addition of PrP^C-GPI increased the PrP^{Sc} content of ScN2a cells without affecting the amount of β-actin (Fig. 2*B*). This effect of PrP^C-GPI was not cell-line specific; the daily addition of 10 ng of PrP^C-GPI also increased the PrP^{Sc} content of ScGT1 and SMB cells (Table 1). The amount of PrP^{Sc} in these cells was not altered by the addition of 10 ng PrP^C-IPG and was reduced following the addition of 10 ng PrP^C-G-lyso-PI. Supernatants were collected to determine whether extracellular PrP^{Sc} had been formed during these experiments. The daily addition of 10 ng PrP^C-GPI increased the amount of extracellular PrP^{Sc}

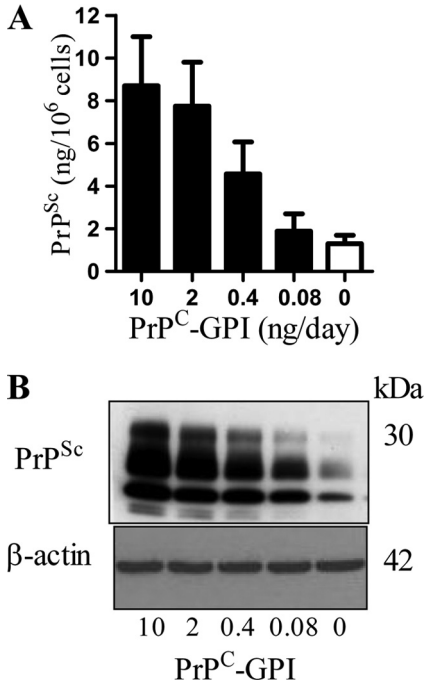

FIGURE 2. **The addition of PrP^C-GPI increased the PrP^{Sc} content of ScN2a cells.** *A*, The amount of PrP^{Sc} in ScN2a cells treated with different concentrations of PrP^C-GPI as shown for 7 days. Values shown are the mean amount of PrP^{Sc} (ng/10^6 cells) ± S.D. from triplicate experiments performed 10 times ($n = 30$). *B*, ScN2a cells were incubated for 7 days with different concentrations of PrP^C-GPI as shown, and cell extracts prepared (± proteinase K digestion) and separated by PAGE and transferred to membranes. β-Actin in cell extracts was detected by immunoblot with clone AC-74, and PrP^{Sc} was detected by immunoblot with mAb ICSM18.

