Glycosylation influences cross-species formation of protease-resistant prion protein

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A key event in the transmissible spongiform encephalopathies (TSEs) is the formation of aggregated and protease-resistant prion protein, PrP-res, from a normally soluble, protease-sensitive and glycosylated precursor, PrP-sen. While amino acid sequence similarity between PrP-sen and PrP-res influences both PrP-res formation and cross-species transmission of infectivity, the influence of co- or post-translational modifications to PrP-sen is unknown. Here we report that, if PrP-sen and PrP-res are derived from different species, PrP-sen glycosylation can significantly affect PrP-res formation. Glycosylation affected PrP-res formation by influencing the amount of PrP-sen bound to PrP-res, while the amino acid sequence of PrP-sen influenced the amount of PrP-res generated in the post-binding conversion step. Our results show that in addition to amino acid sequence, co- or post-translational modifications to PrP-sen influence PrP-res formation *in vitro*. *In vivo*, these modifications might contribute to the resistance to infection associated with transmission of TSE infectivity across species barriers.

Keywords: glycosylation/prion protein/PrP/scrapie

Introduction

The transmissible spongiform encephalopathies (TSEs) are a group of rare, transmissible and neurodegenerative diseases of unusual etiology. They include scrapie in sheep and goats, kuru and Creutzfeldt–Jakob disease (CJD) in man and bovine spongiform encephalopathy (BSE) in cattle. A critical part of TSE pathogenesis involves the mammalian prion protein (PrP). PrP is a cell surface glycoprotein present in a variety of different tissues (Oesch *et al.*, 1985; Caughey *et al.*, 1988; Bendheim *et al.*, 1992) and expressed at particularly high levels in the brain (Bendheim *et al.*, 1992). Normal PrP (PrP-sen) is both detergent soluble and sensitive to digestion with proteinase K. During the course of TSE infection, PrP-sen is converted to an abnormal, detergent-insoluble form that is partially resistant to proteinase K (PrP-res) and which accumulates to high levels in the lymphoreticular and central nervous systems of the infected host.

Initial transmission of the TSE agent between different animal species is inefficient and often requires several serial passages before the infectious agent becomes

adapted to the new host. This initial species-specific resistance to TSE infection is known as the species barrier. Species barriers in TSE diseases are of particular importance given the probability that BSE has infected humans in the UK leading to a new form of human TSE called variant CJD (vCJD) (Will *et al.*, 1996). In the USA, there is concern that chronic wasting disease (CWD), a TSE identified in wild and captive populations of deer and elk in several western states (Williams and Young, 1980, 1982), might cross species barriers and potentially expose the human population to a new TSE infection. Thus, it is important to understand the mechanisms underlying species barriers to infection in the TSEs and to determine how to prevent cross-species transmission of TSE infectivity.

One factor which has been shown to influence TSE species barriers is the primary amino acid sequence of PrP-sen (Scott *et al.*, 1989; Prusiner *et al.*, 1990). In particular, homology between PrP-sen and PrP-res in the central portion of the PrP protein is important in the transmission of TSE infectivity between species (Scott *et al.*, 1992, 1993). In both cell-free and tissue culture-based assays of PrP-res formation, even single amino acid sequence differences between PrP-sen and PrP-res within this region have been shown to influence the efficient generation of protease-resistant PrP (Priola *et al.*, 1994; Kocisko *et al.*, 1995; Priola and Chesebro, 1995; Raymond *et al.*, 1997). These data suggest that one way in which the PrP amino acid sequence might influence TSE pathogenesis is by influencing the efficiency with which PrP-res is formed, thus altering the tempo of the disease.

Although there is a correlation between PrP sequence homology and cross-species transmission of TSE infectivity, the importance of co- or post-translational modifications to PrP-sen on species barriers to infection is unclear. PrP-sen is modified co-translationally by the addition of a glycosyl-phosphatidylinositol (GPI) membrane anchor (Stahl *et al.*, 1987), and post-translationally by the addition of N-linked complex glycans at two asparagine residues near the C-terminus (Hope *et al.*, 1986; Haraguchi *et al.*, 1989). The GPI anchor is thought to localize PrP in the appropriate membrane compartment for PrP-res formation to occur (Taraboulos *et al.*, 1995; Kaneko *et al.*, 1997). However, *in vitro* studies have demonstrated that the GPI anchor is not absolutely required for PrP-res formation (Rogers *et al.*, 1993; Kocisko *et al.*, 1994). While glycosylation can influence the biochemical properties of certain PrP-sen mutants, it also is not absolutely required for PrP-res formation in transgenic mice (DeArmond *et al.*, 1999), murine neuroblastoma cells (Taraboulos *et al.*, 1990; Korth *et al.*, 2000) or cell-free assay systems (Kocisko *et al.*, 1994).

