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Pathogenic mutations in the glycosylphosphatidylinositol signal peptide of PrP modulate its topology in neuroblastoma cells

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Abstract

Point mutations M232R (PrP^{232R}), M232T (PrP^{232T}), and P238S (PrP^{238S}) in the glycosylphosphatidylinositol signal peptide (GPI-SP) of the prion protein (PrP^C) segregate with familial Creutzfeldt-Jakob disease (CJD). However, the mechanism by which these mutations induce cytotoxicity is unclear since the GPI-SP is replaced by a GPI anchor within five minutes of PrP synthesis and translocation into the endoplasmic reticulum (ER). To examine if mutations in this region interfere with translocation of nascent PrP into the ER or anchor addition, the metabolism of PrP^{232R} and PrP^{232T} was investigated in transfected human neuroblastoma cells. In this report, we demonstrate that PrP mutations M232R and M232T do not interfere with GPI anchor addition. Instead, these mutations increase the stability and transport of GPI-SP mediated post-translationally translocated PrP to the plasma membrane, where it is linked to the lipid bilayer in a potentially neurotoxic C-transmembrane (C^{tm}PrP) orientation. Furthermore, we demonstrate that the GPI-SP of PrP functions as an efficient co-translational and inefficient post-translational ER translocation signal when tagged to an unrelated protein, underscoring the functional versatility of this peptide. These data uncover an alternate pathway of ER translocation for nascent PrP, and provide information on the possible mechanism(s) of neurotoxicity by mutations in the GPI-SP.

Keywords

PrP transport; GPI-signal peptide; mutant PrP; C^{tm}PrP; post-translational ER translocation; familial prion disorders

Introduction

More than fifty autosomal dominant mutations in the prion protein (PrP) coding region segregate with familial prion disorders that include familial Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) (Wadsworth et al., 2003). Despite a strong correlation between specific mutations and the disease phenotype, a clear understanding of the underlying mechanism of neurotoxicity in these disorders remains unclear (Prusiner, 1998; Aguzzi and Polymenidou, 2004; Harris and True, 2006). Proposed mechanisms include destabilization of PrP by the mutation and conversion to an aggregated, PrP^{Sc}-like form at advanced age, aberrant processing of mutant PrP, and generation of alternate

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PrP forms including cytosolic and C-transmembrane forms ($C_{tm}PrP$) that are believed to cause cytotoxicity by poorly understood mechanisms (Hegde et al., 1998a, 1999; Haik et al., 2000; Ma et al., 2002; Stewart et al., 2005; Singh et al., 2005; Rambold et al., 2006). Paradoxically, cytosolic PrP has also been demonstrated to serve a protective function (Roucou et al., 2003). In each of the above cases, mutations in the PrP coding region are involved directly or indirectly in mediating cytotoxicity.

Pathogenic mutations M232R (PrP^{232R}), M232T (PrP^{232T}), and P238S (PrP^{238S}) in the glycosylphosphatidylinositol signal peptide (GPI-SP) of PrP are particularly perplexing since this region is replaced by a GPI anchor within five minutes of synthesis and is absent in the mature protein (Kitamoto et al., 1993; Hoque et al., 1996; Windl et al., 1999). Likely scenarios include modulation of events prior to translocation of precursor PrP into the endoplasmic reticulum (ER), or interference with GPI-SP cleavage and anchor addition following ER translocation. In the former case mutations in this region would alter ER translocation or membrane topology of translocated PrP (Kim and Hegde, 2002; Ott et al., 2007), and in the latter cause accumulation of GPI-SP containing mutant PrP in the ER (Moran et al., 1991; Udenfriend and Kodukula, 1995; Jin et al., 2000) or secretion of anchorless PrP into the extracellular milieu (Chesebro et al., 2005).

Support for the first hypothesis comes from a study demonstrating GPI-SP mediated post-translational ER translocation of PrP^C in an *in vitro* transcription-translation reaction. Interestingly, PrP synthesized under these conditions is linked to the lipid bilayer in the C_{tm} orientation (Hölscher et al., 2001), a potentially neurotoxic PrP form (Hegde et al., 1998a; 1999). Although rare, post-translational ER translocation of higher eukaryotic proteins has been described for cystic fibrosis transmembrane conductance regulator, and signal-anchor E3-6.7K protein of adenovirus that adopts different topologies in the lipid bilayer (Schnell and Hebert, 2003; Lu et al., 1998; Moise et al., 2004).

Supporting the second scenario are studies demonstrating inefficient GPI-SP cleavage and anchor addition due to mutations in this region (Udenfriend and Kodukula, 1995). Uncleaved GPI-SP causes retention of the protein in the ER (Field et al., 1994; Jin et al., 2000), while cleavage without anchor addition results in secretion of anchorless protein into the medium (Chesebro et al., 2005; Kiachopoulos et al., 2005).