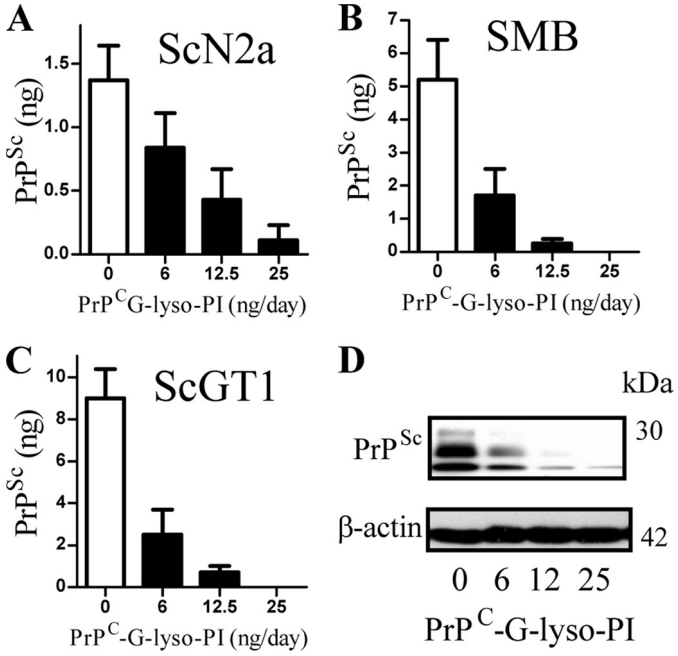

FIGURE 3. **The addition of PrP^C-G-lyso-PI reduced the PrP^{Sc} content of ScN2a cells.** *A*, the amount of PrP^{Sc} in ScN2a cells treated with PrP^C-G-lyso-PI as shown for 7 days. Values shown are the mean amount of PrP^{Sc} (ng/10^6 cells) ± S.D. from triplicate experiments performed 10 times ($n = 30$). *B*, the amount of PrP^{Sc} in SMB cells treated with PrP^C-G-lyso-PI as shown for 7 days. Values shown are the mean amount of PrP^{Sc} (ng/10^6 cells) ± S.D. from triplicate experiments performed 8 times ($n = 24$). *C*, the amount of PrP^{Sc} in ScGT1 cells treated with PrP^C-G-lyso-PI as shown for 7 days. Values shown are the mean amount of PrP^{Sc} (ng/10^6 cells) ± S.D. from triplicate experiments performed 8 times ($n = 24$). *D*, ScGT1 cells were incubated for 7 days with different concentrations of PrP^C-G-lyso-PI as shown, cell extracts prepared (± proteinase K digestion) and separated by PAGE and transferred to membranes. β-Actin in cell extracts was detected by immunoblot with clone AC-74, and PrP^{Sc} was detected by immunoblot with mAb ICSM18.

TABLE 1

PrP^C-G-lyso-PI inhibited the conversion of PrP^C to PrP^{Sc}

Prion-infected neuronal cell lines were treated daily with 10 ng of PrP^C-GPI, PrP^C-IPG, or PrP^C-G-lyso-PI as shown. After 7 days, cells were collected, and the amount of PrP^{Sc} was measured by ELISA. Values shown are the mean amount of PrP^{Sc} (ng/10^6 cells) ± S.D. in ScN2a cells ($n = 28$), ScGT1 cells ($n = 22$), or SMB cells ($n = 18$).

Treatment	PrP^{Sc} (ng/10^6 cells)		
	ScN2a cells	ScGT1 cells	SMB cells
None	1.3 ± 0.4	9.6 ± 1.4	5.8 ± 1.2
PrP^C-GPI	8.7 ± 2.3	19.4 ± 3.1	15.4 ± 3.3
PrP^C-IPG	1.1 ± 0.4	8.6 ± 2.2	5.2 ± 1.3
PrP^C-G-lyso-PI	0.6 ± 0.3	3.8 ± 1.6	1.6 ± 1.1

released from ScN2a cells (1.8 ng of PrP^{Sc} ± 0.67 compared with 0.38 ng ± 0.29, $n = 28$, $p < 0.05$). The addition of 10 ng PrP^C-IPG had no affect (0.41 ng of PrP^{Sc} ± 0.34 compared with 0.38 ng ± 0.29, $n = 28$, $p = 0.7$), and no PrP^{Sc} was detected in the supernatants of ScN2a cells incubated with 10 ng PrP^C-G-lyso-PI (<0.05 ng of PrP^{Sc}).

PrP^C-G-lyso-PI reduced PrP^{Sc} Formation—The inhibitory effect of PrP^C-G-lyso-PI on PrP^{Sc} formation was studied further. We report that PrP^C-G-lyso-PI reduced the PrP^{Sc} content of ScN2a cells (Fig. 3*A*), SMB cells (Fig. 3*B*), and ScGT1 cells in a dose-dependent manner (Fig. 3*C*). Immunoblots confirmed that PrP^C-G-lyso-PI reduced the PrP^{Sc} content of ScGT1 cells without affecting the amount of β-actin (Fig. 3*D*). The addition of 25 ng PrP^C-G-lyso-PI did not affect the survival of ScGT1 cells (101% cell survival ± 9 compared with 100 ± 7, $n = 12$, $p = 0.6$). PrP^C-G-lyso-PI also reduced the infectivity of ScGT1 cells. Groups of mice were injected intra-cerebrally with homogenates from ScGT1 cells treated for 7 days with 25 ng of PrP^C-G-lyso-PI or with a vehicle control. The mean incubation period of mice given the control homogenate was 164 days ± 4 (incubation period ± S.D., $n = 13$). None of the six mice inoculated with homogenates from cells treated with PrP^C-G-lyso-PI had died by day 600, indicating that treatment with PrP^C-G-lyso-PI reduced infectivity.

