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The glycosylphosphatidylinositol anchor is a major determinant of prion binding and replication

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Page heading; Prion infectivity dependent upon the GPI anchor

Synopsis

The prion diseases occur following the conversion of the cellular prion protein (PrP^C) into an alternatively folded, disease-related isoform (PrP^{Sc}). However, the spread of PrP^{Sc} from cell to cell is poorly understood. Here we report that soluble PrP^{Sc} bound to and replicated within both GT1 neuronal cells and primary cortical neurones. The capacity of PrP^{Sc} to bind and replicate within cells was significantly reduced by enzymatic modification of its glycosylphosphatidylinositol (GPI) anchor. Thus, PrP^{Sc} that had been digested with phosphatidylinositol-phospholipase C bound poorly to GT1 cells or cortical neurones and did not result in PrP^{Sc} formation in recipient cells. PrP^{Sc} that had been digested with phospholipase A₂ (PrP^{Sc}-G-lyso-PI) bound readily to GT1 cells and cortical neurones but replicated less efficiently than mock treated PrP^{Sc}. Whereas the addition of PrP^{Sc} increased cellular cholesterol levels and was predominantly found within lipid raft micro-domains, PrP^{Sc}-G-lyso-PI did not alter cholesterol levels and most of it was found outside lipid rafts. We conclude that the nature of the GPI anchor attached to PrP^{Sc} affected the binding of PrP^{Sc} to neurones, its localisation to lipid rafts and its ability to convert endogenous PrP^C.

Key words: Cholesterol, glycosylphosphatidylinositols, inositolphosphoglycans, phospholipases, prion.

Abbreviations. enzyme linked immunoassay (ELISA), glycosylphosphatidylinositol (GPI), inositolphosphoglycan (IPG), phosphatidylinositol-phospholipase C (PI-PLC), phospholipase A₂ (PLA₂), normal host prion protein (PrP^C), disease-associated prion isoform (PrP^{Sc}).

Introduction

Transmissible spongiform encephalopathies, more commonly known as the prion diseases, are mostly fatal neurodegenerative disorders that include scrapie in sheep and goats, bovine spongiform encephalopathy in cattle and kuru and Creutzfeldt-Jakob disease in man. A key event in prion disease is the conversion of a normal host protein (PrP^C) into a disease-associated isoform (PrP^{Sc}), which represents the major component of infectious scrapie prions [1]. The conversion process results in a portion of the α -helix and random coil structure in PrP^C being refolded into a β -pleated sheet in PrP^{Sc} [2]. The newly formed PrP^{Sc} subsequently acts as a template that facilitates conversion of PrP^C to PrP^{Sc}. This conversion is accompanied by changes in biological and biochemical properties of PrP^{Sc} including increased resistance to proteases [3].

Precisely how PrP^{Sc} spreads throughout the brain during infection is poorly understood. PrP^{Sc} can be released from cells in exosomes [4, 5] nanometer-sized particles that contain specific proteins [6] which could infect neighbouring cells or by tunnelling nanotubules [7]. The mechanisms by which PrP^{Sc} infects neuronal cells has been examined *in vitro*; PrP^{Sc} infected a neuronal cell line (SN56), hamster neurones [8] and murine neurones [9]. More recently N2a cells were used to provide a highly sensitive cell based

infectivity assay for mouse scrapie prions [10]. Here we show that PrP^{Sc} bound to and replicated within both GT1 neuronal cells and murine cortical neurones.

Proteins that contain a glycosylphosphatidylinositol (GPI) anchor are rapidly incorporated into both living cells and synthetic membranes [11, 12]. PrP^{Sc} molecules are linked to membranes via a GPI anchor [13] suggesting that the GPI anchor may have a role in the binding of PrP^{Sc} to cells. Previously, the role of GPI anchors in the biological activity of PrP^{Sc} has been difficult to determine as the aggregated forms of PrP^{Sc} were thought to be resistant to digestion with phosphatidylinositol-phospholipase C (PI-PLC) [14, 15]. However, sonication and shaking techniques, originally developed to amplify small amounts of PrP^{Sc}, break up large prion particles into smaller soluble oligomers [16]. In the current study a combination of a shaking technique and soluble PrP^{Sc} released from ScGT1 cells, rather than PrP^{Sc} extracted with strong detergents from infected brains, allowed successful digestion of the PrP^{Sc}-GPI anchor with PI-PLC or phospholipases A₂ (PLA₂). These enzymes target the acyl chains of the GPI anchor (Figure 1). We report that PrP^{Sc} digested with PI-PLC (PrP^{Sc}-inositolphosphoglycan (PrP^{Sc}-IPG)) did not bind readily to GT1 cells or cortical neurones and did not lead to the formation of PrP^{Sc} in recipient cells over the time course of these experiments. Surprisingly, although PrP^{Sc} that had been digested with PLA₂ (PrP^{Sc}-G-lyso-PI) bound readily to GT1 cells and cortical neurones, it had little capacity to convert endogenous PrP^C into PrP^{Sc}.

Experimental.

Cell lines. GT1 cells were grown in Ham's F12 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 200 nM retinoic acid and 2 % foetal calf serum (PAA, Yeovil, UK). GT1 cells were plated in 6 well plates (10⁵ cells/well for PrP^{Sc} replication studies and 10⁶ cells/well for binding studies) and allowed to adhere overnight.

Primary cortical neurones. Cortical neuronal cultures were prepared from the brains of mouse embryos (day 15.5) derived from wildtype (PrP^{+/+}) or PrP knockout mice (PrP^{0/0}) after mechanical dissociation and cell sieving. Neuronal precursors were plated at (10⁶ cells/well) in 6 well plates that had been pre-coated with 5 µg/ml poly-L-lysine, in Ham's F12 containing 5% foetal calf serum for 2 hours. Cultures were shaken (600 r.p.m for 5 minutes) and non-adherent cells removed by 2 washes in phosphate buffered saline (PBS). Neurones were subsequently grown in neurobasal medium containing B27 components (PAA, Yeovil, UK) for 7 days before use. Immunostaining showed that the cells were greater than 97% neurofilament positive. Less than 3% stained for GFAP (astrocytes) or for F4/80 (microglial cells).