We have demonstrated previously that homology between PrP-sen and hamster PrP-res at amino acid

residue 155 was important for the efficient formation of hamster PrP-res (Priola *et al.*, 2001). These studies were carried out using PrP-sen molecules that were largely unglycosylated and that lacked the GPI membrane anchor. Therefore, we asked how homology between PrP-sen and PrP-res at amino acid residue 155 would affect hamster PrP-res formation in the presence of glycosylation and/or the GPI anchor. In the present studies, we show that the presence of a GPI anchor in PrP-sen allowed for a more efficient formation of PrP-res. This effect did not appear to be species specific. In contrast, the presence of N-linked glycans in a non-homologous PrP-sen molecule significantly decreased the amount of protease-resistant PrP generated by hamster PrP-res. Glycosylation affected PrP-res formation by influencing the amount of non-homologous PrP-sen that bound to PrP-res. Glycosylation did not, however, appear to influence how much of the bound, non-homologous PrP-sen subsequently was converted to PrP-res. Our results demonstrate that post-translational modifications to PrP-sen can influence the cross-species formation of PrP-res. Thus, differences in PrP molecules other than at the level of primary amino acid sequence may contribute to species barriers to infection in the TSE diseases.

Results

Factors other than amino acid sequence influence the efficient formation of protease-resistant PrP

A cell-free assay of PrP-res formation is available that models the species-specific formation of PrP-res (Kocisko *et al.*, 1995). Although there is no evidence that TSE infectivity is generated (Hill *et al.*, 1999), PrP-res formation in this assay has been used to model TSE species barriers at the molecular level and has been shown to correlate well with *in vivo* transmissibility data (Raymond *et al.*, 1997). Using the cell-free conversion assay, we previously demonstrated that hamster PrP-res converted Mo3F4 PrP-sen containing a hamster-specific asparagine at residue 154 to protease resistance as efficiently as wild-type HaPrP (Priola *et al.*, 2001). Thus, this residue was important in the cross-species formation of protease-resistant PrP by hamster PrP-res. However, the Mo3F4 recombinant molecules used were largely unglycosylated and contained no GPI anchor. In order to determine if a hamster-specific asparagine at residue 154 was also sufficient for hamster PrP-res to convert fully glycosylated, GPI anchor-positive Mo3F4 PrP-sen to a protease-resistant form, a hamster-specific asparagine was substituted for the mouse-specific tyrosine in glycosylated, GPI anchor-positive Mo3F4 PrP-sen. The resultant clone, Mo3F4-N154, was radiolabeled and formation of protease-resistant, radiolabeled Mo3F4-N154 by hamster PrP-res was assayed using the cell-free conversion assay.

Similarly to our previously published results (Priola *et al.*, 2001; Table I), the conversion of wild-type HaPrP by hamster PrP-res was more efficient than the conversion of Mo3F4 PrP-sen by hamster PrP-res (Figure 1A, compare lanes 4 and 6). This demonstrated the species specificity of the reaction and indicated that glycosylation and/or the GPI anchor did not significantly influence the amount of wild-type HaPrP converted to PrP-res (Table I). However, there was a qualitative difference between the

Table I. Formation of protease-resistant PrP by hamster PrP-res from PrP-sen molecules with or without the GPI anchor

PrP-sen	GPI anchor ^a	
	Present	Absent ^b
Mo3F4	11 ± 1 (<i>n</i> = 10) ^c	9 ± 2 (<i>n</i> = 11) ^c
Mo3F4-N154	15 ± 1 (<i>n</i> = 11) ^c	31 ± 5 (<i>n</i> = 13)
HaPrP	35 ± 3 (<i>n</i> = 8)	28 ± 6 (<i>n</i> = 12)

^aThe presence or absence of the GPI membrane anchor. Numbers shown indicate the percentage of PrP-sen converted to PrP-res and represent the mean ± SEM (*n* is sample size).

^bData from Priola *et al.* (2001).