PrP^C-GPI Increased Amount of Cholesterol in Cells—The amount of cellular cholesterol affects the formation of PrP^{Sc} (11, 12). Here, we show that the addition of 10 ng PrP^C-GPI, but not PrP^C-G-lyso-PI, for 12 h increased the amount of cholesterol in ScN2a cell membranes (Fig. 4*A*) and reduced the amount of cholesterol esters (Fig. 4*B*) suggesting that the increase in cholesterol was partially derived from the hydrolysis of cholesterol esters. Pretreatment of cells with 100 μM diethylumbelliferyl phosphate, which inhibits the hydrolysis of cholesterol esters (21), reduced both the PrP^C-GPI-induced increase in cholesterol (613 ng/10^6 cells ± 38 compared with 727 ± 51, $n = 12$, $p < 0.01$), and prevented the reduction in cholesterol esters (Fig. 4*B*). The addition of 100 μM diethylumbelliferyl phosphate also reduced PrP^{Sc} production in ScN2a cells incubated with 10 ng of PrP^C-GPI (3.8 ng PrP^{Sc} ± 1.4 compared with 8.7 ± 2.3, $n = 12$, $p < 0.01$), indicating that the hydrolysis of cholesterol esters provides cholesterol to facilitate PrP^{Sc} formation.

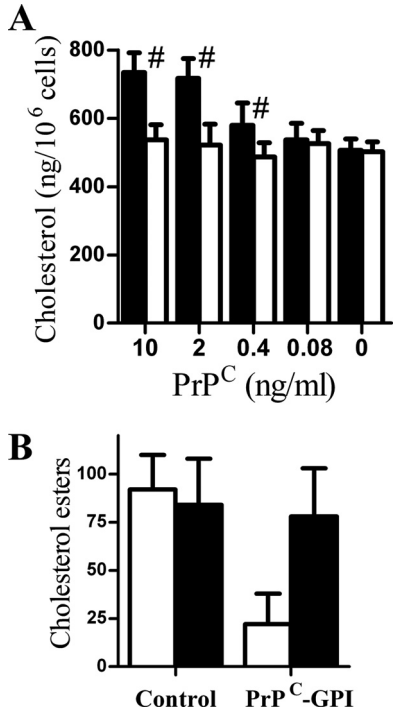

FIGURE 4. **PrP^C-GPI increased the cholesterol content of cell membranes.** *A*, the amount of cholesterol in extracts from ScN2a cells that had been treated for 12 h with different concentrations of PrP^C-GPI (■) or PrP^C-G-lyso-PI (□) as shown. #, amount of cholesterol in cell extracts significantly higher than that of untreated ScN2a cells. Values shown are the mean amount of cholesterol (ng/10^6 cells) ± S.D. from triplicate experiments performed four times ($n = 12$). *B*, the amount of cholesterol esters in extracts from ScN2a cells treated for 12 h with control medium or 10 ng of PrP^C-GPI as shown in the absence (□) or presence (■) of 100 μM diethylumbelliferyl phosphate. Values shown are the mean amount of cholesterol esters (ng/10^6 cells) ± S.D. from triplicate experiments performed three times ($n = 9$).

