Specific binding of normal prion protein to the scrapie form via a localized domain initiates its conversion to the protease-resistant state

Motohiro Horiuchi¹ and Byron Caughey²

Laboratory of Persistent Viral Diseases, Rocky Mountain Laboratories, NIAID, NIH, 903 S 4th Street, Hamilton, MT 59840, USA

¹Permanent address: Department of Veterinary Public Health and Research Center for Protozoan Molecular Immunology, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan

²Corresponding author
e-mail: byron_caughey@nih.gov

In the transmissible spongiform encephalopathies, normal prion protein (PrP^{sen}) is converted to a protease-resistant isoform, PrP^{res}, by an apparent self-propagating activity of the latter. Here we describe new, more physiological cell-free systems for analyzing the initial binding and subsequent conversion reactions between PrP^{sen} and PrP^{res}. These systems allowed the use of antibodies to map the sites of interaction between PrP^{sen} and PrP^{res}. Binding of antibodies (α 219–232) to hamster PrP^{sen} residues 219–232 inhibited the binding of PrP^{sen} to PrP^{res} and the subsequent generation of PK-resistant PrP. However, antibodies to several other parts of PrP^{sen} did not inhibit. The α 219–232 epitope itself was not required for PrP^{res} binding; thus, inhibition by α 219–232 was likely due to steric blocking of a binding site that is close to, but does not include the epitope in the folded PrP^{sen} structure. The selectivity of the binding reaction was tested by incubating PrP^{res} with cell lysates or culture supernatants. Only PrP^{sen} was observed to bind PrP^{res}. This highly selective binding to PrP^{res} and the localized nature of the binding site on PrP^{sen} support the idea that PrP^{sen} serves as a critical ligand and/or receptor for PrP^{res} in the course of PrP^{res} propagation and pathogenesis *in vivo*.

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Introduction

Transmissible spongiform encephalopathies (TSEs) include scrapie in sheep and goats, bovine spongiform encephalopathy in cattle and Creutzfeldt–Jakob disease in humans. TSEs are fatal neurodegenerative diseases which are characterized by vacuolation of neurons, astroglyosis and the accumulation of the abnormal, protease-resistant prion protein (PrP^{res} or PrP^{Sc}) in the central nervous system and lymphoreticular tissues. A variety of studies suggest that PrP^{res} is important in TSE pathogenesis and is a likely component of TSE infectivity; however, the exact nature of the infectious agent is still controversial (reviewed in Prusiner, 1991; Weissmann, 1996; Chesebro and Caughey, 1997).

Studies using scrapie-infected cultured cells revealed

that PrP^{res} is synthesized from the normal, protease-sensitive prion protein (PrP^{sen} or PrP^C) on the cell surface and/or in an endocytic pathway (Caughey and Raymond, 1991; Borchelt *et al.*, 1992). No difference has been observed in the covalent structures which can distinguish these two isoforms (Hope *et al.*, 1986; Stahl *et al.*, 1993). However, the two isoforms differ considerably biochemically and biophysically. PrP^{sen} is soluble in mild detergents and sensitive to proteinase K (PK) digestion, whereas PrP^{res} is insoluble in mild detergents and partially resistant to PK digestion (Meyer *et al.*, 1986). Spectroscopic studies revealed that PrP^{sen} has mainly α -helix and disordered structures (Riek *et al.*, 1996, 1997; Donne *et al.*, 1997; James *et al.*, 1997), whereas PrP^{res} has a much higher β -sheet content (Caughey *et al.*, 1991a; Pan *et al.*, 1993; Safar *et al.*, 1993).