Test PrP^{Sc} preparations. Two distinct PrP^{Sc} preparations were used in these assays. The first consisted of the supernatants collected from ScGT1 cells which were centrifuged (500 x g for 5 minutes) to remove cell debris. Supernatants were digested with 0.2 units/ml PI-PLC (*Bacillus cereus*) or 10 units/ml bee venom PLA₂ (Sigma) with intermittent shaking at 37°C for 24 hours. The enzymatic activity was destroyed by heating at 70°C for 5 minutes. This method eschewed proteinase K digestion in order to preserve protease sensitive forms of PrP^{Sc}. The amount of PrP^{Sc} in these supernatants was determined by enzyme linked immunoassay (ELISA) and diluted to 1 ng/ml for further studies. We also used partially purified PrP^{Sc} preparations isolated from ScGT1 cells homogenised in distilled water. Cell debris and nuclei were removed by centrifugation at 500 x g for 5 minutes. Cell membranes collected from the supernatant by centrifugation (10 minutes at 14,000 x g) and solubilised in buffer containing 10 mM Tris-HCL, 10 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40 and 0.5% sodium deoxycholate. PrP^C and PrP^{Sc} were immunoprecipitated by incubation with 0.5 µg/ml ICSM35 which recognises native PrP^{Sc} (D-Gen, www.d-gen.co.uk) and µMACS protein G microbeads (10 µl/ml) (Miltenyi Biotech, Bisley, UK). Protein G bound antibody complexes were isolated using a µMACS magnetic system (Miltenyi) and digested with 0.2 units/ml PI-PLC or 10 units/ml bee venom PLA₂ with intermittent shaking at 37°C for 24 hours. Precipitates were further digested with 5 µg/ml proteinase K for 1 hour at 37°C to remove PrP^C.

Trifluoroacetic acid was added to achieve a 0.1% final concentration, samples were shaken in a cell disruptor (10 minutes) and applied to C18 columns (Waters, Elstree, UK). PrP^{Sc} was eluted under a gradient of acetonitrile in water and 0.1% trifluoroacetic acid and fractions were tested for PrP by ELISA. PrP positive fractions were pooled and run on second C18 column. Samples containing PrP were pooled, washed twice with PBS and the amount of PrP^{Sc} determined by ELISA. Samples were frozen at -20°C for storage. On the day of use, PrP^{Sc} preparations were diluted to 1 ng/ml and solubilised in culture medium.

Cell treatments. For PrP^{Sc} replication experiments, cells were pulsed with PrP^{Sc} preparations for 24 hours, washed 5 times in warm PBS to remove unbound PrP^{Sc} and incubated in fresh medium for a further 6 days (GT1 cells) or 9 days (cortical neurones). For binding studies, cells were incubated with PrP^{Sc} preparations for 1 or 24 hours. Cells were washed 5 times in warm PBS and cell membrane extracts collected.

Cell membrane extracts. Treated cells were homogenised in an extraction buffer (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS) at 10⁶ cells/ml. Membranes were homogenised and nuclei and large fragments were removed by centrifugation (300 x g for 5 minutes). To determine the amount of PrP^{Sc} in cells these extracts were digested with 5 µg/ml proteinase K for 1 hour at 37°C; digestion was stopped with mixed protease inhibitors. The soluble material was heated to 95°C for 5 minutes to denature PrP^{Sc} and tested in a PrP ELISA. Some samples were mixed with an equal volume of Laemmli buffer (Bio-Rad) and subjected to electrophoresis on a 15 % polyacrylamide gel. Proteins were transferred onto a Hybond-P PVDF or nitrocellulose membranes (Amersham Biotech, UK) by semi-dry blotting. Membranes were blocked using 10 % milk powder in Triz-buffered saline, pH 7.2 containing 0.2 % Tween 20. PrP was detected by incubation with mAb ICSM35 (D-gen), followed by a secondary anti-mouse IgG conjugated to peroxidase. Bound antibody was visualised using an enhanced chemiluminescence kit (Amersham Biotech, UK).

Isolation of GPI anchors. PrP^{Sc} samples were further digested with 100 µg/ml proteinase K at 37°C for 24 hours to release GPI anchors. The released GPIs were extracted with water-saturated butan-1-ol, washed 3 times with ice cold water, lyophilised and dissolved in ethanol. Extracted GPIs were applied to silica gel 60 high performance thin layer chromatography (HPTLC) plates (Whatman) and developed using a mixture of chloroform/methanol/water (4/4/1 - v/v/v). GPI anchors were detected by immunoblotting as described [17]. Briefly, plates were soaked in 0.1 % poly(isobutyl methacrylate) in hexane, dried, and blocked with PBS containing 5 % milk powder. They were probed with 1 µg/ml of a mouse mAb that binds to phosphatidylinositol, washed with PBS-Tween, and incubated with goat anti-mouse IgG conjugated to peroxidase (Sigma) for 1 hr. The bound antibody was washed and visualised using an enhanced chemiluminescence kit (Amersham Biotech, UK).

Isolation of detergent resistant membranes (lipid rafts). Treated cells were lysed in an ice-cold buffer containing 1% Triton X-100, 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA at 10⁶ cells/ml. Membranes were prepared by homogenisation followed by mechanical agitation on a cell disruptor (10 minutes full power), and nuclei and large fragments were removed by centrifugation (300 x g for 5 minutes). The supernatant was incubated on ice for 60 minutes prior to further centrifugation (16,000 x g for 30 minutes at 4°C). The soluble material was reserved as the normal cell membrane. Insoluble proteins were solubilised in extraction buffer (10 mM Tris-HCl, 10 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate and 0.2% SDS and mixed protease inhibitors, centrifuged (10 minutes at 16,000 x g), and the supernatant retained as the lipid raft fraction. Both fractions were digested with proteinase K (5 µg/ml for 1 hour at 37°C) to remove PrP^C, heated to 95°C for 5 minutes to denature PrP^{Sc} and tested in a PrP ELISA.