To distinguish between these possibilities, we evaluated the metabolism of PrP^C, PrP^{232R}, PrP^{232T}, and corresponding proteins lacking the conventional ER targeting N-SP in transfected human neuroblastoma cells. The N-SP deleted forms were included to improve the efficiency of GPI-SP mediated ER translocation and to evaluate the effect of mutations in this region on ER targeting. In this report, we demonstrate that PrP mutations M232R and M232T do not interfere with GPI anchor addition. Instead, the GPI-SP mediates post-translational ER targeting of PrP in the absence of N-SP, and point mutations in this region increase the stability and transport of mutant PrP to the plasma membrane where it is linked in the potentially neurotoxic C_{tm} orientation. Moreover, the GPI-SP of PrP mediates efficient co-translational and inefficient but significant post-translational ER translocation of an unrelated protein, the green fluorescent protein (GFP) when ligated to its N- and C-terminal ends respectively, highlighting the dual function of GPI-SP of PrP and the influence of pathogenic mutations in region on PrP topology.

Materials and Methods

Antibodies and materials

PrP-specific monoclonal antibodies 3F4, 8H4, and 8B4 were obtained from Abcam (Cambridge, MA, USA), and from Man-Sun Sy and Pierluigi Gambetti (Case Western Reserve

University) respectively. Anti-calnexin and anti-GFP antibodies were procured from Stressgen (Ann Arbor, Michigan, USA), and all secondary antibodies were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL). Cell culture supplies were obtained from Invitrogen, hygromycin B from Calbiochem, and Sulfo-N-hydroxysuccinimide (NHS)-biotin and streptavidin-Texas red from Pierce (Rockford, IL). Hoechst dye was obtained from Molecular Probes (Eugene, Oregon), *Tran*³⁵S-label from ICN (Costa Mesa, CA), and protein A-agarose and N-glycosidase F (PNGase-F) from Roche Molecular Biochemicals. All other chemicals were purchased from Sigma.

Generation of mutant PrP cell lines

Human neuroblastoma cells (M17) expressing normal and mutant PrP forms were generated and maintained as described previously (Jin et al., 2000). Briefly, point mutations M232R/T and G123P were created using the QuikChange site-directed mutagenesis kit according to manufacturer's directions (Stratagene, La Jolla, CA). Deletion of the N-SP was carried out using over-lapping primer pairs from either end of PrP. To ligate GPI-SP and N-SP on one or both ends of GFP, specific sequences containing appropriate restriction sites were amplified by PCR and inserted in pEGFP-N1 and pEGFPC3 vectors respectively (Operon Technologies Inc., Alameda, CA). Cells were transfected using the Invitrogen transfection kit and processed 48 hours following transfection.

Immunostaining and fluorescence microscopy

Transfected cells were cultured and processed for immunostaining as described in a previous report (Gu et al., 2002). Anti-PrP antibodies 3F4, 8H4, biotin tagged 8B4, and anti-GFP antibody were used at a dilution of 1:20. Fluorescein- and rhodamine-conjugated secondary antibodies were used at a dilution of 1:40. Biotin tagged 8B4 was detected with streptavidin-conjugated-Texas red (1:40), and nuclei were visualized with Hoechst 33342 (1 µg/ml). All samples were observed using a laser scanning confocal microscope (Bio-Rad MRC 600). A single 5-µm optical section and a composite of several sections were evaluated in each case.

Metabolic labeling, immunoprecipitation and Western blotting

Transfected cells were radiolabeled with 40 µCi/ml of *Tran*³⁵S-label for 15–30 min or for 2 h in DMEM lacking methionine/cysteine and supplemented with 5% dialyzed serum. Labeled cells were washed with PBS and lysed in non-ionic detergents, or chased in normal medium for 1 h before processing for immunoprecipitation as described (Jin et al., 2000). For Western blotting, cells expressing normal or mutant PrP forms were washed in PBS, and lysates were processed for Western blotting as described previously (Gu et al., 2002).

Enzymatic deglycosylation

Radiolabeled, immunoprecipitated proteins were re-precipitated with five volumes of cold methanol and resuspended in denaturing buffer (0.5% SDS, 1% β-mercaptoethanol). Samples were boiled for 10 min and deglycosylated with PNGase-F (1000 units in 1% Nonidet P-40, 25 mM sodium phosphate, pH 7.5) for 1–3 h at 37°C. Deglycosylated proteins were re-precipitated and resolved by SDS-PAGE-fluorography.

Surface biotinylation

Cells expressing desired PrP forms were washed with PBS and incubated with 0.5 mg/ml of sulfo-NHS-biotin in PBS for 15 min on ice. Subsequently, cells were washed with 50 mM glycine in PBS to quench excess biotin, washed three times with cold PBS, and lysed in non-ionic detergents containing protease inhibitors. Biotin-tagged proteins were retrieved with streptavidin-coated beads, and intracellular proteins in the supernatant were precipitated with

cold methanol. Both surface and intracellular proteins were fractionated by SDS-PAGE and immunoblotted with 3F4.

