Effect of Glycosylphosphatidylinositol Anchor-dependent and -independent Prion Protein Association with Model Raft Membranes on Conversion to the Protease-resistant Isoform*

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Prion protein (PrP) is usually bound to membranes by a glycosylphosphatidylinositol (GPI) anchor that associates with detergent-resistant membranes, or rafts. To examine the effect of membrane association on the interaction between the normal protease-sensitive PrP isoform (PrP-sen) and the protease-resistant isoform (PrP-res), a system was employed using PrP-sen reconstituted into sphingolipid-cholesterol-rich raft-like liposomes (SCRLs). Both full-length (GPI*) and GPI-anchor-deficient (GPI–) PrP-sen produced in fibroblasts stably associated with SCRLs. The latter, alternative mode of membrane association was not detectably altered by glycosylation and was markedly reduced by deletion of residues 34–94. The SCRL-associated PrP molecules were not removed by treatments with either high salt or carbonate buffer. However, only GPI* PrP-sen resisted extraction with cold Triton X-100. PrP-sen association with SCRLs was pH-independent. PrP-sen was also one of a small subset of phosphatidylinositol-specific phospholipase C (PI-PLC)-released proteins from fibroblast cells found to bind SCRLs. A cell-free conversion assay was used to measure the interaction of SCRL-bound PrP-sen with exogenous PrP-res as contained in microsomes. SCRL-bound GPI* PrP-sen was not converted to PrP-res until PI-PLC was added to the reaction or the combined membrane fractions were treated with the membrane-fusing agent polyethylene glycol (PEG). In contrast, SCRL-bound GPI– PrP-sen was converted to PrP-res without PI-PLC or PEG treatment. Thus, of the two forms of raft membrane association by PrP-sen, only the GPI anchor-directed form resists conversion induced by exogenous PrP-res.

Prion protein (PrP)1 is a glycoprotein usually bound to membranes by a glycosylphosphatidylinositol (GPI) anchor (1). Like other GPI-anchored proteins, PrP is enriched in sphingolipid- and cholesterol-rich membrane microdomains known as detergent-resistant membranes (DRMs), or rafts (2). Several lines of evidence from biochemical and molecular biological approaches suggest raft association is required for conversion of the normal protease-sensitive isoform (PrP-sen) to the transmissible spongiform encephalopathy-associated protease-resistant isoform (PrP-res) in a cell culture model of infection (2–7). Although cell-free studies using purified PrP molecules have provided new insights into binding and conversion of PrP-sen by PrP-res (reviewed in Ref. 8), few studies have considered the membrane-associated nature of PrP (9–13) and the influence of this association on PrP-sen/PrP-res interactions (14).

Given the complex composition of cellular raft membranes, which contain molecules other than PrP-sen that might influence interactions with PrP-res, investigations into the effect of PrP-sen association with rafts on these interactions would benefit from the use of a defined system that replicated raft membranes in the absence of other raft-associated molecules. One candidate system involves the use of sphingolipid-cholesterol-rich raft-like liposomes (SCRLs) containing phosphatidylycholine, sphingomyelin, brain cerebrosides, and cholesterol. SCRLs have been shown to resemble rafts in several respects, including major lipid composition and low buoyant density, which permits their isolation by floatation through density gradients (15). GPI-anchored proteins reconstituted into SCRLs or related sphingolipid-rich liposomes acquire properties of their cell-associated counterparts, most notably insolubility in cold Triton X-100 (15, 16). Hence, SCRLs serve as a reasonable approximation of the natural membrane environment of PrP in the absence of other raft-associated molecules.

Our previous work examined the effect of PrP-sen association with rafts on interactions with PrP-res using raft membranes prepared from neuroblastoma cells (14). These experiments showed that raft-bound PrP-sen resisted conversion to PrP-res until PrP-sen was released from rafts by phospholipase digestion or the PrP-res was inserted into contiguous membranes. To examine the effect of PrP-sen membrane association on its interactions with PrP-res under more defined conditions and to determine if membrane association itself inhibits conversion of PrP-sen by exogenous PrP-res, we have employed a model system using PrP-sen reconstituted into SCRLs. While developing this system, two groups recently reported a novel property of recombinant PrP-sen expressed in Escherichia coli: binding to model membranes of various compositions (10, 13).

We had also observed this phenomenon in our system using GPI anchor-deficient PrP-sen expressed in mammalian cell

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lines and have further characterized the nature of this binding activity using various forms of PrP-sen that may more closely represent the native state of the molecule, particularly with respect to the addition of N-linked glycans. Furthermore, we have directly tested the effect of the two methods of PrP-sen association with membranes (i.e. GPI anchor-dependent and -independent) on its interactions with exogenous PrP-res molecules by its ability to serve as a substrate for conversion to the protease-resistant state under cell-free conditions. Our results indicate that the method of PrP-sen association with model membranes has strikingly different effects on its ability to interact with PrP-res.