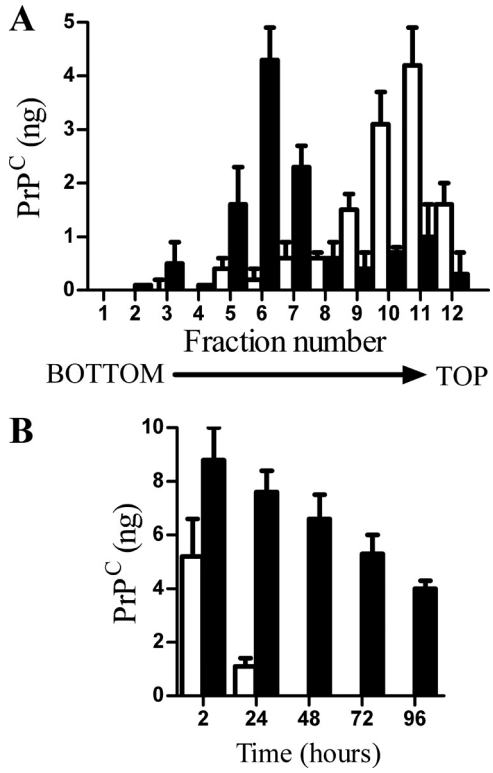

FIGURE 5. **Acylation of GPI anchors affected the targeting of PrP^C to lipid rafts.** *A*, PrP null cortical neurons were incubated with 10 ng of PrP^C-GPI (□) or PrP^C-G-lyso-PI (■) for 2 h. Cell extracts were prepared and separated by centrifugation on a sucrose density gradient, and the amount of PrP^C in each fraction was determined by ELISA. Values shown are the mean amount of PrP^C (ng) ± S.D. from triplicate experiments performed three times ($n = 9$). *B*, PrP null cortical neurons were pulsed with 10 ng of PrP^C-GPI (□) or PrP^C-G-lyso-PI (■) for 2 h. The amount of PrP^C in cells was determined at time points thereafter as shown. Values shown are the mean amount of PrP^C (ng/10^6 cells) ± S.D. from triplicate experiments performed four times ($n = 12$).

TABLE 2

Acylation of the GPI anchor altered the targeting of PrP^C to specific membranes

Cortical neurons derived from PrP null mice were incubated with 10 ng of PrP^C-GPI, PrP^C-IPG, or PrP^C-G-lyso-PI for 2 h. The amounts of PrP^C in whole cell extracts (Total), lipid rafts (detergent-resistant membrane (DRM)), detergent-soluble membranes (DSM), or expressed at the surface of cells were measured by ELISA. Values shown are the mean amount of PrP^C ± S.D. in total cell extracts ($n = 18$), detergent-resistant membranes, or detergent-soluble membranes ($n = 18$) and cell surface PrP^C ($n = 21$).

	PrP^C (ng/10^6 cells)		
	PrP^C-GPI	PrP^C-IPG	PrP^C-G-lyso-PI
Total	9.4 ± 0.9	<0.05	9.2 ± 1.3
DRM	8.2 ± 0.9	<0.05	2.3 ± 0.4
DSM	1.1 ± 0.2	<0.05	7.4 ± 1.1
Cell surface	3.7 ± 0.5	<0.05	8.3 ± 0.8

Acylation of GPI Anchor Altered Membrane Targeting of PrP^C—Cortical neurons derived from PrP null mice were incubated with 10 ng of PrP^C to determine the effect of the GPI anchor on the targeting of PrP^C. PrP^C-GPI and PrP^C-G-lyso-PI bound rapidly to cortical neurons, whereas PrP^C-IPG did not (Table 2). Although PrP^C-GPI was mostly found within detergent-resistant membranes, PrP^C-G-lyso-PI was detergent soluble (Table 2). Acylation of the GPI anchor also affected the expression of PrP^C at the cell surface; the amount of PrP^C-G-lyso-PI at the surface of these cells after 2 h was higher than the amount of PrP^C-GPI (8.3 ng PrP^C ± 0.8 compared with 3.7 ng ± 0.5, $n = 12$, $p < 0.01$). Cortical neurons from PrP null mice were incubated for 2 h with 10 ng of PrP^C preparations, and membrane constituents were separated on sucrose density gradients. Although most of the PrP^C-GPI was found in low density membranes, PrP^C-G-lyso-PI was found in different fractions (Fig. 5*A*). The targeting of proteins to lipid rafts can affect their trafficking within cells and hence their fate. After PrP null neurons were pulsed with 10 ng PrP^C-GPI or PrP^C-G-lyso-PI for 2 h PrP^C-GPI was rapidly cleared from neurons and was absent after 48 h, whereas PrP^C-G-lyso-PI remained in neurons for up to 96 h (Fig. 5*B*).