Results

Majority of PrP^{232R} and PrP^{232T} is processed normally in transfected cells

The position of point mutations M232R and M232T in precursor PrP and their absence in post-translationally processed PrP are depicted diagrammatically. Reactivity of anti-PrP antibodies specific for the N-terminus (8B4), C-terminus (8H4), and residues 109–112 (3F4) overlapping with the TM domain is also shown (Figure 1A).

To evaluate the effect of point mutations M232R and M232T on PrP metabolism, steady state expression of PrP^C, PrP^{232R}, and PrP^{232T} in transiently transfected human neuroblastoma cells was assessed by immunoblotting cell lysates with 3F4 and 8H4. As expected, both antibodies detect PrP^C glycoforms comprising of unglycosylated, mono- and di-glycosylated forms migrating at 27, 30–32, and 37 kDa respectively (Figure 1B, lanes 2 and 6). In addition, C-terminal fragments of 20 and 18 kDa are detected with 3F4 and 8H4 respectively (Figure 1B, lanes 2 and 6) (Gu et al., 2006). Similar evaluation of lysates prepared from PrP^{232R} and PrP^{232T} cells shows full-length and truncated PrP forms that do not differ significantly from PrP^C glycoforms, though there is a slight difference in the glycoform ratio (Fig. 1B, lanes 2–4 and 6–8). Minimal reactivity is observed with either antibody with vector-transfected lysates (Figure 1B, lanes 1 and 5). Quantification of full length and truncated forms after normalizing with β -actin shows similar expression levels in all three cell lines (data not shown). Morphological evaluation by immunostaining non-permeabilized cells with 3F4 shows similar distribution of PrP^C, PrP^{232R}, and PrP^{232T} at the plasma membrane (Figure 1C, panels 1–3), 70% of which is released into the medium in the presence of phosphatidylinositol specific phospholipase C (PI-PLC) that cleaves the GPI anchor (data not shown).

The above results indicate that majority of PrP^{232R} and PrP^{232T} is processed normally in neuroblastoma cells, ruling out the possibility these mutations interfere with GPI anchor addition.

Normal and mutant PrP forms lacking the N-SP are translocated into the ER

To investigate if the GPI-SP mediates ER translocation of PrP, the conventional ER targeting N-SP of PrP comprising of residues 1–22 was deleted, and PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) were expressed in human neuroblastoma cells (Figure 2A). Acquisition of core glycans and transport to the plasma membrane were used as indicators of successful ER translocation. Accordingly, evaluations were carried out within 30 minutes of synthesis and at steady state.

To follow recently synthesized PrP, cells expressing PrP^C, PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) were radiolabeled with *Tran*³⁵S-methionine for 30 minutes, and labeled PrP was immunoprecipitated from lysates with 3F4 followed by SDS-PAGE-fluorography. As reported previously (Gu et al., 2002), PrP^C is localized in the ER within 30 minutes of synthesis and resolves into three distinct bands representing the unglycosylated and two core-glycosylated forms migrating at 27, 30, and 32 kDa respectively (Figure 2B, lane 1). Surprisingly, PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) also show core-glycosylated PrP bands, confirming ER translocation despite the absence of N-SP (Figure 2B, lanes 2–4). Similarly treated mock-transfected M17 cells do not show detectable PrP signal (Figure 2B, lane 5). Whether the GPI-SP is cleaved or GPI anchor added to Δ N-SP forms following ER translocation is unclear from our data, since the slightly slower migration of these

forms compared to PrP^C may reflect either absence of the GPI anchor or presence of uncleaved GPI-SP (Gu et al., 2003).

The above results confirm that PrP lacking the N-SP is translocated into the ER lumen. Subsequent investigations were therefore focused on evaluating if the GPI-SP mediates ER translocation of PrP, and whether mutations in this region modulate this function.

GPI-SP of PrP functions as an alternate ER-targeting signal

As a first step towards confirming the role of GPI-SP in ER targeting, this region was ligated to the N-terminus of green fluorescent protein (GFP) (Figure 3A), and the cellular distribution of GPI-SP^{GFP} was evaluated in transfected M17 cells. A comparison of intracellular and secreted pools using Western blot analysis shows mainly intracellular localization of GFP with minimal secretion into the medium (Figure 3B, lanes 1 and 3). In contrast, ~40% of GPI-SP^{GFP} is secreted into the medium (Figure 3B, lanes 2 and 4).