EXPERIMENTAL PROCEDURES

Cells and Purification of $^{35}$S-Labeled PrP-sen—Hamster PrP-sen was derived from mouse fibroblast cell lines expressing either full-length (GPI-PrP-sen) or GPI anchor-deficient (GPI−PrP-sen) wild-type (wt) PrP-sen (17, 18). The corresponding N-terminal hamster PrP-sen deletion mutants were derived from cell lines created by Lawson and co-workers (19). Wild-type GPI mouse PrP-sen was isolated from a mouse neuroblastoma cell line described elsewhere (20). PrP-sen molecules were immunoprecipitated from cells metabolically labeled with $^{[35]$S]methionine as described previously (21). Mouse PrP-sen was immunoprecipitated with 3F4 monoclonal antibody (23) or rabbit polyclonal antiserum (R20) raised against a C-terminal (amino acids 218–232) PrP synthetic peptide (22). The R20 antiserum was used specifically to isolate PrP-sen molecules (both wt and mutants) for experiments involving the N-terminal deletion mutants, because one mutant (Δ124) lacks the 3F4 epitope. Cell culture supernatants were immunoprecipitated using rabbit antiserum (R30) against a PrP synthetic peptide (amino acids 89–103) (22). Hamster PrP-sen was immunoprecipitated with 3F4 monoclonal antibody (23) or rabbit polyclonal antiserum (R20) raised against a C-terminal (amino acids 218–232) PrP synthetic peptide (22). The R20 antiserum was used specifically to isolate PrP-sen molecules (both wt and mutants) for experiments involving the N-terminal deletion mutants, because one mutant (Δ124) lacks the 3F4 epitope. Cell culture supernatants containing phosphatidylinositol-specific phospholipase C (PI-PLC)-released proteins from metabolically labeled cells expressing GPI−PrP-sen were prepared as described previously (14). Where indicated, PI-PLC-released hamster PrP-sen (referred to as GPI−PrP-sen) was immunoprecipitated from the supernatants as described elsewhere with 3F4 or R20 (14). For some experiments, PI-PLC culture supernatant proteins were deglycosylated with PNGase F (New England BioLabs) as per the manufacturer’s instructions prior to PrP-sen immunoprecipitation.

Preparation of Liposomes—Sphingolipid-cholesterol-rich liposomes (SCRLs) were prepared essentially as described previously (15) with mice neuroblastoma cells and have further characterized the nature of this binding activity using various forms of PrP-sen that may more closely represent the native state of the molecule, particularly with respect to the addition of N-linked glycans. Furthermore, we have directly tested the effect of the two methods of PrP-sen association with membranes (i.e. GPI anchor-dependent and -independent) on its interactions with exogenous PrP-res molecules by its ability to serve as a substrate for conversion to the protease-resistant state under cell-free conditions. Our results indicate that the method of PrP-sen association with model membranes has strikingly different effects on its ability to interact with PrP-res.

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gradient. GPI anchor-deficient (GPI- ) PrP-sen was mixed with SCRLs in the presence of 0.08% Sarkosyl followed by immediate dialysis. The dialyzed sample was fractionated by flotation on five-step Optiprep gradients as described under “Experimental Procedures.” Fraction numbers for fractions collected from the gradients are indicated above each lane. Molecular mass markers are indicated in kDa on the left. The data are representative of several experiments.

RESULTS

PrP-sen Reconstitution into SCRLs—We adapted a previously described technique (15) to allow reconstitution of PrP-sen into SCRLs. Hamster PrP-sen was expressed in fibroblasts as either full-length GPI-anchored protein (GPI-PrP) or a GPI anchor-deficient (GPI′-PrP) form lacking the GPI anchor addition sequence. Purified PrP-sen was mixed with SCRLs in the presence of a low concentration (≤0.08%) of Sarkosyl and immediately dialyzed against buffered saline. SCRLs and SCRL-bound PrP-sen were then isolated by flotation through a density gradient. GPI-PrP-sen stably associated with SCRLs in the low density fractions (1–4) of the gradient (Fig. 1A). Surprisingly, control experiments with GPI′-PrP-sen revealed that this derivative also avidly associated with SCRLs (Fig. 1B). When fractionated on gradients in the absence of SCRLs, all of the PrP-sen (both GPI′ and GPI″) was found in the bottom fraction (fraction 6) indicating that flotation of PrP-sen in the gradient is dependent upon SCRLs (data not shown). To verify the SCRL association was not due to trapping of PrP-sen inside the liposomes, we incubated the PrP-containing SCRLs with proteinase K (PK). As shown in Fig. 2, the vast majority (90%) of SCRL-bound PrP-sen was susceptible to PK digestion without detergent-mediated disruption of the liposomes, indicating that the bulk of the protein was surface-accessible. These data show that PrP-sen exhibits an alternative, GPI anchor-independent method of associating with model membranes, a conclusion consistent with other recent studies (10, 13).