^cSignificantly different from HaPrP control. *P* < 0.001 by one-way ANOVA repeated measures test with Dunnett's post-test.

reactions that was species specific. In the reaction with either Mo3F4 PrP-sen or Mo3F4-N154, the glycosylated 34–40 kDa forms of PrP-res were under-represented when compared with the wild-type HaPrP reaction (Figure 1A, arrow). Quantitation of the reaction products not only confirmed that glycosylated PrP-res forms were significantly fewer for Mo3F4 and Mo3F4-N154 when compared with HaPrP but also demonstrated that significantly more of the 25 kDa unglycosylated PrP-res form was present (Figure 1B, right panel). These differences were not due to differences in the input level of the different PrP-sen glycoforms, which were the same for all constructs tested (Figure 1B, left panel). This suggested that, in the context of the Mo3F4 amino acid sequence, glycosylated PrP-sen was converted by hamster PrP-res less efficiently than unglycosylated forms of PrP-sen.

Conversion of the mutant Mo3F4-N154 by hamster PrP-res yielded a pattern of protease-resistant products similar to that of newly formed protease-resistant HaPrP (Figure 1A, compare lanes 5 and 6), although, as detailed above, the 34–40 kDa forms of PrP-res were present at slightly lower levels (Figure 1B). Quantitatively, however, Mo3F4-N154 was converted to protease resistance less efficiently than wild-type HaPrP and only slightly better than Mo3F4 PrP-sen (Figure 1A, compare lanes 4–6). This was in contradiction to our previously published data (Priola *et al.*, 2001), which demonstrated that a hamster-specific asparagine at amino acid residue 154 allowed mouse PrP-sen without a GPI anchor to be converted efficiently to protease-resistance by hamster PrP-res (Table I). Recombinant PrP-sen molecules expressed without the GPI anchor are largely unglycosylated whereas GPI anchor-positive PrP-sen is mostly glycosylated (Kocisko *et al.*, 1994). The primary differences between the Mo3F4-N154 clone in the current study and our previous study (Priola *et al.*, 2001) are the presence of the GPI anchor and the level of glycosylation. Thus, our data suggested that in addition to amino acid sequence homology, co- or post-translational modifications to PrP-sen could influence species-specific formation of protease-resistant PrP.

Removal of the GPI anchor decreases protease-resistant PrP formation

In order to determine if the presence of the GPI anchor could account for the observed difference in conversion

Fig. 1. Sequence homology to hamster PrP-res at Mo3F4 PrP-sen codon 154 influences formation of protease-resistant PrP. Cell-free conversion of radioimmunoprecipitated Mo3F4, Mo3F4-N154 or HaPrP by 263K-derived hamster PrP-res. (A) A representative experiment is shown. The name of the construct is indicated above each lane. Lanes 1–3 show 1/10 of the total reaction in the absence of PK treatment (–PK) and demonstrate that the input amount of radiolabeled PrP-sen was similar for each reaction. Lanes 4–6 show the remaining 9/10 of the reaction following digestion with PK (+PK). The brackets designate the bands that were quantified and indicate the fully glycosylated, partially glycosylated and unglycosylated forms of PrP. Molecular mass markers in kDa are indicated on the right side of the panel. The arrow indicates the fully glycosylated form of PrP-res, which migrates from ~34 to 40 kDa. (B) Quantitation of glycosylated 30–40 kDa (open circle) versus 25 kDa unglycosylated (closed circle) forms of PrP-sen (left panel) and PrP-res (right panel) from the experiments represented in (A). The data shown represent the mean $\pm$ SD for several independent experiments and multiple samples ($n = 6$ –12). Where standard deviation bars are not visible, the deviation from the mean was $<0.5\%$. * $P < 0.001$ when compared with HaPrP using Bonferroni's multiple comparison test.

levels, radiolabeled GPI-anchored PrP-sen molecules were removed from the cell surface using phosphatidylinositol-specific phospholipase C (PIPLC) and isolated by immunoprecipitation. The resultant PrP-sen molecules were cleaved at the phosphatidylinositol moiety of the GPI anchor but were still fully glycosylated. These molecules were then tested in the cell-free conversion assay with hamster PrP-res. Cleavage of the GPI anchor with PIPLC did not increase the amount of Mo3F4 or Mo3F4-N154 converted to protease resistance when compared with

Fig. 2. Treatment of recombinant PrP-sen with PIPLC or tunicamycin influences formation of protease-resistant PrP. (A) Radiolabeled, recombinant PrP-sen was released from the cell surface using PIPLC and purified by immunoprecipitation as described in Materials and methods. The resultant PrP-sen is missing the phosphatidylinositol moiety of the GPI anchor. (B) Radiolabeled, recombinant PrP-sen was immunoprecipitated from cells that had been treated with tunicamycin as described in Materials and methods. The resultant PrP-sen is unglycosylated and migrates as a single band (lanes 1–3). For both panels, the name of the construct is indicated above each lane. Lanes 1–3 show 1/10 of the total reaction in the absence of PK treatment (–PK) and demonstrate that the input amount of radiolabeled PrP-sen was similar for each reaction. Lanes 4–6 show the remaining 9/10 of the reaction following digestion with PK (+PK). The brackets designate the bands that were quantified. Molecular mass markers in kDa are indicated on the right side of each panel.