PrP^C-G-lyso-PI reduced activation of cPLA$_2$ in Prion-infected Cells—As PLA$_2$ is involved in prion formation (22), the effects of PrP^C-G-lyso-PI on cPLA$_2$ were studied. The addition of 25 ng of PrP^C-G-lyso-PI for 3 h reduced the amount of activated cPLA$_2$ in ScN2a cells (55 units ± 16 compared with 100 ± 12, $n = 12$, $p < 0.01$), ScGT1 cells (38 units ± 14 compared with 100 ± 12, $n = 12$, $p < 0.01$), or SMB cells (48 units ± 15 compared with 100 ± 12, $n = 15$, $p < 0.01$). Upon activation, cPLA$_2$ migrates to specific membranes utilizing a Ca^{2+}-dependent lipid binding domain (23). Sucrose density gradients showed that 3 h after the addition of 25 ng of PrP^C-G-lyso-PI to ScN2a cells, a proportion of cPLA$_2$ had relocated (Fig. 6*A*).

Prior studies showed that cPLA$_2$ was found within PrP^{Sc}-containing lipid rafts (20). We report that pretreatment of ScN2a cells with 25 ng of PrP^C-G-lyso-PI for 3 h did not affect

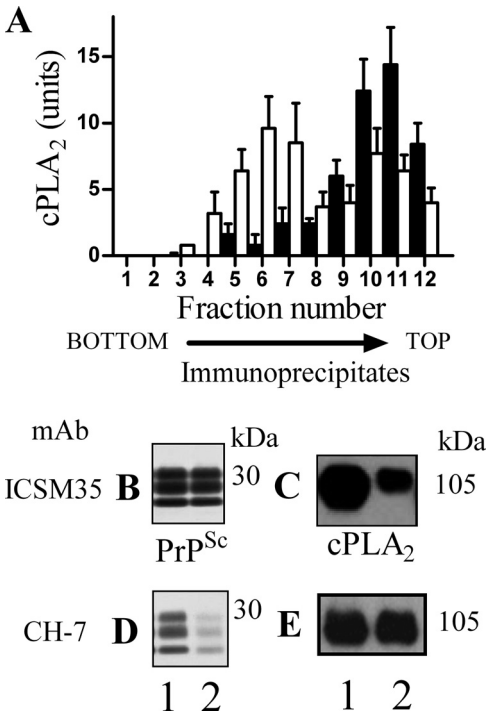

FIGURE 6. **PrP^C-G-lyso-PI reduced the co-precipitation of $cPLA_2$ with PrP^{Sc}.** *A*, membrane extracts from ScN2a cells that had been treated for 3 h with control medium (■) or 25 ng of PrP^C-G-lyso-PI (□) were separated by centrifugation on a sucrose density gradient, and the amount of $cPLA_2$ in each fraction was determined by ELISA. Values shown are the mean amount of $cPLA_2$ (units) ± S.D. from an experiment run in triplicate. Cell extracts were prepared from control ScN2a cells (*fraction 1*) or ScN2a cells that had been pretreated with 25 ng of PrP^C-G-lyso-PI (*fraction 2*). Membranes were isolated and incubated with either mAb ICSM35 (anti-PrP) or mAb CH-7 (anti-$cPLA_2$) and protein G magnetic beads. Immunoprecipitates were prepared (± proteinase K digestion), separated by PAGE, and transferred to membranes. The presence of PrP^{Sc} was detected by mAb ICSM18, and $cPLA_2$ was detected by mAb CH-7. Immunoblots show the amount of PrP^{Sc} (*B*) and $cPLA_2$ (*C*) precipitated by mAb ICSM35 (anti-PrP) or the amount of PrP^{Sc} (*D*) and $cPLA_2$ (*E*) precipitated by mAb CH-7 (anti-$cPLA_2$).