Interactions between PrP^{sen} and PrP^{res} appear to be important in TSE pathogenesis and the conversion of PrP^{sen} to PrP^{res}. Studies in scrapie-infected animals and tissue culture cells indicated that sequence compatibility between PrP^{sen} and PrP^{res} molecules is necessary for efficient PrP^{res} formation (Prusiner *et al.*, 1990; Scott *et al.*, 1992; Carlson *et al.*, 1994; Priola *et al.*, 1994). The importance and specificity of direct interactions between PrP^{sen} and PrP^{res} in PK-resistant PrP formation has been demonstrated in *in vitro* studies using largely purified forms of PrP^{res} and PrP^{sen}. These studies showed that PrP^{res} can induce the conversion of PrP^{sen} to a partially PK-resistant state that is characteristic of TSE-associated PrP^{res} (Kocisko *et al.*, 1994; Bessen *et al.*, 1995). Furthermore, the PrP sequence specificities of the conversion reaction correlate with inter- and intra-species TSE transmissibilities *in vivo* (Kocisko *et al.*, 1995; Bossers *et al.*, 1997; Raymond *et al.*, 1997). Although the previous studies have focused on the generation of the final PK-resistant products, a recent study showed that there can be two kinetically separable steps in the PrP^{res}–PrP^{sen} interactions that can result in conversion of PrP^{sen} to the PK-resistant state (DeBurman *et al.*, 1997): first, the binding of PrP^{sen} to PrP^{res} and secondly, the conversion of PrP^{sen} to the PK-resistant state. The initial binding between PrP^{sen} and PrP^{res} is of interest as: (i) a critical aspect of the overall conversion mechanism; (ii) a potential mode of interaction between exogenous PrP^{res} and an uninfected cell; (iii) a potential determinant of interspecies transmissibilities of TSE agents; and (iv) a possible target for therapeutic intervention with binding inhibitors.

To study the binding and conversion of PrP^{sen} to the PK-resistant state under near physiological conditions, we have developed a cell-free system that substitutes NaCl or KCl for the chaotropic ions or non-mammalian chaperone proteins that were required for efficient conversion in previous studies (Kocisko *et al.*, 1994; DeBurman *et al.*,

Fig. 1. The generation of PK-resistant [35 S]PrP in the presence of physiological salts. (A) Effect of KCl. One-tenth of the reaction mixture was loaded without PK treatment [PK(-)], whereas the remaining nine-tenths of the reaction mixture were loaded after PK treatment [PK(+)]. (B) Effect of NaCl. (C) Summary of the effects of KCl and NaCl. Averages and standard deviations are indicated ($n = 3$). [35 S]PrP-sen in the PK(-) samples and the PK-resistant [35 S]PrP bands indicated by the bracket in the PK(+) lanes were quantified by PhosphorImager (Molecular Dynamics) and conversion percentages [% conversion in (C), (D) and (E)] were calculated as follows: (integrated intensity of PK-resistant PrP)/(integrated intensity of PK-untreated GPI(-)PrP-sen) $\times 100$. (D) Effect of pH. The cell-free reactions were performed as described in Materials and methods, except for buffers. Buffers used are: pH 5 and 6, 50 mM citric acid-sodium citrate buffer; pH 7, 50 mM phosphate buffer; pH 8, 50 mM boric acid-borax buffer; pH 9 and 10, 50 mM sodium carbonate-sodium bicarbonate buffer ($n = 3$). (E) Comparison of salt-stimulated conversion reaction [GdnHCl(-), at 200 mM KCl] with GdnHCl-containing conversion reaction [GdnHCl(+)] as a function of PrP-res concentration ($n = 3$). Molecular mass markers are marked in kDa on the left.

1997). This system has allowed us to use various anti-PrP antibodies to map the sites of binding between the two PrP isoforms that precede conversion. In addition, we have assessed the selectivity of PrP-res in binding PrP-sen from complex mixtures of proteins under non-denaturing conditions. The results indicate that the PrP-res-binding site of PrP-sen is highly localized and the selectivity of PrP-res in binding of PrP-sen rivals that of anti-PrP antibodies.