HaPrP (Figure 2A). In fact, overall conversion levels significantly decreased for all constructs tested when compared with untreated controls (Figure 3). This suggested that an intact GPI anchor was actually beneficial to the formation of protease-resistant PrP. Therefore, the presence of a GPI anchor could not account for the observed decrease in conversion by hamster PrP-res of GPI anchor-positive Mo3F4-N154 versus GPI anchor-negative Mo3F4-N154.

Glycosylation influences the formation of protease-resistant PrP from heterologous PrP-sen

In order to determine if glycosylation influenced the conversion of Mo3F4-N154 to PrP-res, cells were radiolabeled in the presence of tunicamycin. The resultant PrP-sen, which was unglycosylated but still contained the GPI anchor, was then tested in the cell-free conversion assay. As expected from previous studies, unglycosylated PrP-sen was converted primarily to the predicted 17 kDa protease-resistant form as well as several smaller protease-resistant products (Figure 2B). While removal of the sugars had no effect on the overall conversion of homologous HaPrP, unglycosylated Mo3F4 PrP-sen was converted more efficiently than glycosylated Mo3F4 by hamster PrP-res (Figure 3). Removal of the sugars had the most dramatic effect on the formation of PrP-res from Mo3F4-N154. The level of PrP-res generated from unglycosylated Mo3F4-N154 was more than double that of fully glycosylated Mo3F4-N154 and was indistinguishable from the amount formed by either glycosylated or unglycosylated hamster PrP (Figures 2B and 3). These results were consistent with our previous data, which demonstrated that homology at amino acid residue 154 in

Mo3F4 (155 in HaPrP) was important in the efficient formation of protease-resistant PrP by hamster PrP-res. Overall, our results demonstrate that the conversion of recombinant Mo3F4 PrP-sen by hamster PrP-res was strongly influenced by PrP-sen glycosylation.

Glycosylation influences binding of heterologous PrP-sen to hamster PrP-res

The *in vitro* conversion of PrP-sen to protease resistance encompasses two broadly delineated steps: (i) binding of PrP-sen to PrP-res; and (ii) a post-binding, poorly under-

Fig. 3. Co- and post-translational modifications influence conversion of PrP-sen to protease resistance. The percentage conversion of recombinant PrP-sen to protease resistance by hamster PrP-res is shown. Data for untreated samples (hatched bars) are from the 'GPI anchor present' column of Table I. The data for PIPLC-treated (open bars, $n = 5-8$) and tunicamycin-treated samples (black bars, $n = 5-9$) are from several independent experiments. The percentage conversion was calculated as described in Materials and methods. Statistical comparisons were made between the treated and untreated samples for each group using the one-way ANOVA repeated measures test with Dunnett's post-test. Each column represents the mean while the error bars show the SEM. * $P = 0.05$; ** $P < 0.001$.

stood conversion event (Bessen *et al.*, 1995; DebBurman *et al.*, 1997; Horiuchi *et al.*, 1999). The presence of N-linked sugars could influence one or both of these events. Therefore, we assayed the influence of glycosylation on PrP-sen-PrP-res binding and the subsequent formation of protease-resistant PrP.

In order to determine whether N-linked sugars influenced the ability of PrP-sen to aggregate, Mo3F4, Mo3F4-N154 and HaPrP isolated from tunicamycin-treated or untreated cells were incubated in a cell-free reaction in the absence of PrP-res. As expected, in the absence of PrP-res, no PrP-sen was converted to protease resistance (Figure 4A) and only low levels of the input, radiolabeled PrP-sen pelleted (Figure 4B). Thus, the presence or absence of N-linked sugars did not significantly influence the ability of any of the PrP-sen molecules tested to form aggregates or convert to protease resistance in the absence of input PrP-res.