PrP ELISA. The amount of PrP in cell extracts/supernatants was determined by ELISA as described [18]. Maxisorb Immunoplates (Nunc, Roskilde, Denmark) were coated with 0.5 µg/ml a capture mAb

(ICSM18 (D-Gen)) which recognises amino acids 146 to 159 of murine PrP. Samples were applied and detected with biotinylated mAb ICSM35 (D-Gen) (which recognises an epitope between amino acids 91 and 110). Biotinylated mAb was detected using extravidin-alkaline phosphatase and 1 mg/ml 4-nitrophenyl phosphate (Sigma). Absorbance was measured at 450 nm and the amount of PrP in cell extracts was calculated by reference to a standard curve of recombinant murine PrP (Prionics, Zurich, Switzerland).

Cholesterol Assay. The amount of cholesterol was measured using the Amplex Red cholesterol assay kit (Invitrogen, Paisley, UK). Cholesterol is oxidised by cholesterol oxidase to yield hydrogen peroxide that reacts with 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red reagent) to produce highly fluorescent resorufin; measured by excitation at 550 nm and emission detection at 590 nm. The amount of cholesterol was calculated by reference to cholesterol standards.

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

Results

Partial purification of phospholipase digested PrP^{Sc} on C18 columns. Digestion with PI-PLC results in the removal of diacylglycerol from GPI anchors. As shown in Figure 1 the digestion of PrP^{Sc} with PI-PLC resulted in PrP^{Sc}-IPG. Whereas mock-treated PrP^{Sc} (PrP^{Sc}-GPI) bound to C18 columns and was eluted in fractions containing between 65 and 85% acetonitrile, PrP^{Sc}-IPG did not bind to C18 columns and was eluted in the void volume (Figure 2A). PrP^{Sc} digested with PLA₂ (PrP^{Sc}-G-lyso-PI) bound to C18 columns and was eluted in fractions containing between 35 and 50% acetonitrile. Positive fractions were pooled, the amount of PrP^{Sc} measured by ELISA and the pool diluted to 1 ng/ml for future assays. The amount of PrP^{Sc} in cell preparations was not significantly altered following by digestion with either PLA₂ (4.8 ng/ml ± 0.4 compared with 4.6 ± 0.5, n=9, P=0.6) or PI-PLC (4.5 ng/ml ± 0.3 compared with 4.6 ± 0.5, n=9, P=0.8). Western blot analysis did not detect an observable difference in the apparent molecular weight of these preparations (Figure 2B). However, HPTLC analysis of GPI anchors isolated from phospholipase digested PrP^{Sc} preparations showed that the GPI isolated from PrP^{Sc} digested with PLA₂ or PI-PLC had different migration patterns to those isolated from mock treated PrP^{Sc} (Figure 2C).

Phospholipase digestion of PrP^{Sc} reduced infectivity for GT1 cells. In the first set of experiments GT1 cells were pulsed with different amounts of mock-treated, or phospholipase digested ScGT1 supernatants (range 1 to 0.016 ng) for 24 hours. We report that mock treated supernatants infected GT1 cells and that the PrP^{Sc} content of recipient GT1 cells was dependent upon the dose of the original inoculum (Figure 3A). In contrast, recipient GT1 cells pulsed with 1 ng of PI-PLC digested ScGT1 supernatants did not contain detectable levels of PrP^{Sc} (less than 0.05 ng). GT1 cells pulsed with PLA₂ digested ScGT1 supernatants contained less PrP^{Sc} than GT1 cells pulsed with mock treated supernatants.

GT1 cells were also pulsed with partially purified PrP^{Sc} preparations. The addition of PrP^{Sc}-GPI resulted in a dose-dependent increase in the amount of PrP^{Sc} in recipient GT1 cells (Figure 3B). The addition of 1 ng PrP^{Sc}-GPI did not significantly affect cell survival, as measured by thiazyl blue tetrazolium. We did not detect PrP^{Sc} in GT1 cells pulsed with 1 ng PrP^{Sc}-IPG (less than 0.05 ng). GT1 cells pulsed with 1 ng PrP^{Sc}-G-lyso-PI contained significantly less PrP^{Sc} than GT1 cells pulsed with 1 ng PrP^{Sc}-GPI (0.28 ng PrP^{Sc} ± 0.25 compared with 6.1 ng PrP^{Sc} ± 1.12, n=27, P<0.01). Western blots confirmed that after 7 days GT1 cells pulsed with PrP^{Sc}-GPI contained greater amounts of PrP^{Sc} than did cells pulsed with PrP^{Sc}-G-lyso-PI or PrP^{Sc}-IPG (Figure 3C).

Phospholipase digestion reduced PrP^{Sc} infectivity for cortical neurones. To confirm the GT1 cell data in non-dividing cells primary cortical neurones were pulsed with ScGT1 supernatants at different

concentrations as shown. We report that mock treated ScGT1 supernatants infected cortical neurones; there was a dose-dependent increase in the PrP^{Sc} content of recipient cortical neurones (Figure 4A). Cortical neurones pulsed with PLA₂ digested ScGT1 supernatants also contained PrP^{Sc}. However, digestion with PLA₂ significantly reduced the infectivity of ScGT1 supernatants. We did not detect PrP^{Sc} in cortical neurones pulsed with ScGT1 supernatants containing 1 ng PrP^{Sc} that had been digested with PI-PLC (less than 0.05 ng PrP^{Sc}).

The addition of partially purified PrP^{Sc}-GPI resulted in a dose-dependent increase in the amount of PrP^{Sc} in cortical neurones (Figure 4B). Thus, the addition of 1 ng PrP^{Sc}-GPI was amplified to 44 ng PrP^{Sc} ± 6.7, n=22 after 10 days in cortical neurones. We were unable to detect PrP^{Sc} in cortical neurones pulsed with 1 ng PrP^{Sc}-IPG. Cortical neurones pulsed with 1 ng PrP^{Sc}-G-lyso-PI contained 3.63 ng PrP^{Sc} ± 2, n=22. The PrP^{Sc} in these cells bound to C18 columns and was eluted in fractions containing greater than 70% acetonitrile indicating that the PrP^{Sc} contained a GPI anchor with 2 acyl chains. Thus, this PrP^{Sc} had been synthesised by the recipient cortical neurones and was not a remnant of the original inoculum. Primary cortical neurones derived from PrP knockout mice were also pulsed with ScGT1 supernatants containing 1 ng PrP^{Sc} or 1 ng partially purified PrP^{Sc}-GPI for 24 hours and incubated for a further 9 days. We were unable to detect PrP^{Sc} in these cells using either ELISA (less than 0.05 ng) or immunoblot (Figure 4C).