PrP-sen Associates with SCRLs via Hydrophobic Interactions—The nature of the binding interactions of the PrP-sen molecules with SCRLs was characterized by subjecting the PrP-bound SCRLs to various extraction conditions. Treatments with either high salt (1 m NaCl (Fig. 3A) or 3 m NaCl (data not shown)) or 0.1 m sodium carbonate (pH 11.5) (Fig. 3B), which extract peripheral membrane proteins, failed to remove either type of PrP-sen molecule from SCRLs. A significant fraction (40–50%) of GPI′-PrP-sen resisted extraction with Triton X-100 at 4 °C (Fig. 3C, lanes 1 and 2 versus lane 6) but not 37 °C (Fig. 3D, lanes 1 and 2 versus lane 6), resembling the behavior of GPI-anchored proteins in rafts (14, 26). Treatment of the cold-Triton X-100-extracted SCRLs with PI-PLC to cleave the GPI anchors caused the release of a majority (60%) of the formerly GPI-anchored PrP-sen molecules from the SCRLs upon re-extraction with cold Triton (data not shown). However, GPI′-PrP-sen was readily extracted with cold Triton X-100 without PI-PLC treatment (Fig. 3C, lanes 7 and 8 versus lane 12), suggesting that this GPI anchor-independent method of attachment was mediated by hydrophobic protein-lipid interactions. A very small proportion of GPI′-PrP-sen apparently resisted extraction by cold Triton X-100, possibly due to use of an insufficient amount of detergent or inversion of the liposomes during extraction leading to trapping/reorienting of PrP-sen inside the liposomes. In support of this proposal, we have observed a small decrease (5%) in the amount of surface-localized PrP-sen in GPI-PrP-sen-containing SCRLs after extraction with cold Triton X-100 (data not shown).

Because others have reported various factors affecting the interaction of PrP-sen with membranes using model systems different from ours, we examined the effect of some of these factors on the binding observed in our system. Given evidence PrP may exhibit preferential binding to specific lipids (12, 27), we tested the incorporation efficiency of PrP-sen into modified SCRLs. No specific interactions with any SCRL lipids were detected as both types of PrP-sen incorporated similarly into...
modified SCRLs lacking any of the individual lipid components or sphingolipids (i.e. PC/Chol liposomes) (data not shown). Other investigators have shown a pH-dependent binding of recombinant PrP-sen to liposomes (10, 13). We determined the incorporation efficiency over a range of pH in our system. Association of both GPI and GPI anchor-deficient PrP-sen was pH-independent over the range of pH 5–7.8 (data not shown). Therefore, the factors influencing PrP-sen binding to membranes in our SCRL system differ from those reported previously.

**PrP-sen Binds SCRLs in the Absence of Detergent**—Given the reconstitution protocol involved the incubation of PrP-sen with SCRLs in the presence of a low concentration of detergent, it was possible that the GPI anchor-independent binding was mediated by a minor, transient perturbation of the liposomes by the detergent. To address this issue, binding studies were conducted by directly mixing GPI PrP-sen with SCRLs in the absence of detergent followed by SCRL isolation by floatation. Under these conditions, GPI PrP-sen readily associated with SCRLs (Fig. 4A). Kinetic analysis of the association within the time constraints of the floatation assay indicated this binding occurred rapidly, achieving near maximal levels with a 5-min incubation (41% of input PrP-sen) (Fig. 4A), although there was an additional ~20-min delay before initiating the centrifugation due to the time required to prepare the density gradients. Extending the incubation time for the reaction to 60 min increased the recovery of GPI PrP-sen in the SCRL fractions to 64% of the PrP-sen input into the reaction (Fig. 4A). Also, as described above, the binding was pH-independent (Fig. 5), and bound protein was only removed by extraction with cold Triton X-100 (data not shown), suggesting the binding is mediated by hydrophobic protein-lipid interactions. Therefore, the GPI anchor-independent SCRL binding by GPI PrP-sen was not dependent on incubation in the presence of detergent. Nevertheless, unless otherwise indicated, all remaining studies with GPI anchor-deficient PrP-sen were performed using these binding conditions in the absence of detergent.

On the contrary, the transient Sarkosyl treatment had a clear effect on the nature of GPI PrP-sen association with SCRLs. When incubated with SCRLs in the absence of Sarkosyl, GPI PrP-sen efficiently bound to the SCRLs but, similar to the behavior of GPI PrP-sen, was also readily extracted by treatment with cold Triton X-100 (data not shown). This shows that GPI PrP-sen can exhibit GPI anchor-independent-like SCRL binding, however, this can be clearly distinguished from GPI anchor-dependent binding by extracting the PrP-bound SCRLs with cold Triton X-100.

**Glycosylated, GPI Anchor-deficient PrP-sen Binds to SCRLs**—The GPI PrP-sen used above was generated by deletion of the GPI anchor addition sequence and is synthesized in a predominantly unglycosylated form. GPI anchor-deficient PrP-sen can also be generated by treatment of cells with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove the diacylglycerol moiety of the GPI anchor, which releases some PrP-sen from the cell surface where it is found in a predominantly fully glycosylated form. This allowed us to test the effect of glycosylation on GPI anchor-independent SCRL binding. Purified PI-PLC-released PrP-sen (GPI PI-PLC PrP-sen) bound to SCRLs with similar kinetics and efficiency as GPI PrP-sen with 48% or 62% of the input GPI PI-PLC PrP-sen being recovered in the SCRL fractions after a 5- or 60-min incubation, respectively (Fig. 4B). This indicates that glycosylation does not detectably influence SCRL binding in our assay.