In order to determine the effect of glycosylation on binding and protease-resistant PrP formation in the presence of PrP-res, hamster 263K-derived PrP-res was added to the reaction and the experiment was repeated. The amount of PrP-sen which pelleted for the conversion reactions shown in Figure 5A is shown in Figure 5B. A significant amount of radiolabeled PrP-sen pelleted, indicating binding of PrP-sen to PrP-res as previously reported (Horiuchi *et al.*, 2000). The fully glycosylated forms (Figure 5B, arrow) of both Mo3F4 and Mo3F4-N154 PrP-sen were under-represented in the bound, pelleted fraction while the less glycosylated forms appeared to be over-represented (Figure 5B, lane 1 versus lane 3; lane 7 versus lane 9). In contrast, all forms of HaPrP were present at similar levels in both the bound and unbound fractions (Figure 5B, compare lanes 13 and 15). These results

Fig. 4. Recombinant deglycosylated PrP-sen does not pellet or acquire protease resistance in the absence of PrP-res. Radioimmuno-precipitated Mo3F4, Mo3F4-N154 or HaPrP from cells that had been treated with or without tunicamycin were incubated in the absence of PrP-res for 48 h at 37°C under the reaction conditions used for the cell-free conversion assay. The amount of PrP-sen converted to protease resistance (A) and the amount that pelleted (B) was assayed as described in Materials and methods. The lanes designated -PK show 1/10 of the total reaction in the absence of PK treatment and demonstrate that the input amount of radiolabeled PrP-sen was similar for each reaction. The lanes designated +PK show the remaining 9/10 of the reaction following digestion with PK. Whether or not the sample was from tunicamycin-treated cells is indicated. In (B), the fractions analyzed following centrifugation are shown above each lane: S = supernatant; W = pellet wash; P = pellet. The data for both panels are from the same experiment. Molecular mass markers in kDa are indicated on the right side of each panel.

Fig. 5. Binding and conversion of recombinant deglycosylated PrP-sen in the presence of hamster PrP-res. Radioimmunoprecipitated Mo3F4, Mo3F4-N154 or HaPrP from cells that had been treated with or without tunicamycin were incubated in the presence of hamster PrP-res for 48 h at 37°C under the reaction conditions used for the cell-free conversion assay. The amount of PrP-sen converted to protease resistance (**A**) and the amount that pelleted (**B**) was assayed as described in Materials and methods. The lanes designated -PK show 1/10 of the total reaction in the absence of PK treatment and demonstrate that the input amount of radiolabeled PrP-sen was similar for each reaction. The lanes designated +PK show the remaining 9/10 of the reaction following digestion with PK. Whether or not the sample was from tunicamycin-treated cells is indicated. The fractions analyzed after centrifugation are shown above each lane: S = supernatant; W = pellet wash; P = pellet. The data for both panels are from the same experiment. Molecular mass markers in kDa are indicated on the right side of each panel. Arrows = 34–40 kDa glycosylated PrP; asterisks = 33 and 30 kDa partially glycosylated PrP; closed circle = 25 kDa unglycosylated PrP.

suggested that hamster PrP-res bound most efficiently to the less glycosylated forms of Mo3F4 and Mo3F4-N154. Indeed, when the N-linked sugars were removed from either Mo3F4 or Mo3F4-N154 PrP-sen, both recombinants bound to hamster PrP-res similarly to wild-type HaPrP (Figures 5B and 6). Thus, the presence of N-linked glycans strongly influenced the ability of hamster PrP-res to bind to a PrP molecule derived from a different species.

The amount of bound PrP-sen converted to PrP-res was also determined. For each construct, the percentage of bound PrP-sen converted to PrP-res was the same regardless of whether or not the PrP-sen was glycosylated (Figure 6). For both HaPrP and Mo3F4-N154, the amount of PrP-res formed (Figure 6) was approximately equal to the amount of PrP-sen bound. Therefore, the lower amount of PrP-res generated from glycosylated versus unglycosylated Mo3F4-N154 was due solely to the presence of N-linked sugars affecting the amount of Mo3F4-N154 PrP-sen that bound to hamster PrP-res. In contrast, while almost all of the bound Mo3F4-N154 was converted to PrP-res, a little less than half of the Mo3F4 PrP-sen bound to PrP-res was converted to protease resistance (Figure 6). Thus, while glycosylation influenced the amount of PrP-sen bound, it was homology at residue 155 that determined the overall efficiency of conversion of the bound PrP-sen to PrP-res.