Since GPI-SP is normally attached to the C-terminus of proteins, cellular localization of GFP tagged with GPI-SP at the C-terminus (GFP^{GPI-SP}) was assessed using co-localization with the ER marker protein protein disulfide isomerase (PDI) as an indication of successful ER translocation. Thus, cells expressing GFP^{GPI-SP} were immunostained with PDI followed by TRITC-tagged secondary antibody and observed under a confocal microscope (Figure 3C). In addition to a prominent green signal in the nucleus (Figure 3C, panel 1), a significant amount of GFP^{GPI-SP} is noted in vesicular structures that co-localize with PDI (Figure 3C, panels 1–3, arrows).

To characterize the signaling function of GPI-SP further, N-SP and GPI-SP were ligated to the N-terminus of GFP^{GPI-SP} (N-SPGFP^{GPI-SP} and GPI-SPGFP^{GPI-SP} respectively), and efficiency of ER translocation was evaluated by immunostaining with anti-GFP antibody followed by anti-mouse-TRITC. The latter approach enables identification of transfected GFP regardless of autofluorescence (Figure 3D). Both N-SPGFP^{GPI-SP} and GPI-SPGFP^{GPI-SP} are detected on the plasma membrane and in the Golgi region of transfected cells (Figure 3D, panels 1–4). However, if N-SP is ligated to the C-terminus of GFP, only a small amount of GPI-SPGFP^{N-SP} is translocated into the ER as evidenced by co-localization with PDI (Figure 3D, panels 5–9). In the absence of GPI-SP at the N-terminus, all of GFP^{N-SP} accumulates in the cytosol in aggregate-like structures (Figure 3E, panels 1–3, arrow).

To rule out the involvement of transmembrane domain (TM) of PrP in mediating ER translocation, point mutation G123P was introduced in PrP^C lacking the N-SP (PrP^{123P}(ΔN-SP)) to abolish synthesis of transmembrane PrP forms (Hegde et al., 1998a) (Figure 4A). Translocation of PrP^{123P}(ΔN-SP) into the ER was evaluated using metabolic labeling. Thus, cells expressing PrP^C, PrP^C(ΔN-SP), and PrP^{123P}(ΔN-SP) were labeled with *Tran*³⁵S-methionine for 15 minutes and PrP was immunoprecipitated with 3F4. An aliquot of PrP^{123P}(ΔN-SP) sample was deglycosylated with PNGase-F to confirm the presence of N-linked glycans, and all samples were fractionated by SDS-PAGE followed by fluorography. As expected, PrP^C resolves into unglycosylated and core-glycosylated forms migrating between 27 and 32 kDa (Figure 4B, lane 1). Notably, PrP^C(ΔN-SP) and PrP^{123P}(ΔN-SP) show partially glycosylated forms that co-migrate with unglycosylated PrP following deglycosylation with PNGase-F (Figure 4B, lanes 2–4). The relative paucity of core-glycosylated forms of PrP^C(ΔN-SP) and PrP^{123P}(ΔN-SP) compared to PrP^C indicates a lag in ER translocation and/or maturation following synthesis. This lag becomes more apparent when the duration of pulse is reduced from 30 to 15 min (compare Figures 2B and 4B).

Together, the above results demonstrate that the GPI-SP of PrP is an efficient co- and post-translational ER targeting signal for PrP, an efficient GPI addition signal, and an inefficient

post-translational ER targeting signal for GFP. These results also highlight the predilection of N-SP to induce aggregation of GFP in the cytosol if not translocated co-translationally into the ER. When considered in the context of PrP, molecules that engage with the ER translocon unsuccessfully and therefore lack the N-SP (Hölscher et al., 2001; Rane et al., 2004) would undergo successful post-translational ER translocon mediated by the GPI-SP. Others that contain uncleaved N-SP would most likely aggregate in the cytosol.

PrP^{232R} and PrP^{232T} are linked to the plasma membrane in the C_{tm} orientation

To assess the transport of Δ N-SP forms to the cell surface, intracellular, plasma membrane, and secreted pools of PrP^C, PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) were evaluated. Quantitative assessment of PrP^C in the intracellular, surface, and accumulated secreted pool (collected after 18 hours) shows a distribution of 55, 45, and 80% respectively (Figure 5A, lanes 1, 5 and 9, and Figure 5B and 5C). In contrast, PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) reveal only the unglycosylated form at steady state that shows differential cellular distribution in the three cell lines. Thus, majority of PrP^C(Δ N-SP) is localized in the intracellular pool, whereas PrP^{232R}(Δ N-SP) and PrP^{232T}(Δ N-SP) are detected increasingly on the surface and in the secreted pool (Figure 5A, lanes 2–4, 6–8, and 10–12). Quantitative estimation of intracellular and surface pools reveals a distribution of 72 and 28% for PrP^C(Δ N-SP), 50 and 50% for PrP^{232R}(Δ N-SP), and 45 and 55% for PrP^{232T}(Δ N-SP) (Figure 5B). Secreted pool collected over 18 hours represents 38, 42, and 78% of the total cellular pool of PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) respectively (Figure 5C) (the sum total of three pools is >100 since the secreted pool contains proteins collected over an 18 hour period). Re-probing with anti-calnexin antibody shows a detectable signal only in the intracellular sample, excluding possible contamination of cell surface proteins with the intracellular pool (Figure 5A, lanes 1–8). Similar intensity of calnexin signal in PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) samples indicates equivalent protein loading, providing confidence that the differences noted above are not an artifact of protein loading (Figure 5A, lanes 2–4).