The binding of PrP^{Sc} to cells is reduced by phospholipase digestion. To determine if GPI anchors affected the binding of PrP^{Sc} to recipient cells, GT1 cells were incubated with 1 ng partially purified PrP^{Sc} preparations for 1 or 24 hours, washed and the amount of cell-associated PrP^{Sc} was measured. After 1 hour there was no significant difference in the amount of PrP^{Sc}-GPI and PrP^{Sc}-G-lyso-PI that had bound (0.95 ng ± 0.1 compared with 0.93 ± 0.12, n=12, P=0.7) (Figure 5A). After 1 hour incubation we were unable to detect any PrP^{Sc}-IPG that had bound to cells (less than 0.05 ng) indicating that acylation of the GPI anchor facilitates the efficient binding of PrP^{Sc} to neurones. However, increasing the incubation period to 24 hours resulted in a small amount of cell associated PrP^{Sc}-IPG (128 pg ± 42, n=12).

Phospholipase A₂ digested PrP^{Sc} does not target lipid rafts. Next we sought to determine why it was that although PrP^{Sc}-G-lyso-PI bound readily to cells, only low amounts of PrP^{Sc} were produced in recipient cells. PrP^{Sc} production is thought to be dependent on the formation or function of cholesterol-sensitive lipid rafts. While the addition of 1 ng PrP^{Sc}-GPI significantly increased the amount of cholesterol in GT1 cell membranes (573 ng/10⁶ cells ± 57 compared to 395 ± 38, n=8, P<0.01), the addition of 1 ng PrP^{Sc}-G-lyso-PI had no significant effect (402 ng/10⁶ cells ± 47 compared to 395 ± 38, n=8, P=0.78). PrP^{Sc} is found within lipid rafts that are defined by their resistance to solubilisation by non-ionic detergents [19]. We report that greater than 90% of PrP^{Sc}-GPI was found in the detergent resistant (lipid raft) fraction of GT1 cell membranes. In contrast, less than 10% of PrP^{Sc}-G-lyso-PI was found in the lipid raft fraction (Figure 5B). Similar results were obtained when these preparations were added to cortical neurones; PrP^{Sc}-GPI was mainly found within lipid rafts and PrP^{Sc}-G-lyso-PI within the normal cell membrane.

Discussion

The molecular mechanisms by which prions spread throughout an infected brain remain poorly understood. In this study we demonstrate the role of acyl chains of the GPI anchor on the ability of PrP^{Sc} to bind neuronal cells and to replicate. PrP^{Sc} with a conventional GPI anchor (PrP^{Sc}-GPI) was able to bind and replicate within both GT1 neuronal cells and primary cortical neurones. As expected, PrP^{Sc} did not replicate within cortical neurones derived from PrP knockout mice. Modification of the GPI anchor reduced the infectivity of PrP^{Sc}; PrP^{Sc}-IPG did not bind to cells and although PrP^{Sc}-G-lyso-PI did bind to cells it did not readily convert PrP^C to PrP^{Sc}.

The partial purification of phospholipase digested PrP^{Sc} molecules was facilitated by using PrP^{Sc} isolated from ScGT1 cells rather than from infected brains. PrP^{Sc} exists as aggregates of different sizes [20] and large aggregates of PrP^{Sc} are thought to cause steric hindrance of PI-PLC. In this study vigorous shaking increased the susceptibility of PrP^{Sc} to phospholipases, presumably because it reduced the size of PrP^{Sc} aggregates. Digested products were isolated by differential binding and elution from C18 columns in a gradient of acetonitrile and water. Western blot analysis of PI-PLC or PLA₂ digested preparations showed no apparent difference in molecular weight. This result was not surprising considering digestion with PLA₂ would reduce the molecular weight by only 0.2 kDa and PI-PLC digestion by 0.4 kDa. In contrast the removal of the whole GPI anchor would be expected to reduce the molecular weight by approximately 5 kDa. However, HPTLC analysis showed that digestion with either PI-PLC or PLA₂ altered the GPI anchors of these preparations.

Whereas PrP^{Sc}-GPI bound rapidly to neurones, PrP^{Sc}-IPG did not indicating that the acylation of the GPI anchor affected PrP^{Sc} binding to cells. The binding of PrP^{Sc} to cells is complex, and although there are many reports of specific PrP^{Sc}-protein interactions [21], the nature and presence of specific protein receptors for PrP^{Sc} is yet to meet an agreed consensus. Our observation that the removal of both acyl chains greatly reduced the binding of PrP^{Sc} to cells shows that they contribute strongly to the binding of PrP^{Sc} to cells. Such results do not necessarily exclude prion-protein or prion-glycan interactions which may be facilitated after PrP^{Sc} has inserted into the membrane. We conclude that in these studies PrP^{Sc}-IPG in the supernatant had limited interactions with cell-associated PrP^C.