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**Fig. 3.** PrP-sen associates with SCRLs via hydrophobic interactions. SCRLs containing reconstituted [35S]PrP-sen as described in Fig. 1 were treated with 1 M NaCl (A), 0.1 M sodium carbonate (Na2CO3, pH 11.5) (B), 1% Triton X-100 at 4 °C (C), or 1% Triton X-100 at 37 °C (D) and fractionated by floatation on five-step Optiprep gradients. The results are representative of an experiment performed in duplicate.

**Fig. 4.** GPI anchor-deficient PrP-sen binds to SCRLs in the absence of detergent. [35S]PrP-sen lacking a GPI anchor either due to expression in a form deleted for the GPI anchor addition sequence (GPI PrP-sen) (A) or to PI-PLC digestion (GPI PI-PLC PrP-sen) (B) was mixed with SCRLs in the absence of detergent, incubated for the time indicated, and fractionated by floatation on three-step density gradients. No increase in binding was observed after overnight incubation (not shown). The data are representative of two independent experiments, each performed in duplicate.
Residues from the N-terminal Half of PrP Contribute to GPI-PrP-sen Binding to SCRLs—PrP-sen contains a hydrophobic stretch of amino acids from residues 112 to 135 that comprises a transmembrane domain in certain rare forms of PrP (28) and was a candidate for the region mediating the hydrophobic, GPI anchor-independent SCRL association. To evaluate the role of N-terminal residues of GPI PrP-sen in SCRL binding and to determine the contribution of this hydrophobic region, we tested the binding of two readily available mutants containing deletions from amino acid residues 34–94 (Δ94) and 34–124 (Δ124). Both mutants bound SCRLs less efficiently than the wild-type GPI PrP-sen control, the Δ124 mutant showing a slightly reduced binding compared with the Δ94 mutant (Fig. 6A). Western blot analysis of the PrP-sen samples verified equivalent amounts of each type of PrP-sen were used in the binding reactions (Fig. 6B). Because these deletions failed to completely inhibit binding, our data suggest that residues outside of this region may also contribute to SCRL binding.

SCRL Binding Is Specific to PrP-sen and a Subset of PI-PLC-released/secreted Proteins—To determine if GPI anchor-independent membrane binding might be an activity common among GPI-anchored proteins, we incubated SCRLs with culture supernatants from PI-PLC-treated fibroblast cells expressing GPI PrP-sen to simultaneously assay the complement of (formerly) GPI-anchored proteins for SCRL-binding activity. Only a small subset of PI-PLC-released proteins and proteins secreted during the PI-PLC treatment period were found to bind SCRLs (Fig. 7A, fractions 1–3), an observation more easily visualized for gradient fractions deglycosylated with PNGase F prior to SDS-PAGE (Fig. 7B, fractions 1–3). Interestingly, one of these proteins (indicated by a bracket in Fig. 7A and by an arrow in Fig. 7B) had an apparent molecular mass and glycoform profile (as revealed by PNGase F digestion) similar to PrP-sen. By immunoprecipitation from the gradient fractions with anti-PrP monoclonal antibody (3F4), one of the proteins was identified as PrP-sen (Fig. 7C). We also confirmed that the PI-PLC-released proteins were binding to the SCRLs in a fashion similar to purified PrP-sen by concentrating the combined protein-containing SCRL fractions by centrifugation, extracting the samples with cold Triton X-100 or 1 M NaCl, and re-floating the samples on a new density gradient. As with purified PrP-sen, the bound proteins were largely removed by treatment with cold Triton X-100 (Fig. 7D) but not high salt (data not shown). No evidence of SCRL binding of proteins corresponding to PrP-sen was observed when PI-PLC culture supernatants from cells expressing the Δ94 (Fig. 7E) or Δ124 (Fig. 7F) mutant PrP-sen molecules were used, consistent with the poor binding observed using the purified proteins (Fig. 6A). To control for the possibility that SCRL-binding activity was simply due to an excess of PrP-sen relative to other proteins in the supernatant, we tested a generic protein (purified recombinant murine/human Fab fragment D13) for SCRL binding. Even when incubated with SCRLs at a protein/lipid molar ratio >20-fold higher than the PrP-sen/lipid ratio present in PrP-containing reactions, the recombinant Fab fragment failed to bind to SCRLs (data not shown). Altogether, these data demonstrate GPI anchor-independent SCRL binding is not a ubiquitous property of GPI-anchored proteins and that SCRL binding is not an artifact of using purified PrP-sen.