In a previous report, post-translational ER translocation of PrP in an *in vitro* system has been shown to result in increased synthesis of potentially pathogenic C_{tm}PrP form (Hölscher et al., 2001). To evaluate the orientation of PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP) and PrP^{232T}(Δ N-SP) at the plasma membrane, transfected cells were sequentially immunoreacted with 8H4 and 8B4 to visualize specific epitopes in the C- and N-terminal regions of PrP using green and red fluorophores respectively. Under these conditions, full-length PrP^C at the plasma membrane can be distinguished from C_{tm} and N_{tm} PrP forms based on dual reactivity of PrP^C with both antibodies (yellow, *), and single reactivity of C_{tm}PrP with 8H4 (green, arrow) and N_{tm}PrP with 8B4 (red, arrow-head) (Figure 6A, schematic). Thus, fixed cells were immunoreacted with 8H4-anti-mouse-FITC followed by mild permeabilization with Triton X-100 and immunoreaction with biotin-tagged 8B4 and Texas-red-streptavidin. As expected, full length PrP^C at the plasma membrane immunoreacts with 8H4 (arrows) and 8B4 (arrow-heads) and appears yellow (Figure 6A, panels 1–3, stars). In contrast, PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP) and PrP^{232T}(Δ N-SP) show strong reactivity with 8H4 (arrows) and limited reaction with 8B4 (arrow-heads), though strong 8B4 reactivity is detected intracellularly (Figure 6A, panels 4–12). In addition, vesicular structures reacting with both antibodies are detected in PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP) and PrP^{232T}(Δ N-SP) expressing cells (Figure 6A, panels 4–12).

Exclusive reactivity with 8H4 in non-permeabilized cells in certain areas of the plasma membrane (panels 4–12, arrow) suggests either the presence of N-terminally truncated fragments, or C_{tm} orientation of PrP that exposes only the C-terminus on the exoplasmic face of the plasma membrane. However, the former possibility can be ruled out since only full-length PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP) and PrP^{232T}(Δ N-SP) are detected on the plasma

membrane (Figure 5A above), suggesting Ctm orientation for the majority of N-SP deleted molecules on the plasma membrane. It is notable that the amount of PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) far exceeds PrP^C(ΔN-SP), most of which is retained intracellularly (Figure 6A, panels 4–6), corroborating our results in Figure 5A.

To further confirm the above results, cells expressing PrP^C, PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) were radiolabeled with *Tran*³⁵S-methionine for 2 hours followed by a chase of 1 hour, and PrP was immunoprecipitated from lysates with 3F4 followed by SDS-PAGE-fluorography. As expected, the three glycoforms of PrP^C migrating at 27, 32, and 37 kDa are detected with 3F4 (Figure 6B, lane 1). In contrast, PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) lysates show mostly the unglycosylated form migrating a little slower than unglycosylated PrP^C (Figure 6B, lanes 2–4). Notably, PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) lysates show a prominent 20 kDa band that is barely detectable in PrP^C and PrP^C(ΔN-SP) samples (Figure 6B, lanes 1 and 2, arrow). Since the 3F4 reactive 20 kDa fragment is believed to be a metabolic product of C^{tm}PrP (Gu et al., 2002, 2003), these results suggest an upregulation of this form in cells expressing PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP), more so in the latter.

Discussion

In this report, we demonstrate that the GPI-SP of PrP functions as an ER targeting signal for PrP and GFP in transfected human neuroblastoma cells. More importantly, pathogenic mutations PrP^{232R} and PrP^{232T} in the GPI-SP increase the stability and transport of mutant PrP to the plasma membrane, where it is linked in the Ctm orientation. Below we discuss this unusual feature of PrP biogenesis and its implications on the pathogenesis of familial CJD associated with point mutations in the GPI-SP.

The versatile nature of the GPI-SP of PrP

It is interesting to note that in addition to functioning as a signal for GPI anchor addition, the GPI-SP of PrP functions as an efficient ER targeting signal. Two key observations from our data support this conclusion: 1) PrP forms lacking the conventional ER targeting N-SP and disabled transmembrane domain are translocated into the ER with almost the same efficiency as N-SP containing PrP, and 2) ligation of GPI-SP of PrP to the N-terminus of GFP results in efficient ER translocation in transfected cells. Thus, precursor PrP has the potential for both co- and post-translational ER translocation, a rare example of a mammalian GPI-linked cell surface protein.