At first glance our results appear to contradict studies from transgenic mice producing PrP^C without a GPI anchor which produced high amounts of PrP^{Sc} and infectivity [22]. However, in these transgenic mice anchorless PrP^C was rapidly secreted from neurones. Moreover, a recent study showed that cell lines producing anchorless PrP^C were resistant to scrapie infection [23]. A direct comparison with these results is invalid considering that the removal of the entire GPI anchor in these mice affected the glycosylation of PrP^C and its expression at the surface of neurones. As the glycosylation status of PrP^C can affect PrP^{Sc} formation [24] it is not clear whether the effects of removing the entire GPI anchor was a direct effect of the loss of the GPI anchor, or an indirect effect resulting from altered glycosylation. Another study in which recombinant PrP that did not contain a GPI anchor induced prion disease in transgenic mice [25] suggests that the GPI anchor is not essential for infectivity. Our studies suggest that acylation of the GPI anchor facilitates PrP^{Sc} binding but is probably not the sole factor involved in infectivity. Our results also differ from a study which reported that removal of the GPI anchor from PrP^{Sc} by cathepsin D did not affect prion infectivity [26]. However, digestion with cathepsin D removes the whole GPI anchor as well as a fragment of the C terminus, whereas phospholipases simply modify the GPI anchor by the removal of 1 or both acyl chains as shown in Figure 1. Several technical differences are also apparent in the 2 studies, those authors used PrP^{Sc} derived from infected brain homogenates which was extracted using strong detergents and incubated uninfected cells with PrP^{Sc} for 3 days. We used two PrP^{Sc} preparations, the first (ScGT1 supernatants) contained PrP^{Sc} that had been released from ScGT1 cells without the use of detergents and which had not been protease treated. Since protease sensitive forms of PrP^{Sc} have been identified [27] our preparations overcame the problem of protease digestion reducing the infectivity of PrP^{Sc}. Although the second PrP^{Sc} preparation was extracted using a mild detergent, most of the detergent was removed by the subsequent passage through C18 columns prior to the addition of PrP^{Sc} to cells. The lack of detergent in our PrP^{Sc} preparations is a possible explanation of the differences between our studies and previous reports.

The amount of PrP^{Sc}-G-lyso-PI and PrP^{Sc}-GPI that had bound to recipient cells after 1 hour was not significantly different indicating that a single acyl chain is sufficient to facilitate the binding of PrP^{Sc} to neuronal cells. Crucially, although PrP^{Sc}-G-lyso-PI bound to cells, it did not readily convert endogenous PrP^C. While these preparations contained mostly PrP^{Sc}-G-lyso-PI we cannot exclude the possibility that they also contained a small amount of undigested PrP^{Sc}-GPI which was responsible for PrP^{Sc} formation in

recipient cells. Most GPI-anchored proteins are targeted to cholesterol dense lipid rafts and the conversion of PrP^C to PrP^{Sc} is cholesterol sensitive [28, 29]. GPI-anchors contain predominantly unsaturated acyl chains which encourage the solubilisation of cholesterol [30]. In this study the addition of PrP^{Sc}-GPI significantly increased the cholesterol content of cells whereas the addition of PrP^{Sc}-G-lyso-PI had no effect. The amount of cholesterol in cell membranes affects lipid raft formation and greater than 90% of PrP^{Sc}-GPI was targeted to lipid rafts. In contrast; less than 10% of PrP^{Sc}-G-lyso-PI was found within lipid rafts indicating that these molecules were mostly in the normal cell membrane. The localisation of PrP^{Sc}-G-lyso-PI to non-raft membranes may restrict its interactions with endogenous PrP^C which is localised to lipid rafts. Since lipid rafts can regulate cell signalling [31] the localisation of PrP^{Sc}-GPI to lipid rafts may enhance cell signalling pathways involved in PrP^C conversion. In contrast, the localisation of PrP^{Sc}-G-lyso-PI to non-raft membranes may reduce cell signalling involved in PrP^C conversion.

In summary we showed that PrP^{Sc} was able to bind and replicate in both GT1 neuronal cells and primary cortical neurones. A combination of phospholipase digestion and shaking allowed the role of the GPI anchor on PrP^{Sc} binding and replication to be determined. PI-PLC digestion of PrP^{Sc} reduced its ability to bind to cells. Although PLA₂ digested PrP^{Sc} bound to neuronal cells, it was not found within lipid rafts and infected cells contained only low amounts of PrP^{Sc}. Such studies show that the GPI anchor attached to PrP^{Sc} affects the targeting of PrP^{Sc} to specific membranes and its ability to either interact with, or convert, endogenous PrP^C. We speculate that compounds that alter GPI anchors may provide a novel therapeutic approach to combat prion disease.

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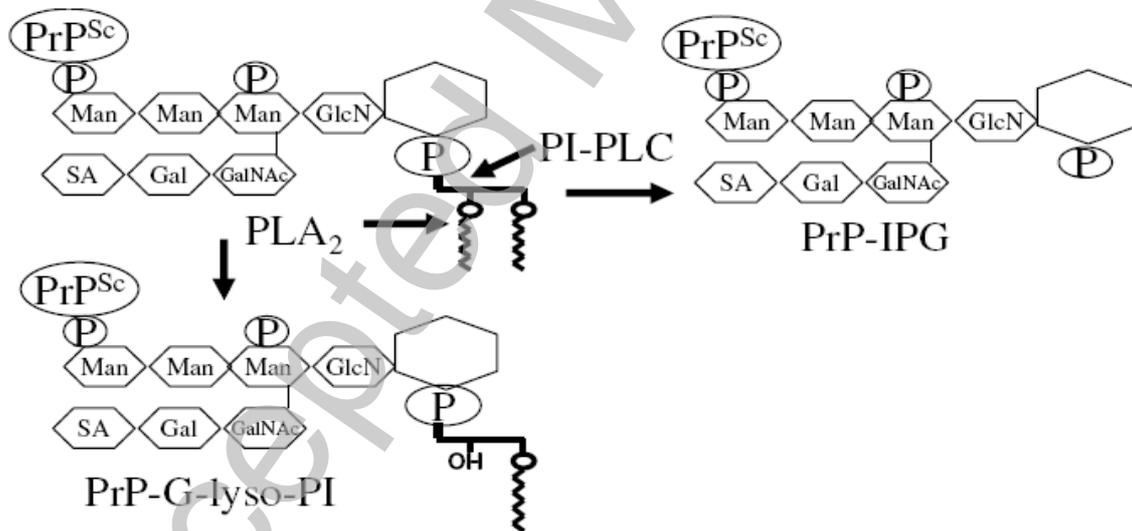


Figure 1. Phospholipase digestion of PrP^{Sc} GPI anchors. Cartoon showing the cleavage sites of PLA₂ and PI-PLC on the putative GPI anchor attached to PrP^{Sc}. Glycan residues shown include mannose (Man), sialic acid (SA), galactose (Gal), N-acetyl galactosamine (GalNAc) and glucosamine (GlcN) as well as phosphate (P). Also shown are the predicted digested products (PrP^{Sc}-IPG and PrP^{Sc}-G-lyso-PI).