Cell-free Conversion of SCRL-associated GPI-PrP-sen by Exogenous PrP-res—To measure the effect of the different types of membrane association on the interaction of PrP-sen with exogenous PrP-res, we used a previously described near-physiological cell-free conversion assay (14) containing a crude brain microsome fraction from 263K scrapie-infected hamsters as a source of PrP-res. This assay is capable of generating new protease-resistant PrP exhibiting the 6–8-kDa decrease in apparent molecular mass after PK digestion that is characteristic of PrP-res synthesized in vivo. The fate of the input [35]S-labeled PrP-sen in the reactions is specifically monitored by autoradiography. For reactions using GPI PrP-sen, the PrP-sen was reconstituted into the SCRLs in the presence of Sarkosyl followed by extraction with cold Triton X-100 to ensure that the PrP-sen was associated with the SCRLs in a GPI anchor-dependent manner. As observed with DRMs prepared
from a mouse PrP-overexpressing neuroblastoma cell line (14), SCRL-bound GPI \( ^{14} \) PrP-sen was not converted to PrP-res until PI-PLC, which cleaves the GPI anchor of PrP-sen but not PrP-res molecules (1, 29–30), was added to the reaction (Fig. 8A, lane 10), or the combined membrane fractions were treated with a high concentration (30%) of the membrane-fusing agent polyethylene glycol (PEG) (Fig. 8A, lane 9). Reactions assisted by PI-PLC often exhibited a higher conversion efficiency than reactions with 30% PEG (27% versus 9.1% conversion for the experiment shown in Fig. 8A), possibly due to a higher efficiency of PI-PLC cleavage than PEG-assisted membrane fusion. When compared with PEG-assisted reactions, the PI-PLC-assisted conversion products exhibited a slightly reduced electrophoretic mobility (Fig. 8A, lane 10 versus lane 9), suggesting the PI-PLC-assisted converting species lacked a GPI

**Fig. 7.** Binding of PI-PLC-released/secreted proteins to SCRLs. Culture supernatants from metabolically labeled, PI-PLC-treated fibroblast cells expressing either full-length (A–D) or N-terminally-deleted (E and F) GPI \(^{14} \) PrP-sen were incubated with SCRLs in the absence of detergent and fractionated on three-step density gradients. Fractions were treated with PNGase F where indicated. A and B, the bracket and arrow indicate bands (most abundant in fraction 2) with apparent molecular mass and glycoform pattern similar to PrP-sen. C, gradient fractions were immunoprecipitated with anti-PrP monoclonal antibody prior to SDS-PAGE. The arrow indicates the band corresponding to PrP-sen. D, SCRL fractions (1–3) as in A were combined, pelleted, extracted with cold 1% Triton X-100, and fractionated by floatation on a second density gradient. Fractions from this second gradient are shown. E and F, lanes labeled “PrP-sen” correspond to PrP-sen immunoprecipitated from a PNGase F-treated aliquot of PI-PLC culture supernatant equivalent to one-tenth the amount added to the SCRL binding reactions. Arrows indicate bands corresponding to PrP-sen.

**Fig. 8.** PEG and PI-PLC assist cell-free conversion of SCRL-associated GPI \(^{14} \) PrP-sen. Cell-free conversion reactions were performed as described under “Experimental Procedures” using either hamster (A) or mouse (B) \(^{35} \)SGPI \(^{14} \) PrP-sen reconstituted into SCRLs as in Fig. 1 and isolated by floatation on density gradients after extraction with cold 1% Triton X-100. Samples were treated with various concentrations of PEG or PI-PLC as indicated. PK \(^{14} \) lanes contain a one-tenth aliquot of each reaction mixture before PK digestion. PK\(^{+}\) lanes contain a nine-tenths aliquot of each reaction mixture after PK digestion. A, samples were deglycosylated with PNGase F after conversion to aid visualization of PrP bands. Arrows indicate newly generated PK-resistant PrP-res bands (lanes 9 and 10). The upper two arrows correspond to incompletely deglycosylated conversion products. Results are representative of two independent experiments each performed in duplicate. B, all reactions contained PrP-res. Brackets indicate PrP-sen (PK\(^{+}\) panel) and newly generated PrP-res (PK\(^{+}\) panel). Results are representative of a single experiment performed in duplicate.
efficiencies were comparable to reactions containing free GPI. In contrast to GPI

chor-independent form of PrP-sen association with SCRLs on Exogenous PrP-res

Next, we tested the effect of the GPI anchor (14, 29, 31). A similar requirement for 30% PEG or PI-PLC was observed in reactions using SCRL-reconstituted mouse GPI PrP-sen and brain microsomes from a mouse infected with the murine-adapted scrapie strain 87V (Fig. 8B, lanes 7 and 8). These data show that SCRL-incorporated GPI PrP-sen behaves similarly to GPI PrP-sen in cellular raft membranes in being resistant to conversion by exogenous PrP-res molecules without the assistance of PI-PLC or a membrane-fusogenic concentration of PEG (14, 32, 33). Our data also show that the requirements for conversion of membrane-associated GPI PrP-sen are conserved between PrP-res molecules associated with two different strains of scrapie agent, adapted to either hamsters (263K) or mice (87V).