Based on the kinetics of core glycosylation of normal, N-SP deleted, and transmembrane domain ablated PrP within 30 minutes of synthesis, it appears that the N- and GPI-SP sequences mediate ER translocation with almost equal efficiency. Extrapolating these findings to the *in vivo* situation, PrP forms that fail to undergo co-translational ER translocation through the N-SP would have a second chance for post-translational translocation through the GPI-SP. The most likely candidates for this mode of translocation include cytosolic PrP forms that arise due to the ‘weak’ ER translocation function of N-SP, and can amount to as much as 20% of total synthesized PrP (Hölscher et al., 2001; Rane et al., 2004). The percentage of such molecules increases with ER stress (Orsi et al., 2006), adding to the total pool of PrP likely to undergo GPI-SP mediated translocation. A significant proportion of these molecules lack the N-SP due to its abortive interaction with the ER translocon (Rane et al., 2004), and would be perfect substrates for GPI-SP mediated ER translocation. It is interesting to note that although PrP translocated *via* the GPI-SP acquires core glycans, only unglycosylated forms are detected at steady state. It is possible that the absence of GPI anchor interferes with the stability and maturation of N-SP deleted forms, with the result that mostly unglycosylated PrP is released into the medium (Chesebro et al., 2005; Rogers et al., 1993). The transmembrane orientation

of PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP), and PrP^{232T}(ΔN-SP) with the plasma membrane appears to be transient in the absence of the GPI anchor, an observation that has been noted in a previous study (Stewart and Harris, 2003).

It is interesting to note that the GPI-SP of PrP functions as an efficient co-translational but inefficient post-translational ER targeting signal for GFP, an unrelated cytosolic protein used extensively for evaluating the sub-cellular targeting function of peptides. Different constructs of GFP containing N- and GPI-SP of PrP at the N- and C-terminal ends of GFP and *vice versa*, or only one of the signals at each end were tested. When the N- and GPI-SP were ligated to the N- and C-terminal ends (N-SPGFP^{GPI-SP}), or when the GPI-SP was ligated to both ends (GPI-SPGFP^{GPI-SP}), efficient translocation was observed and both forms were linked to the plasma membrane with the GPI anchor. Similarly, N- and GPI-SP sequences tethered to the N-terminus of GFP resulted in efficient ER translocation and secretion into the medium. However, ligation of N- or GPI-SP to the C-terminus (GFP^{N-SP} and GFP^{GPI-SP}) resulted in aggregation and inefficient ER translocation respectively. Thus, the GPI-SP is similar to N-SP in all necessary characteristics required for ER translocation, but the two peptides are not interchangeable since the N-SP cannot mediate GPI-anchor addition and induces aggregation in the cytosol. This observation contrasts with a previous report where the N-SP of Decay accelerating factor (DAF) mediates efficient GPI anchor addition when ligated to the C-terminus of DAF, though the ER translocation role of GPI-SP of DAF was not checked in this study (Caras et al., 1989). The relatively higher efficiency of ER translocation of PrP lacking the N-SP compared to GFP^{GPI-SP} is probably due to the presence of additional ER targeting signals in PrP, or a difference in the overall biochemical and biophysical characteristics of the two proteins. However, the former possibility is unlikely since PrP lacking the N- and GPI-SP is not translocated into the ER and accumulates in the cytosol of different cell lines and transgenic mouse lines (Ma et al., 2002, our unpublished observations). It is therefore likely that in addition to the ER targeting signal, substrate and its interaction with other factors, both *cis*- and *trans*-acting, are important for ER translocation (Hegde et al., 1998b; Ott and Lingappa, 2002, 2004).

Implications for disease pathogenesis

How might mutations in the GPI-SP induce neurotoxicity? Our data do not answer this question conclusively, but provide clues about how such mutations might compromise cell health. First, our data indicate that mutations in the GPI-SP increase the amount of potentially neurotoxic C^{tm}PrP on the plasma membrane and in the medium. C^{tm}PrP is known to be neurotoxic (Hegde et al., 1999; Chabry et al., 2003), though the mechanism by which it induces such a signal remains unclear. Second, post-translationally translocated PrP is likely to contain uncleaved N-SP in some molecules that is likely to induce cytotoxicity by interacting with the ER membrane (Heller et al., 2003), and mutations in the GPI-SP increase the proportion of this fraction. Third, ER stress has been reported to weaken co-translational translocation of PrP in transfected cells and primary neurons (Orsi et al., 2006), thereby increasing the proportion of PrP that could undergo post-translational translocation. And finally, complex glycosylation of post-translationally translocated PrP is inhibited, an event that promotes conversion to the aggregated, cytotoxic form (Winklhofer et al., 2003).