Discussion

PrP-res formation across species is strongly influenced by amino acid sequence homology between PrP-sen and PrP-res in the central portion of the PrP protein (Scott *et al.*, 1992, 1993). Our data provide the first evidence at the molecular level that glycosylation of PrP-sen, and not just the amino acid sequence, can also influence cross-species

Fig. 6. PrP-sen glycosylation influences the binding of hamster PrP-res to non-homologous PrP-sen molecules. The amount of PrP-sen converted to protease resistance and the amount that pelleted was quantified as described in Materials and methods for five independent experiments. In order to correct for non-specific pelleting of radiolabeled PrP-sen, the volume of radiolabeled PrP in the pellet in the absence of PrP-res (e.g. Figure 4, 'P' lanes) was subtracted from the volume of radiolabeled PrP in the pellet in the presence of PrP-res (e.g. Figure 5, 'P' lanes). The columns and error bars show the mean $\pm$ SEM, respectively. The unpaired Student's *t*-test was used to compare conversion (C) or binding (B) in untreated (hatched bars) versus tunicamycin-treated samples (black bars) for each construct. **P* = 0.02; ***P* = 0.0001.

formation of PrP-res. Mechanistically, we have shown that glycosylation can modulate PrP-res formation by significantly affecting the binding of heterologous PrP-sen and PrP-res molecules. Since binding of PrP-sen to PrP-res precedes the formation of new protease-resistant PrP (Bessen *et al.*, 1995; DebBurman *et al.*, 1997; Horiuchi *et al.*, 1999), less efficient binding between heterologous PrP-sen and PrP-res molecules would result in less efficient PrP-res formation and, presumably, a delayed disease onset *in vivo*. Thus, our results suggest that regions of amino acid sequence homology between heterologous PrP-sen and PrP-res molecules cannot be the sole criterion

used to predict either how efficiently PrP-res is formed or how efficiently TSE infectivity can be transmitted across species barriers.

The fact that hamster PrP-res can bind homologous and heterologous PrP-sen similarly is consistent with previously reported data using largely unglycosylated, GPI anchor-negative PrP-sen (Horiuchi *et al.*, 2000). However, two observations in the current study suggest that glycosylation can affect PrP-res–PrP-sen binding in a species-specific manner. First, the binding of hamster PrP-res to Mo3F4 or Mo3F4-N154 PrP-sen increased 2- to 3-fold if the PrP-sen was unglycosylated. Conversely, removal of the sugars did not significantly affect the amount of HaPrP bound to hamster PrP-res (Figure 6). Secondly, when compared with the unbound fraction, fully glycosylated Mo3F4 and Mo3F4-N154 were clearly under-represented in the fraction of PrP-sen bound to hamster PrP-res while less glycosylated forms were over-represented (Figure 5B, arrows). In contrast, little or no difference was observed in the distribution of the different glycoforms between the unbound and bound fractions of HaPrP. These data suggest that, for glycosylated PrP-sen molecules, the primary sequence of PrP can affect the accessibility and/or affinity of PrP-res for a binding site or sites. For example, amino acid differences between mouse and hamster PrP-sen might lead to minor conformational changes that allow N-linked glycans partially to obscure the binding site in mouse PrP-sen used by hamster PrP-res. When the sugars are removed from mouse PrP-sen, the binding site becomes more accessible to hamster PrP-res and PrP-sen–PrP-res binding increases.

The conversion of PrP-sen to PrP-res is a sequential process that begins with an initial PrP-sen–PrP-res interaction followed by conversion of the bound PrP-sen to protease resistance (DebBurman *et al.*, 1997). The percentage of bound PrP-sen converted to PrP-res would therefore indicate the efficiency of the conversion reaction (Horiuchi *et al.*, 2000). While glycosylation clearly influenced the initial binding step, it did not appear to influence the conversion efficiency (Figure 6). It is important to note, however, that only overall conversion levels were assayed. Thus, it is still possible that individual PrP-sen glycoforms convert to protease resistance with different efficiencies. In fact, there is some evidence to suggest that this is the case. PrP-res isolated from the brains of animals infected with different TSE strains can be distinguished based on the relative amounts of the different PrP-res glycoforms (Collinge *et al.*, 1996; Parchi *et al.*, 1997; Somerville *et al.*, 1997). These differences could be explained if TSE strains converted the various PrP glycoforms with different efficiencies. Some tissue culture models of PrP-res formation have suggested that the less glycosylated forms of PrP-sen are converted preferentially to PrP-res (Caughey and Raymond, 1991; Korth *et al.*, 2000). Our data are consistent with this hypothesis. However, further studies will be necessary to determine whether or not individual PrP-sen glycoforms can actually be converted preferentially to protease resistance under the cell-free assay conditions used in the current report.