the amount of PrP^{Sc} precipitated by ICSM35 (Fig. 6*B*) but reduced the amount of co-precipitated $cPLA_2$ (Fig. 6*C*), indicating that PrP^C-G-lyso-PI caused the dissociation of some $cPLA_2$ from PrP-containing membranes. Conversely, an mAb to $cPLA_2$ (CH-7) precipitated similar amounts of $cPLA_2$ from extracts from control cells and cells incubated with 25 ng of PrP^C-G-lyso-PI (Fig. 6*E*). However, it precipitated less PrP^{Sc} from ScN2a cells treated with 25 ng of PrP^C-G-lyso-PI than from control cells (1.6 ng of PrP^{Sc} ± 1.2 compared with 5.2 ng ± 1.4, $n = 9$, $p < 0.01$) and (Fig. 6*D*). These studies were conducted on cells 3 h after the addition of PrP^C-G-lyso-PI and before any differences in the PrP^{Sc} content of cells were observed.

DISCUSSION

A cell painting technique was used to demonstrate the effect of the GPI anchor on the targeting of PrP^C to specific membranes, cell activation, and on the conversion of PrP^C to PrP^{Sc}. While treating prion-infected cells with PrP^C increased PrP^{Sc} formation, this effect was lost after the removal of acyl chains from the GPI anchor attached to PrP^C. Moreover, the addition of high amounts of PrP^C-G-lyso-PI altered membrane domains surrounding PrP^{Sc}, reducing the activation of $cPLA_2$, PrP^{Sc} formation, and infectivity.

The addition of PrP^C-IPG did not affect PrP^{Sc} formation in cells, a surprising observation considering of a report that transgenic mice producing anchorless PrP^C produced large amounts of PrP^{Sc} (14). However, the PrP^{Sc} produced in those mice was extracellular, and cells producing anchorless PrP^C are resistant to scrapie infection (15). It should also be noted that PrP^C-IPG contains the glycan component of the GPI anchor, whereas the transgenic mice produced PrP^C lacking the whole GPI anchor. Although PrP^C-G-lyso-PI was readily taken up by cells, we found no evidence that it was converted to PrP^{Sc}. Once incorporated into recipient cells, the effects and fate of PrP^C-GPI and PrP^C-G-lyso-PI were different. Prion formation is dependent upon the amount of cholesterol in cell membranes and whereas the addition of PrP^C-GPI increased the amount of cholesterol in membranes, consistent with reports that GPI anchors contain mostly saturated fatty acids, which help to solubilize cholesterol (24), the addition of PrP^C-G-lyso-PI had no effect. The increase in cholesterol following the addition of PrP^C-GPI was reduced by an inhibitor of cholesterol esterase, showing that it was partly derived from the hydrolysis of cholesterol esters. This release of this cholesterol was also necessary for the PrP^C-GPI induced PrP^{Sc} formation.

The amount of cholesterol in cell membranes is important for the formation and function of lipid rafts (25). Although both detergent resistance assays and sucrose density gradients showed that PrP^C-GPI was found within lipid rafts, most of the PrP^C-G-lyso-PI molecules were found within non-raft membranes. The presence of a GPI anchor targets PrP^C to cholesterol-dense lipid rafts (12, 26), which is essential for PrP^C to PrP^{Sc} conversion (11, 12). Thus, the targeting of PrP^C-G-lyso-PI to non-raft membranes suggested that it limited interactions between raft-associated PrP^{Sc} and PrP^C-G-lyso-PI.

PrP^C interacts with other membrane molecules, including glycosaminoglycans (27), the laminin receptor precursor (28), the low density lipoprotein receptor related protein-1 (29), or glypican-1 (30). The targeting of PrP^C-G-lyso-PI to the normal cell membrane may reduce interactions with these proteins, or facilitate interactions with other proteins that alter its trafficking. Differences in the composition of the GPI anchor affect the trafficking of proteins (31). Thus, greater amounts of PrP^C-G-lyso-PI than PrP^C-GPI were expressed at the cell surface, and although PrP^C-GPI was rapidly removed from these cells, PrP^C-G-lyso-PI remained in neurons for longer. One explanation of these results is that PrP^C-G-lyso-PI targets a different membrane domain to PrP^C-GPI and consequently most of the PrP^C-G-lyso-PI molecules traffic via a pathway that is physically segregated from PrP^{Sc}.