Cell-free Conversion of SCRL-associated GPI PrP-sen by Exogenous PrP-res—Next, we tested the effect of the GPI anchor-independent form of PrP-sen association with SCRLs on conversion. In contrast to GPI PrP-sen, GPI PrP-sen pre-bound to SCRLs in the presence or absence of Sarkosyl was converted to new PrP-res without any additional treatments to the reactions (Fig. 9A, lanes 8 and 12). Overall, the conversion efficiencies were comparable to reactions containing free GPI PrP-sen without SCRLs (Fig. 9B). One potential exception involved GPI PrP-sen bound to SCRLs in the absence of Sarkosyl, which converted with a similar efficiency to free GPI PrP-sen after a 1-day reaction but was marginally more efficient after a 2-day reaction (Fig. 9B). In addition, GPI PrP-sen bound to SCRLs in the presence of Sarkosyl was converted with a slightly reduced efficiency compared with free GPI PrP-sen after a 1-day reaction but not a 2-day reaction. Hence, we cannot exclude the possibility that Sarkosyl modifies the binding of GPI PrP-sen to SCRLs in a subtle way not detected by our salt/detergent treatments to extract PrP-sen above. Nevertheless, it is notable that conversion products were generated in all of these reactions. Thus, the mode of membrane association (GPI anchor-dependent versus -independent) had dramatically different effects on the ability of PrP-sen to serve as a substrate in the conversion reaction.

DISCUSSION

We have examined the interaction of PrP-sen with model raft liposomes and described two modes by which this association can occur. The first is mediated by insertion of the GPI anchor of PrP-sen into the liposome membranes after reconstitution in the presence of detergent (Fig. 10B). The second is a newly described method that does not require a GPI anchor (Fig. 10A) but can also occur when GPI PrP-sen is incubated with SCRLs in the absence of detergent (Fig. 10C) and may provide a dual mode of membrane attachment for GPI PrP-sen (not depicted).

This alternative, GPI anchor-independent mode of membrane association was detected in control experiments with GPI PrP-sen during our attempts to reconstitute GPI PrP-sen in SCRLs. These reconstitution experiments included the mixing of PrP-sen with SCRLs in the presence of a low concentration of Sarkosyl as transient exposure to detergent is commonly required to permit insertion of GPI-anchored proteins into liposomes (15, 16, 34, 35). Presumably, the detergent is required to disrupt the pseudomicelles formed by GPI-anchored proteins in the absence of detergent (36) and/or to transiently perturb the liposome membranes to facilitate insertion of the GPI anchor. The acquisition of resistance to solubilization in cold Triton X-100 by a significant fraction of GPI PrP-sen indicates that we were successful in reconstituting this type of PrP-sen in a GPI anchor-directed fashion. This was confirmed by the fact that PI-PLC pretreatment allowed the solubilization in cold Triton of a majority of these formerly Triton X-100-resistant molecules (data not shown). Because treatment of GPI PrP-sen-containing, cold Triton-extracted SCRLs with PI-PLC alone released only a small percentage of the PrP-sen (data not shown), simultaneous association via GPI anchor-dependent and -independent methods might be possible. Alternatively, there may be re-binding of PrP-sen by

Fig. 9. Cell-free conversion of SCRL-associated GPI PrP-sen. Cell-free conversion reactions were incubated for 1 or 2 days using [35S]GPI PrP-sen either in the absence of SCRLs (free) or pre-bound to SCRLs in the presence (as in Fig. 1) or absence (as in Fig. 4) of Sarkosyl. A, representative images of conversion reactions using each type of GPI PrP-sen after conversion for 1 day. Brackets indicate newly generated PrP-res (lanes 4, 8, and 12). B, quantitation of newly generated PrP-res. Results are expressed as mean percent conversion ± S.D. (n = 3).

Fig. 10. Summary of modes of PrP-sen association with SCRLs. A, GPI anchor-deficient PrP-sen likely associates with SCRLs by partial insertion into the lipid bilayer. B, GPI-anchored PrP-sen can associate with SCRLs by insertion of the GPI anchor into the lipid bilayer when reconstituted in the presence of detergent. C, when bound to SCRLs in the absence of detergent, GPI-anchored PrP-sen binds SCRLs in a manner not directed by the GPI anchor similar to GPI anchor-deficient PrP-sen. The GPI anchors may self-associate as pseudomicelles and be unavailable for insertion into SCRLs.
the GPI-independent mechanism after release by PI-PLC cleavage. The extraction of some of the GPI+ PrP-sen in cold Triton X-100 (Fig. 3C, lane 6) may indicate that our protocol used suboptimal levels of Sarkosyl to promote GPI anchor-directed insertion into the SCRLs and that this extractable population was associated solely via the GPI anchor-independent mode. Arguably, the presence of the detergent could have artifically assisted the association of GPI+ PrP-sen with the SCRLs. However, the occurrence of GPI+ PrP-sen binding to SCRLs in the absence of detergent with a comparable efficiency with respect to the input PrP-sen showed that detergent was not required for the effect. Furthermore, we have demonstrated the specificity of the SCRL binding activity of GPI anchor-deficient PrP-sen by showing only very few PI-PLC-released or secreted proteins share this activity (Fig. 7, A, B, E, and F).