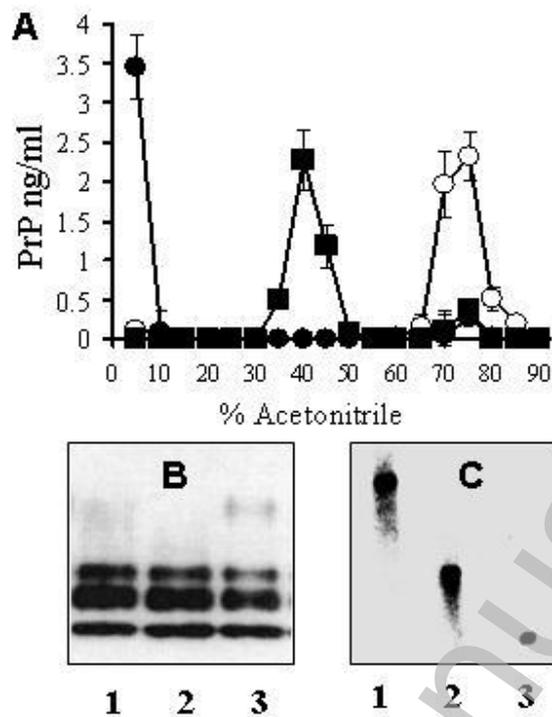


Figure 2. Phospholipase digestion of PrP^{Sc} alters C18 binding. (A) The amount of PrP in fractions eluted by reverse phase chromatography under a gradient of acetonitrile in water from C18 columns loaded with mock digested PrP^{Sc} (PrP^{Sc}-GPI) (○), PrP^{Sc} digested with PI-PLC (PrP^{Sc}-IPG) (●) or PrP^{Sc} digested with PLA₂ (PrP^{Sc}-G-lyso-PI) (■). Values shown are the mean average amount of PrP^{Sc} (ng/ml) ± SD, n=9. (B) Immunoblots showing PrP^{Sc}-GPI (1), PrP^{Sc}-G-lyso-PI (2) or PrP^{Sc}-IPG (3). (C) HPTLC analysis of GPI anchors isolated from PrP^{Sc}-GPI (1), PrP^{Sc}-G-lyso-PI (2) and PrP^{Sc}-IPG (3).

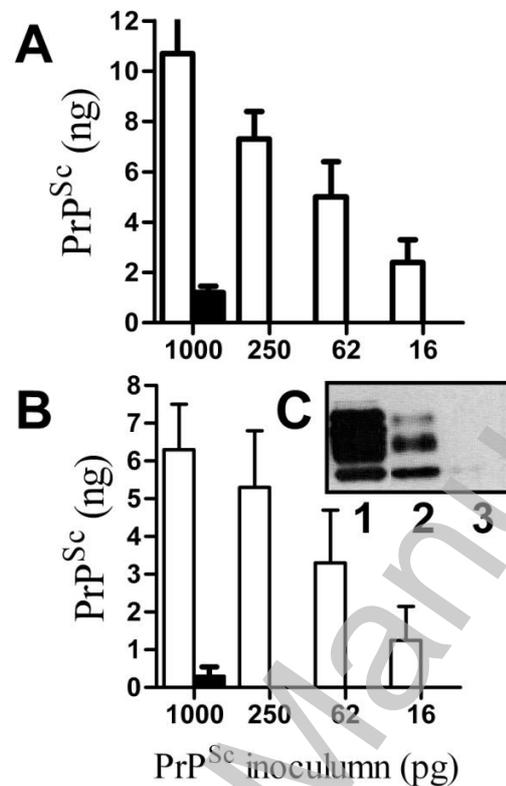


Figure 3. PrP^{Sc} replicates within GT1 cells. (A) The amount of PrP^{Sc} in GT1 cells pulsed for 24 hours with different concentrations (inoculum) of mock treated ScGT1 supernatants (□) or PLA₂ digested ScGT1 supernatants (■) as shown and incubated for a further 6 days. Values show are the mean average amount of PrP^{Sc} (ng/10⁶ cells) ± SD, n=9. (B) The amount of PrP^{Sc} in GT1 cells pulsed for 24 hours with different amounts of partially purified PrP^{Sc}-GPI (□) or PrP^{Sc}-G-lyso-PI (■) as shown and incubated for a further 6 days. Values show are the mean average amount of PrP^{Sc} (ng/10⁶ cells) ± SD, n= 27. (C) Immunoblots showing the amount of PrP^{Sc} in GT1 cells had been pulsed or 24 hours with 1 ng PrP^{Sc}-GPI (1), PrP^{Sc}-G-lyso-PI (2) or PrP^{Sc}-IPG (3) and grown for a further 6 days.