Although these theories explain why PrP^C-G-lyso-PI was not readily converted to PrP^{Sc}, a more refined hypothesis is required to explain why the addition of high amounts of PrP^C-G-lyso-PI reduced PrP^{Sc} production and infectivity. One possibility is that PrP^C-G-lyso-PI competed with endogenous PrP^C for endocytic partner proteins and consequently altered the trafficking of endogenous PrP^C and hence PrP^{Sc} formation. Another possibility is that PrP^C binds to PrP^{Sc} and modifies lipid rafts. The composition and hence function of lipid rafts is

dynamic and controlled by an induced fit model (32). Because PrP^{C}-G-lyso-PI did not sequester cholesterol, the membrane surrounding a complex between PrP^{Sc} and PrP^{C}-G-lyso-PI would contain less cholesterol than membranes formed following the interaction between PrP^{Sc} and PrP^{C}-GPI. Thus, the binding of PrP^{C}-G-lyso-PI to PrP^{Sc} may reduce the cholesterol content of local membranes to a level below that required for the conversion of PrP^{C} to PrP^{Sc}. This hypothesis is consistent with observations that formation of PrP^{Sc} was affected by the lipid composition of membranes (33) and that lipids were essential co-factors in prion formation (34). Finally, it is possible that PrP^{C}-G-lyso-PI is converted to PrP^{Sc}-G-lyso-PI, which in turn acts as an inefficient template for PrP^{C} to PrP^{Sc} conversion (35).

Lipid rafts are enriched with signaling molecules, which suggests that they act as domains in which the GPI anchors attached to PrP^{C} interact with cell signaling pathways (36). The activation of PLA_2 is required for PrP^{Sc} formation (22), and in prion-infected cells, $cPLA_2$ co-localized with PrP^{Sc}-containing lipid rafts (20). Such observations suggest that PrP^{C}-GPI binds to PrP^{Sc} in cholesterol-dense lipid rafts, where it activates $cPLA_2$ and facilitates the conversion of PrP^{C} to PrP^{Sc}. The addition of PrP^{C}-G-lyso-PI reduced activation of $cPLA_2$ in prion-infected cell lines. Notably, the addition of PrP^{C}-G-lyso-PI reduced the amount of $cPLA_2$ within PrP^{Sc}-containing lipid rafts. We propose that the binding of PrP^{C}-G-lyso-PI to PrP^{Sc} changed the composition of the underlying membrane so that it no longer captured $cPLA_2$. Upon activation, $cPLA_2$ migrated to intracellular membranes utilizing a Ca^{2+}-dependent lipid binding domain (23). It is thought that the targeting of $cPLA_2$ to membranes containing their endogenous substrates can regulate cell signaling. Thus, the targeting of $cPLA_2$ to specific membranes is essential for the formation of second messengers such as platelet-activating factor that facilitate PrP^{Sc} formation (22). Here, we showed that the addition of PrP^{C}-G-lyso-PI to prion-infected cells altered the location of $cPLA_2$. More specifically, it caused the dissociation of $cPLA_2$ from PrP^{Sc}, reduced activation of $cPLA_2$ and hindered the conversion of PrP^{C} to PrP^{Sc}. The activation of $cPLA_2$ is essential to the maintenance of the Golgi network (37), which is involved in the trafficking of a GFP-tagged PrP^{C} (38). Thus, altering the GPI anchor attached to PrP^{C} may reduce the activation of $cPLA_2$ and alter the trafficking of PrP^{C} away from specific sites conducive to prion formation.

In conclusion, we showed that the addition of PrP^{C}-G-lyso-PI reduced the activation of $cPLA_2$ and PrP^{Sc} formation in prion-infected cells. We propose that the chemical composition of the GPI anchor is a factor that targets PrP^{C} to sites conducive to conversion to PrP^{Sc}. Moreover, these results raise the possibility that drugs, which alter the structure of the PrP^{C}-GPI anchor, may provide novel treatments for prion diseases.

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