Consistent with our specificity studies, detergent-assisted reconstitution of another GPI-anchored protein (bovine intestine alkaline phosphatase) into raft-like liposomes was shown to be GPI anchor-dependent (34). Admittedly, we cannot entirely rule out the possibility of another PI-PLC-released protein indirectly facilitating the SCRL binding of PI-PLC-released PrP-sen, although our experiments with purified PrP-sen would indicate that other accessory proteins are not required for this binding activity.

Two other groups have recently published studies of binding of GPI anchor-deficient PrP-sen to liposomes, but with significant differences in experimental design that complicate comparisons to the present study (10, 13). The previous studies relied predominantly on spectroscopic techniques to assay binding activity, whereas we have used a simple direct binding assay by floatation on density gradients. Perhaps most importantly, both of the previous studies made use of recombinant PrP-sen derived from E. coli and refolded in the absence of copper. We have used forms of PrP-sen expressed in mammalian cells (mouse fibroblasts). This allowed us the advantage of using a source of PrP-sen that is synthesized and folded under native conditions and, when expressed with a GPI-anchor, also contains the appropriate N-glycosylation of the native protein. Furthermore, this expression method also allowed the use of cell surface PrP-sen as released into culture medium (phosphate-buffered balanced salts solution) without purification. The binding of this PI-PLC-released PrP-sen to SCRLs (Fig. 7, A–C) shows the binding is not an artifact of using purified PrP-sen that may contain conformational alterations not detected by low resolution structural analyses (e.g. circular dichroism).

Morillas and co-workers (10) showed full-length (amino acids 23–231) human PrP-sen binds to acidic lipid-containing liposomes (phosphatidylcholine/phosphatidylserine (PC/PS)) with the strongest binding occurring at acidic pH. Using two truncated forms of human PrP-sen, a pH-dependent component of the binding was localized to residues 90–231, whereas pH-independent binding was observed for a fragment consisting of residues 23–145 (10). These data are consistent with our results in detecting pH-dependent liposome binding (Fig. 5 and data not shown) and localizing a portion of PrP-sen membrane binding to the N terminus (Fig. 6A), although we did not observe a requirement for acidic lipids.

The second study made use of liposomes with sphingomyelin and cholesterol that are related to SCRLs but with minor differences (13). In comparison to SCRLs, these liposomes lacked cerebrosides and contained a phospholipid:sphingolipid:cholesterol ratio differing from that found in rafts isolated from cells (26). These differences or perhaps one of those described above, might account for their observation of binding of PrP-sen to raft-like liposomes only at neutral pH (13). However, Sanghera and Pinheiro only studied the binding of the 90–231 fragment of hamster PrP-sen at pH 5.0 and 7.0. The most closely related hamster PrP-sen species in our study, lacking residues 34–94 (Δ34), was only tested for binding at pH 6.0 and showed residual binding activity (Fig. 6A). It is possible then that binding of PrP-sen to raft-like liposomes may consist of a pH-dependent component via the C terminus and a pH-independent component directed by the N terminus. Despite the differences, both this and a previous study have provided independent evidence for the hydrophobic nature of the binding of GPI anchor-deficient PrP-sen to raft-like liposomes (13).

Membrane insertion activity is usually restricted to proteins with specialized functions like pore-forming toxins or viral fusion proteins, for example. Hence, the hydrophobic nature of the GPI anchor-independent membrane association is particularly interesting, suggesting that the binding is mediated by at least partial insertion of PrP-sen into the membrane and raising the possibility of a mechanism for direct cytotoxicity of PrP in some conformation, as proposed recently (13). Indeed, Sanghera and Pinheiro (13) obtained spectroscopic evidence for such activity. The involvement of residues 34–94, which contain the glycosaminoglycan-binding (37–40) and copper-binding octapeptide repeats (41–48), suggest that the SCRL binding affected by this region of PrP-sen may be regulated by interactions with glycosaminoglycans and/or copper, although this has yet to be tested. Obviously, this is a model system, and the extent to which this form of GPI anchor-independent binding occurs in cells remains to be determined. However, these observations may provide an explanation for the observation of incomplete release of cell-surface PrP-sen from cultured cells treated with PI-PLC. Armed with a better understanding of how this binding is regulated using our model system, we will be better equipped to investigate these processes directly in cells.