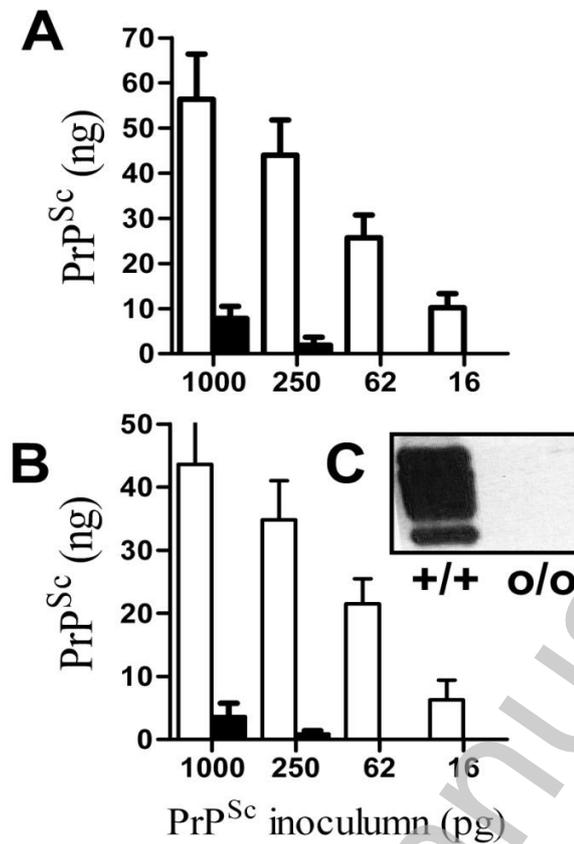


Figure 4. Phospholipase digestion reduced PrP^{Sc} replication in cortical neurones. (A) The amount of PrP^{Sc} in cortical neurones pulsed for 24 hours with different concentrations (innoculum) of mock-treated ScGT1 cell supernatants (□) or PLA₂ digested ScGT1 cell supernatants (■) and incubated for a further 9 days. Values show are the mean average amount of PrP^{Sc} (ng/10⁶ cells) ± SD, n=9. (B) The total amount of PrP^{Sc} in primary cortical neurones pulsed with different amounts of partially purified PrP^{Sc}-GPI (□) or PrP^{Sc}-G-lyso-PI (■) for 24 hours and incubated for a further 9 days. Values show are the mean average amount of PrP^{Sc} (ng/10⁶ cells) ± SD, n=22. (C) Immunoblots showing the PrP^{Sc} content of primary cortical neurones derived from PrP wildtype (+/+) or PrP knockout (o/o) mice that had been pulsed for 24 hours with 1 ng PrP^{Sc}-GPI and incubated for a further 9 days.

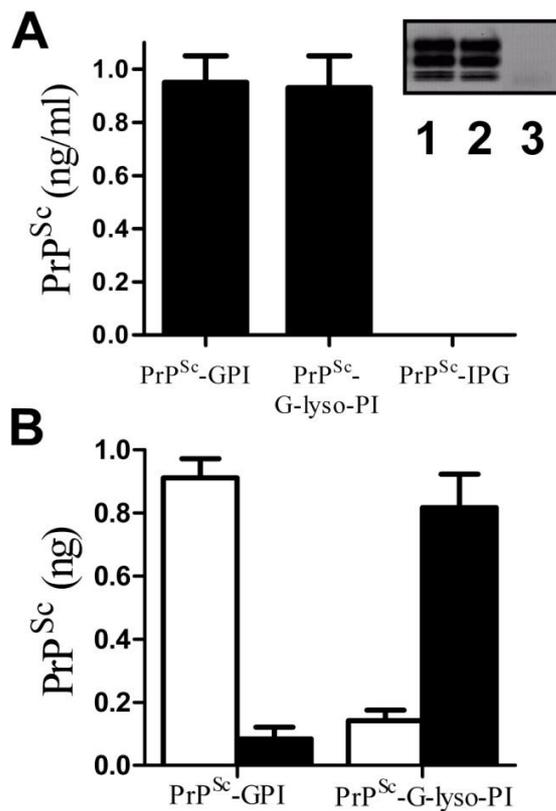


Figure 5. Phospholipase A₂ digestion of the PrP^{Sc}-GPI anchors affects detergent solubility. (A) The amount of PrP^{Sc}-GPI, PrP^{Sc}-G-lyso-PI or PrP^{Sc}-IPG found in the in whole cell extracts from GT1 cells after 1 hour. Values shown are the mean average amount of PrP^{Sc} (ng) \pm SD, n=12. Inset - Immunoblot showing the amount of PrP^{Sc} in GT1 cells had been pulsed for 1 hour with 1 ng PrP^{Sc}-GPI (1), PrP^{Sc}-G-lyso-PI (2) or PrP^{Sc}-IPG (3). (B) The amount of PrP^{Sc}-GPI or PrP^{Sc}-G-lyso-PI found in the in detergent-resistant membranes (lipid rafts) (□) or the detergent soluble membrane (■) in GT1 cells after 24 hours. Values shown are the mean average amount of PrP^{Sc} (ng) \pm SD, n=12.

References

- 1 Prusiner, S. B. (1998) Prions. *Proc.Natl.Acad.Sci.U.S.A.* **95**, 13363-13383
- 2 Pan, K. M., Baldwin, M., Nguyen, J., Gasset, M., Serban, A., Groth, D., Mehlhorn, I., Huang, Z., Fletterick, R. J. and Cohen, F. E. (1993) Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc.Natl.Acad.Sci.U.S.A.* **90**, 10962-10966
- 3 Prusiner, S. B., McKinley, M. P., Bowman, K. A., Bolton, D. C., Bendheim, P. E., Groth, D. F. and Glenner, G. G. (1983) Scrapie prions aggregate to form amyloid-like birefringent rods. *Cell.* **35**, 349-358
- 4 Fevrier, B., Vilette, D., Archer, F., Loew, D., Faigle, W., Vidal, M., Laude, H. and Raposo, G. (2004) Cells release prions in association with exosomes. *Proc.Natl.Acad.Sci.U.S.A.* **101**, 9683-9688
- 5 Vella, L. J., Sharples, R. A., Lawson, V. A., Masters, C. L., Cappai, R. and Hill, A. F. (2007) Packaging of prions into exosomes is associated with a novel pathway of PrP processing. *J Pathol.* **211**, 582-590
- 6 de Gassart, A., Geminard, C., Hoekstra, D. and Vidal, M. (2004) Exosome Secretion: The Art of Reutilizing Nonrecycled Proteins? *Traffic.* **5**, 896-903