Several observations from our data suggest that post-translationally translocated PrP is inserted in the lipid bilayer in the C^{tm} orientation. First, selective immunoreaction of the plasma membrane with antibodies specific for C- and N-terminal regions of PrP shows reactivity mainly for the C-terminus although full length PrP is detected by biotinylation. Second, radiolabeling for two hours followed by a chase of one hour shows the increasing presence of 3F4 reactive 20 kDa C-terminal fragment that is barely detectable in PrP^C cells, and increases in intensity in PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) cells. This fragment is believed to arise

from proteolytic cleavage of C^{tm} PrP at the ER membrane (Gu et al., 2002, 2003). And finally, limited PK digestion of microsomes prepared from cells expressing PrP^C(Δ N-SP) spares the 3F4 reactive 20 kDa C-terminal fragment of PrP that is believed to be confirmatory evidence for the C^{tm} orientation, and the amount of this fragment increases in cells expressing PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) (unpublished observations). Whether cytotoxicity is initiated by C^{tm} PrP, uncleaved N-SP of post-translationally translocated PrP, aggregation or secretion of unglycosylated PrP, or by other undefined mechanisms remains an unresolved question.

In conclusion, our data provide convincing evidence for the ER targeting function of the GPI-SP of PrP, and suggests the involvement of C^{tm} PrP in neurotoxicity associated with familial CJD segregating with mutations in the GPI-SP. Although the mechanism of cytotoxicity in these cases remains unclear, our data highlight the unusual biogenesis of PrP and its implications on the pathobiology of prion disorders.

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Figure 1. Steady state expression of PrP^C, PrP^{232R} and PrP^{232T} in M17 cells

(A) Diagrammatic representation of precursor and post-translationally processed PrP^C, PrP^{232R} and PrP^{232T}. (B) Lysates of cells expressing vector, PrP^C, PrP^{232R} and PrP^{232T} were immunoreacted with 3F4, and the membrane was re-probed with 8H4 followed by β -actin. PrP^C lysates show the expected unglycosylated, mono- and di-glycosylated forms of PrP with both 3F4 and 8H4 (lanes 2 and 6). Similar glycoforms are detected in PrP^{232R} and PrP^{232T} lysates though the ratio is altered (lanes 3, 4, 7, 8). No reaction is detected in vector transfected lysates (lanes 1 and 5). (C) Immunoreaction of non-permeabilized cells with 3F4 followed by anti-mouse FITC shows normal distribution of PrP^C, PrP^{232R} and PrP^{232T} on the cell surface. Bar: 10 μ m.

Figure 2. PrP forms lacking the N-SP are core-glycosylated

(A) Diagrammatic representation of precursor and post-translationally processed PrP^C, PrP^{232R} and PrP^{232T} lacking the N-SP. (B) PrP^C, PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) cells were pulsed with *Tran*³⁵S-methionine for 30 min and radiolabeled PrP was isolated by immunoprecipitation with 3F4 and analyzed by SDS-PAGE-fluorography. PrP^C resolves as unglycosylated and two core-glycosylated forms migrating at 27, 30, and 32 kDa respectively (lane 1). Similar core glycosylated forms are immunoprecipitated from PrP^C(Δ N-SP), PrP^{232R}(Δ N-SP), and PrP^{232T}(Δ N-SP) samples despite the absence of N-SP, though the glycoform ratio is altered (lanes 1–4). No PrP signal is detected in similarly processed mock-transfected immunoprecipitates (lane 5).

Figure 3. GPI-SP of PrP is an ER targeting signal

(A) Diagrammatic representation of GPI-SP ligated to the N-terminus of GFP. (B) Overnight cultures of cells expressing GFP or $\text{GPI-SP}^{\text{GFP}}$ were processed to estimate cell associated and secreted pools of GFP. Immunoblotting of lysate and medium samples with anti-GFP shows the expected intracellular localization of GFP with minimal signal in the medium (lanes 1 and 3). In contrast, a significant amount of $\text{GPI-SP}^{\text{GFP}}$ is secreted in the medium (lanes 2 and 4). ($\text{GPI-SP}^{\text{GFP}}$ migrates slower than GFP in the lysates sample due to the presence of GPI-SP, which is cleaved in the secreted protein). (C) Cells expressing $\text{GFP}^{\text{GPI-SP}}$ were permeabilized and immunoreacted with anti-PDI-anti-mouse-TRITC. Limited co-localization of GFP is seen with PDI, indicating translocation into the ER. In addition, nuclear location of untranslocated $\text{GFP}^{\text{GPI-SP}}$ is observed (panels 1–3). Bar: 10 μm . (D) Cells expressing $\text{N-SP}^{\text{GFP}}\text{GPI-SP}$, $\text{GPI-SP}^{\text{GFP}}\text{GPI-SP}$, and $\text{GPI-SP}^{\text{GFP}}\text{N-SP}$ were either fixed and processed (panels 1, 3, and 5), or permeabilized with Triton X-100 (panels 2, 4, and 6) before immunostaining with anti-GFP followed by anti-mouse-TRITC. Cells expressing $\text{N-SP}^{\text{GFP}}\text{GPI-SP}$ and $\text{GPI-SP}^{\text{GFP}}\text{GPI-SP}$ show uniform expression of these proteins on the plasma membrane (panels 1 and 3) and limited reaction in the Golgi area following permeabilization (panels 2 and 4). In contrast, $\text{GPI-SP}^{\text{GFP}}\text{N-SP}$ is absent from the plasma membrane and is detected in intracellular vesicular structures and as small aggregates (panels