Although conversion efficiency was not influenced detectably by glycosylation, it appeared to be strongly influenced by the amino acid sequence of PrP. Regardless

of glycosylation, ~40% of the Mo3F4 PrP-sen bound to hamster PrP-res was converted to protease resistance. In contrast, essentially all of the Mo3F4-N154 bound to hamster PrP-res was converted to protease resistance (Figure 6). The only difference between Mo3F4 and Mo3F4-N154 is the substitution of the hamster-specific asparagine for the mouse-specific tyrosine at codon 154 (codon 155 in hamster PrP). This single amino acid substitution was sufficient to increase the efficiency of conversion of Mo3F4 by hamster PrP-res from ~40% to almost 100% (Figure 6). Thus, conversion efficiency was dependent upon homology between PrP-sen and PrP-res at codon 155, a residue we have identified previously as important in the formation of hamster PrP-res (Priola *et al.*, 2001).

The fact that all of the PrP-sen bound to PrP-res was converted is in contrast to previously published data showing that, even in a homologous conversion reaction, less than half of the PrP-sen bound is converted to protease resistance (Horiuchi *et al.*, 2000). There are several factors which may account for this difference. The current studies utilized low levels of guanidine hydrochloride (<1.0 M) and sarkosyl (1.25%) to enhance the conversion process. The previous studies utilized similar levels of sarkosyl but no guanidine hydrochloride (Horiuchi *et al.*, 2000). It is possible that the use of guanidine hydrochloride leads to a more efficient conversion by allowing PrP-sen to unfold partially and assume a more flexible conformation. Alternatively, guanidine hydrochloride may prevent PrP-sen from binding to the hypothesized 'non-convertible' binding sites (Horiuchi *et al.*, 2000), especially if these sites have a lower binding affinity. Finally, unlike the previous studies, our studies utilized GPI anchor-positive and fully glycosylated PrP-sen. Either of these modifications could influence protein conformation and/or stabilize protein structure (O'Conner and Imperiali, 1996).

A co-translational addition to PrP-sen, the GPI anchor, also had an effect on the conversion process. This effect, however, was not species specific. For all of the recombinant PrP-sen molecules tested, cleavage of the phosphatidylinositol moiety of the GPI anchor led to a decrease in the amount of PrP-res formed (Figure 3). Although this effect may be specific to the reaction conditions used, previous *in vitro* studies have suggested that localization of PrP-sen in the cell membrane via the GPI anchor is important in the formation of PrP-res in scrapie-infected tissue culture cells (Taraboulos *et al.*, 1995; Kaneko *et al.*, 1997). The current results are consistent with these studies and demonstrate that, even in the absence of cell membrane components, the GPI anchor is beneficial to the generation of protease-resistant PrP. The GPI anchor may be important not only in anchoring PrP in the cellular membrane but also in keeping PrP-sen in a preferred conformation, which in turn allows it to interact more efficiently with PrP-res during the conversion process.

Materials and methods

Reagents

The anti-hamster PrP-specific mouse monoclonal antibody 3F4 recognizes an epitope within hamster PrP which includes the hamster-specific methionines at positions 109 and 112 (Kascsak *et al.*, 1987;

Bolton *et al.*, 1991). Normal mouse PrP-sen is not recognized by the 3F4 antibody (Kascsak *et al.*, 1987). Substitution of the leucine and valine residues at the equivalent mouse positions (residues 108 and 111) with methionine results in the expression of the 3F4 epitope in mouse PrP (Chesebro *et al.*, 1993). In order to isolate recombinant PrP-sen from the 3F4-negative, endogenous mouse PrP-sen expressed in our cell lines, all of the recombinant hamster and mouse PrP-sen molecules utilized in this study expressed the 3F4 antibody epitope.

Clones

Mouse PrP-sen mutated to contain the 3F4 antibody epitope and a unique *NaeI* restriction endonuclease site (Mo3F4) and normal hamster PrP (HaPrP) with a unique *BsrEII* restriction endonuclease site have been described previously (Chesebro *et al.*, 1993; Priola and Chesebro, 1995). A Mo3F4 mutant containing a hamster-specific asparagine in place of the mouse-specific tyrosine at codon 154 was derived using a series of 10 overlapping oligonucleotides containing the desired mutation (Priola and Chesebro, 1995). These oligonucleotides spanned the region of Mo3F4 from the *NaeI* site to the *BsrEII* site (nucleotides 436–660). Following annealing of the 10 fragments, the resulting 224 bp DNA was subcloned at the *NaeI* and *BsrEII* restriction endonuclease sites of Mo3F4 cloned into a Bluescript vector (Stratagene, La Jolla, CA) from which the vector-specific *NaeI* site had been removed. Following confirmation by DNA sequencing, the mutant Mo3F4 PrP was subcloned into the retroviral expression vector pSFF. The resultant clone, Mo3F4-N154, was transfected into a 1:1 mixture of the retroviral packaging cell lines PA317 and Ψ 2 and expressed (Chesebro *et al.*, 1993; Caughey *et al.*, 1999). The uncloned Ψ 2/PA317 culture, in which >95% of the cells were positive by cell surface immunofluorescence for Mo3F4-N154 expression (data not shown), was used as a source of recombinant PrP-sen for the cell-free conversion assay. Ψ 2/PA317 cells expressing Mo3F4 or HaPrP have been described previously (Priola and Chesebro, 1998; Priola *et al.*, 2001).