The observation of conformational changes in PrP-sen occurring on binding to liposomes (10, 13) only emphasizes the importance of understanding the effects of PrP-sen membrane association in its various forms on interactions with PrP-res. We have directly evaluated the effect of both GPI anchor-dependent and -independent methods of PrP-sen SCRL binding on the ability of PrP-sen to serve as a substrate for conversion to PrP-res and shown that the method of membrane association strongly influences the conversion reaction. Our previous work suggested that association of murine PrP-sen with DRM prepared from a mouse PrP-overexpressing neuroblastoma cell line inhibited conversion by exogenous murine PrP-res as contained in a crude brain microsome fraction from scrapie-infected mice (14). Generation of new PrP-res in these DRM-based reactions required the addition of PI-PLC or pulse treatment with 30% PEG to induce membrane fusion, suggesting either removal of PrP-sen from membranes or insertion of PrP-res into contiguous membranes was a prerequisite for conversion. We have recapitulated these results using GPI+ PrP-sen reconstituted into SCRLs with two different scrapie strains, showing that the lack of conversion of DRM-bound PrP-sen by exogenous PrP-res was not due to inhibition by other DRM-associated molecules and that these observations are common to multiple scrapie strains.

Binding of recombinant PrP-sen to raft-like liposomes of PC/SM/Chol leads to a higher content of α-helical structure and has been proposed to stabilize PrP-sen in an α-helical conformation (13). Although we lack sufficient protein to perform a structural determination of SCRL-bound PrP-sen in this study, our data show no evidence for a dramatic effect of pre-association of PrP-sen with SCRLs on conversion (Fig. 9B). Thus, if any conformational changes in PrP-sen occurred on binding
SCRLs, they did not detectably influence the conversion reaction under the conditions used here. The clearly contrasting effects of GPI anchor-dependent versus -independent membrane association of PrP-sen on conversion demonstrate that it is not membrane binding per se but the mode of membrane binding that controls the interaction with exogenous PrP-res. Our observations support the use of SCRLs as model raft membranes and suggest that membrane association specifically directed by the GPI anchor, the PrP-res binding site on PrP-sen is occluded and incapable of binding to PrP-res in a manner leading to conversion until the PrP-res is inserted into a membrane contiguous with PrP-sen. Consistent with this proposal, previous studies have localized residues clustered near the C terminus in the NMR structure of PrP-sen as potentially contributing to the PrP-res binding site (8, 24, 49–51). When PrP-sen is modeled in a GPI anchor-directed membrane-bound state (11), it is conceivable that the PrP-res binding region is not readily accessible for interaction with large aggregates of exogenous PrP-res. Future studies will address whether conversion of raft-bound GPI PrP-sen by exogenous PrP-res is blocked at the level of binding or acquisition of protease resistance (24).

Our study of GPI anchor-deficient forms of PrP-sen led to the detection of an alternative mode by which PrP-sen associates with membranes. Although we have found that GPI PrP-sen can also exhibit this alternative membrane-binding mode under certain circumstances, the characterization of this binding activity is obviously simplified by the use of GPI anchor-deficient forms of PrP-sen where we can definitively rule out any contribution from the GPI anchor to any effects we observe. However, given that the majority of PrP-res generated in infected animals contains a GPI anchor (1), it is not immediately clear whether GPI anchor-deficient forms of PrP-sen themselves play any role in PrP biology.

On the other hand, several studies support the possibility that GPI-anchorless forms of PrP are physiologically important. The release of apparently soluble forms of PrP from cells was first demonstrated many years ago (29, 53, 55). Borchelt and co-workers (56) later showed that low levels of GPI forms of PrP-sen (comprising <10% of total PrP-sen) are generated in various cultured cells and hamster brain homogenates. More recently, similar results have been obtained using splenocytes or cerebellar granule neurons from PrP-overexpressing transgenic mice (57). Furthermore, Stahl et al. (54) reported that 10–20% of PrP-res molecules purified from infected hamsters are truncated at glycine-228, similar to the GPI anchorless forms of PrP-sen used in the present study, which is truncated at residue 231 (18). Although it is unclear whether this small fraction of PrP-res molecules was generated from a GPI PrP-sen precursor, GPI PrP-sen has been shown to be capable of acting as a precursor for the synthesis of new PrP-res in scrapie-infected mouse neuroblastoma cells, albeit at a significantly reduced apparent efficiency compared with the wild-type protein (52). When considered together with our data, these observations suggest possible scenarios whereby GPI forms of PrP-sen might participate in the initiation of infection of cells. Because insertion of exogenous PrP-res into contiguous membranes appears to be a prerequisite for conversion of membrane-associated forms of GPI PrP-sen (14 and this study) but not membrane-associated forms of GPI PrP-sen, exogenous PrP-res molecules might initially interact with and induce the conversion of membrane-bound GPI PrP-sen. This process could promote the association of the PrP-res aggregates with host cell membranes and might facilitate the insertion of the PrP-res molecules, the majority of which are GPI-anchored, into the membrane where they would then become competent to convert the GPI PrP-sen molecules to new PrP-res. Alternatively, the newly converted GPI PrP-res might directly interact with and seed the conversion of GPI PrP-sen molecules thereby acting as a “bridge” between exogenous PrP-res and cell-associated GPI PrP-sen. Whether any of these scenarios are important in transmissible spongiform encephalopathy infections remains to be determined.
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Effect of Glycosylphosphatidylinositol Anchor-dependent and -independent Prion Protein Association with Model Raft Membranes on Conversion to the Protease-resistant Isoform
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