5–6). A significant proportion of vesicles co-localize with PDI (panels 7–9). Bar: 10 μ m. (E) GFP^{N-SP} accumulates exclusively in aggresome-like structures (panels 1–3). Bar: 10 μ m.

Figure 4. The transmembrane domain of PrP does not mediate ER targeting

(A) A diagrammatic representation of precursor and post-translationally processed N-SP deleted PrP^C and PrP^{123P}. (B) Following a 15 min pulse with *Tran*³⁵S-methionine, PrP was immunoprecipitated from PrP^C, PrP^C(ΔN-SP) and PrP^{123P}(ΔN-SP) lysates with 3F4 and resolved by SDS-PAGE-fluorography. As expected, PrP^C resolves into core-glycosylated forms migrating between 27 and 32 kDa (lane 1). PrP^C(ΔN-SP) and PrP^{123P}(ΔN-SP), on the other hand, show unglycosylated and partially glycosylated forms that migrate with unglycosylated PrP following deglycosylation with PNGase-F (lanes 2–4).

Figure 5. Mutations in the GPI-SP influence the stability and transport of PrP

(A) Cells expressing PrP^C, PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) were cultured in serum free medium for 18 h and secreted proteins were collected. Subsequently, cell surface proteins were biotinylated, separated from the intracellular pool using streptavidin beads, and the intracellular, surface, and secreted pools were processed for Western blotting and probed with 3F4. PrP^C resolves into three distinct glycoforms migrating between 27–37 kDa in the intracellular, cell surface, and secreted pools (lanes 1, 5, and 9). In contrast, PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP), and PrP^{232T}(ΔN-SP) comprise mostly of unglycosylated forms in all three pools (lanes 2–4, 6–8, 10–12). Re-probing with anti-calnexin antibody shows a detectable signal only in the intracellular sample. (B) Quantitative estimation of intracellular and cell surface pools shows a distribution of 55 and 45% for PrP^C, 72 and 28% for PrP^C(ΔN-SP), 50 and 50% for PrP^{232R}(ΔN-SP), and 45 and 55% for PrP^{232T}(ΔN-SP) respectively. Data represents mean ±SD of three independent evaluations. (C) Secreted PrP collected over 18 h represents 80, 38, 42, and 78% of total PrP^C, PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP), and PrP^{232T}(ΔN-SP) respectively. Data represents mean ±SD of three independent evaluations.

Figure 6. (A) PrP forms lacking the N-SP assume C_{tm} orientation in the lipid bilayer

Cells expressing PrP^C, PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) were fixed with paraformaldehyde and immunoreacted with 8H4-anti-mouse-FITC (green). Subsequently, plasma membrane was permeabilized with mild triton treatment and cells were reacted with biotin-tagged 8B4 followed by Texas-red conjugated streptavidin (red). A yellow reaction is detected on PrP^C cells, indicating the presence of full-length PrP containing both epitopes on the cell surface (panels 1–2, *). Green reaction on the plasma membrane of PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) cells indicates the presence of C_{tm}PrP with exposed C-terminus and intracellular N-terminus (panels 4–12, arrow). Predominantly intracellular red reaction indicates inefficient transport of PrP to the plasma membrane (panels 4–12, arrow-

head), though limited reaction is detected on the plasma membrane that co-localizes with the green signal (panels 4–12, *). Inserts magnified 4x. Bar: 10µm. **(B)**) Cells expressing PrP^C, PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP), and PrP^{232T}(ΔN-SP) were pulsed with *Tran*³⁵S-methionine for 2 h and chased for 1 h. Labeled PrP was immunoprecipitated with 3F4 and analyzed by SDS-PAGE-fluorography. Labeled PrP^C resolves as three distinct glycoforms as expected (lane 1), while PrP^C(ΔN-SP), PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) show mainly the unglycosylated full length form and a 20 kDa fragment that is significantly more in PrP^{232R}(ΔN-SP) and PrP^{232T}(ΔN-SP) lysates compared to PrP^C and PrP^C(ΔN-SP) lysates.