Tunicamycin and PIPLC treatment of radiolabeled cells

Confluent 25 cm² flasks of a Ψ 2/PA317 cell culture expressing the desired recombinant PrP-sen were labeled with 1.5 mCi of [³⁵S]methionine/cysteine (Tran³⁵S, NEN) in the presence or absence of 2.5 µg/ml tunicamycin, or were treated with PIPLC as described previously (Kocisko *et al.*, 1994; Priola *et al.*, 1995). Radiolabeled recombinant PrP-sen was immunoprecipitated from cell lysates with the hamster-specific mouse monoclonal antibody 3F4 as described previously (Caughey *et al.*, 1999).

Cell-free conversion assay

The *in vitro* conversion of PrP-sen to protease-resistant PrP has been described (Kocisko *et al.*, 1994; Caughey *et al.*, 1999). Briefly, 200 ng of hamster PrP-res purified from brains of Syrian hamsters infected with the hamster scrapie strain 263K (Caughey *et al.*, 1999) was partially unfolded in 2.5 M guanidine hydrochloride and mixed with 20 000 c.p.m. (~2 ng) of radiolabeled, immunoprecipitated PrP-sen. The reaction was incubated in reaction buffer (0.75 M guanidine hydrochloride, 1.25% sarkosyl, 5 mM cetyl pyridinium chloride and 50 mM sodium citrate buffer pH 6.0) at 37°C for 2 days. After incubation, 1/10 of the reaction was precipitated in methanol (total PrP). The remaining 9/10 was treated with 12 µg/ml of proteinase K (PK) for 1 h at 37°C. PK was inactivated by the addition of protease inhibitors and the protein was methanol precipitated (PK-resistant PrP). Radiolabeled protease-resistant products were analyzed by SDS–PAGE. The amount of total radiolabeled PrP and protease-resistant PrP was determined using the Molecular Dynamics Storm Phosphor-Imager system. Bands were quantified in terms of the integrated peak volume and the percentage conversion calculated using the formula:

$$\text{Percentage conversion} = \frac{(\text{volume of PK-resistant PrP})}{(\text{volume of total PrP})(10)} \times 100$$

Binding assay

Cell-free conversion assays were prepared as described above except that two reactions (40 µl total) were used per reaction tube. After incubation at 37°C for 48 h, one reaction volume (20 µl) was removed, fractions were treated with or without PK, and the percentage conversion calculated as described above. The second reaction volume (20 µl) was spun at

14 000 r.p.m. at room temperature for 10 min and the supernatant transferred to a new tube. The pellet was washed briefly with 100 µl of wash buffer (1.25% sarkosyl, 50 mM sodium citrate pH 6.0, 5 mM cetyl pyridinium chloride) and spun at 14 000 g for 10 min at room temperature. The wash supernatant was removed to a new tube and 20 µl of fresh wash buffer was added back to the pellet. Thyroglobulin (10 µg) was added as a carrier protein to each of the three fractions (supernatant, wash and pellet), followed by four volumes of cold methanol. Following precipitation overnight at 4°C, samples were pelleted at 14 000 r.p.m. for 20 min at 4°C, the supernatants aspirated and 10 µl of 1× sample buffer (63 mM Tris–HCl pH 6.8, 5% glycerol, 3 mM EDTA, 5% SDS, 4% 2-mercaptoethanol and 0.02% bromophenol blue) was added to the pellets. Samples were boiled for 5 min and loaded on 16% Tris/glycine preformed gels (Invitrogen). The bands were visualized with the Molecular Dynamics Storm PhosphorImager system. Bands were quantified in terms of the integrated peak volume. In order to correct for non-specific pelleting of radiolabeled PrP-sen, the volume of radiolabeled PrP in the pellet in the absence of PrP-res was subtracted from the volume of radiolabeled PrP in the pellet in the presence of PrP-res. The percentage of radiolabeled PrP in the pellet was then calculated using the corrected volumes and the formula:

$$\frac{(\text{volume of PrP in pellet})}{(\text{volume of PrP in supernatant} + \text{wash} + \text{pellet})} \times 100$$

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