- 7 Gousset, K., Schiff, E., Langevin, C., Marijanovic, Z., Caputo, A., Browman, D. T., Chenouard, N., de Chaumont, F., Martino, A., Enninga, J., Olivo-Marin, J. C., Mannel, D. and Zurzolo, C. (2009) Prions hijack tunnelling nanotubes for intercellular spread. *Nat Cell Biol.* **11**, 328-336
- 8 Magalhaes, A. C., Baron, G. S., Lee, K. S., Steele-Mortimer, O., Dorward, D., Prado, M. A. M. and Caughey, B. (2005) Uptake and Neuritic Transport of Scrapie Prion Protein Coincident with Infection of Neuronal Cells. *J Neurosci.* **25**, 5207-5216
- 9 Cronier, S., Laude, H. and Peyrin, J. M. (2004) Prions can infect primary cultured neurons and astrocytes and promote neuronal cell death. *Proc Natl Acad Sci U S A.* **101**, 12271-12276
- 10 Mahal, S. P., Baker, C. A., Demczyk, C. A., Smith, E. W., Julius, C. and Weissmann, C. (2007) Prion strain discrimination in cell culture: The cell panel assay. *Proc Natl Acad Sci USA.* **104**, 20908-20913
- 11 Legler, D. F., Doucey, M. A., Schneider, P., Chapatte, L., Bender, F. C. and Bron, C. (2005) Differential insertion of GPI-anchored GFPs into lipid rafts of live cells. *FASEB J.* **19**, 73-75
- 12 Premkumar, D. R., Fukuoka, Y., Sevlever, D., Brunschwig, E., Rosenberry, T. L., Tykocinski, M. L. and Medof, M. E. (2001) Properties of exogenously added GPI-anchored proteins following their incorporation into cells. *J Cell Biochem.* **82**, 234-245
- 13 Stahl, N., Borchelt, D. R., Hsiao, K. and Prusiner, S. B. (1987) Scrapie prion protein contains a phosphatidylinositol glycolipid. *Cell.* **51**, 229-240
- 14 Caughey, B., Neary, K., Buller, R., Ernst, D., Perry, L. L., Chesebro, B. and Race, R. E. (1990) Normal and scrapie-associated forms of prion protein differ in their sensitivities to phospholipase and proteases in intact neuroblastoma cells. *J. Virol.* **64**, 1093-1101
- 15 Stahl, N., Borchelt, D. R. and Prusiner, S. B. (1990) Differential release of cellular and scrapie prion proteins from cellular membranes by phosphatidylinositol-specific phospholipase C. *Biochemistry.* **29**, 5405-5412
- 16 Atarashi, R., Wilham, J. M., Christensen, L., Hughson, A. G., Moore, R. A., Johnson, L. M., Onwubiko, H. A., Priola, S. A. and Caughey, B. (2008) Simplified ultrasensitive prion detection by recombinant PrP conversion with shaking. *Nat Methods.* **5**, 211-212
- 17 Bate, C. and Williams, A. (2004) Role of glycosylphosphatidylinositols in the activation of phospholipase A₂ and the neurotoxicity of prions. *J. Gen. Virol.* **85**, 3797-3804
- 18 Wadsworth, J. D. F., Joiner, S., Linehan, J. M., Cooper, S., Powell, C., Mallinson, G., Buckell, J., Gowland, I., Asante, E. A., Budka, H., Brandner, S. and Collinge, J. (2006) Phenotypic heterogeneity in inherited prion disease (P102L) is associated with differential propagation of protease-resistant wild-type and mutant prion protein. *Brain.* **129**, 1557-1569
- 19 Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S. J., Smart, E. J., Anderson, R. G., Taraboulos, A. and Prusiner, S. B. (1996) Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 14945-14949
- 20 Silveira, J. R., Raymond, G. J., Hughson, A. G., Race, R. E., Sim, V. L., Hayes, S. F. and Caughey, B. (2005) The most infectious prion protein particles. *Nature.* **437**, 257-261
- 21 Lee, K. S., Linden, R., Prado, M. A. M., Brentani, R. R. and Martins, V. R. (2003) Towards cellular receptors for prions. *Reviews in Medical Virology.* **13**, 399-408
- 22 Chesebro, B., Trifilo, M., Race, R., Meade-White, K., Teng, C., LaCasse, R., Raymond, L., Favara, C., Baron, G., Priola, S., Caughey, B., Masliah, E. and Oldstone, M. (2005) Anchorless Prion Protein Results in Infectious Amyloid Disease Without Clinical Scrapie. *Science.* **308**, 1435-1439
- 23 McNally, K. L., Ward, A. E. and Priola, S. A. (2009) Cells Expressing Anchorless Prion Protein are Resistant to Scrapie infection. *J. Virol.*, 02412-02408
- 24 Tuzi, N. L., Cancellotti, E., Baybutt, H., Blackford, L., Bradford, B., Plinston, C., Coghill, A., Hart, P., Piccardo, P., Barron, R. M. and Manson, J. C. (2008) Host PrP Glycosylation: A Major Factor Determining the Outcome of Prion Infection. *PLoS Biol.* **6**, e100
- 25 Legname, G., Baskakov, I. V., Nguyen, H. O., Riesner, D., Cohen, F. E., DeArmond, S. J. and Prusiner, S. B. (2004) Synthetic mammalian prions. *Science.* **305**, 673-676

- 26 Lewis, P. A., Properzi, F., Prodromidou, K., Clarke, A. R., Collinge, J. and Jackson, G. S. (2006) Removal of the glycosylphosphatidylinositol anchor from PrP(Sc) by cathepsin D does not reduce prion infectivity. *Biochem J.* **395**, 443-448
- 27 Tzaban, S., Friedlander, G., Schonberger, O., Horonchik, L., Yedidia, Y., Shaked, G., Gabizon, R. and Taraboulos, A. (2002) Protease-Sensitive Scrapie Prion Protein in Aggregates of Heterogeneous Sizes. *Biochemistry.* **41**, 12868-12875
- 28 Bate, C., Salmona, M., Diomede, L. and Williams, A. (2004) Squalastatin cures prion-infected neurones and protects against prion neurotoxicity. *J.Biol.Chem.* **279**, 14983-14990
- 29 Taraboulos, A., Scott, M., Semenov, A., Avrahami, D., Laszlo, L., Prusiner, S. B. and Avraham, D. (1995) Cholesterol depletion and modification of COOH-terminal targeting sequence of the prion protein inhibit formation of the scrapie isoform *J.Cell Biol.* **129**, 121-132
- 30 Schroeder, R., London, E. and Brown, D. (1994) Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior. *Proc.Natl.Acad.Sci.U.S.A.* **91**, 12130-12134
- 31 Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat.Rev.Mol.Cell Biol.* **1**, 31-39

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