Results

Stimulation of the PrP-res-induced conversion reaction with physiological salts

To obtain more physiological conditions for the cell-free reaction, we examined whether KCl and NaCl could stimulate the conversion of PrP-sen into a PK-resistant state. As observed previously (Kocisko *et al.*, 1994), the [35 S]glycophosphatidylinositol (GPI)(-)PrP-sen appeared as two bands of 28 and 32 kDa, corresponding to non-glycosylated and glycosylated forms, respectively. After the conversion reaction with PrP-res, however, PK-resistant [35 S]PrP bands were 6–7 kDa lower in molecular mass than the PrP-sen (Figure 1A and B, indicated by parentheses), which are characteristic of scrapie-associated PrP-res (Oesch *et al.*, 1985) and the expected products of the conversion reaction (Kocisko *et al.*, 1994). Without salt, the efficiency of conversion to PK-resistant [35 S]PrP was poor (<5% of the input [35 S]PrP-sen); however, a much higher efficiency (up to 25%) was obtained in the presence of 100–500 mM KCl (Figure 1A and C). The

effect of KCl increased with concentration up to 200 mM and then reached a plateau (Figure 1C). NaCl also stimulated the generation of PK-resistant [35 S]PrP (Figure 1B and C). However, the reaction was optimal at 100 mM NaCl and decreased with higher concentrations of NaCl. A pH of 6 was optimal for the conversion reaction at 200 mM KCl (Figure 1D). These results indicated that PK-resistant [35 S]PrP can be generated from [35 S]PrP-sen in the presence of PrP-res and the absence of GdnHCl, albeit at an efficiency that was 50–75% of that of the reaction containing GdnHCl (Figure 1E). No PK-resistant [35 S]PrP was obtained with either 200 mM KCl (Figure 1E) or 100 mM NaCl (data not shown) in the absence of PrP-res. In the subsequent experiments, the conversion reaction was performed in the presence of 200 mM KCl, 5 mM MgCl_2 , 0.625% Sarkosyl and 50 mM citrate buffer (pH 6.0), unless specified otherwise.

Binding of PrP-sen to PrP-res precedes the conversion reaction

In order to characterize further the interaction between PrP-sen and PrP-res under the conditions described above, we examined the rates at which [35 S]PrP-sen is bound to PrP-res and converted to the PK-resistant state (Figure 2A and B). PrP-res was incubated with [35 S]PrP-sen and then was pelleted, along with any bound [35 S]PrP-sen, by centrifugation. More than 95% of the PrP-res pelleted in these experiments (data not shown), while in the absence of PrP-res, <10% of the [35 S]PrP-sen appeared in the pellet fraction (Figure 2A). Thus, we regarded PrP-res-dependent increases in [35 S]PrP in the pellet as indicative

Fig. 2. Kinetics of binding of PrP-sen to PrP-res and generation of PK-resistant [35 S]PrP in the presence of KCl. (A) Binding analysis. Both unbound and pellet fractions (see Materials and methods) were electrophoresed, and 35 S-labeled PrP in both fractions was quantified by PhosphorImager analysis. The graph shows the percentage of the 35 S-labeled PrP detected in the pellet fraction. Averages and standard deviations are indicated ($n = 3$). The recoveries of intact [35 S]PrP-sen bands under these conditions were 80–90% throughout the time course. (B) The generation of PK-resistant [35 S]PrP. The graph shows the average percentages of conversion (as described in the legend to Figure 1) in two independent experiments. Time points: 0, 1, 2, 4, 8, 24 and 48 h.

of binding to PrP-res. Without KCl, ~50% of input [35 S]PrP-sen bound to PrP-res within 1 h and, after that, no significant increase of the binding was observed up to 48 h. In contrast, in the presence of 200 mM KCl, the rate of the binding of [35 S]PrP-sen to PrP-res in the early phase was slower than that observed in the absence of KCl; however, the binding increased up to 48 h until 80% of input [35 S]PrP-sen was bound to PrP-res (Figure 2A). Despite the rapid binding of [35 S]PrP-sen to PrP-res in the absence of KCl, only a small amount of PK-resistant [35 S]PrP was detected after 48 h incubation (Figure 2B). In contrast, in the presence of 200 mM KCl, PK-resistant [35 S]PrP was detected after a lag phase of 4 h and increased gradually up to 48 h (Figure 2B). These results indicated that although KCl does not accelerate the initial binding of [35 S]PrP-sen to PrP-res, it increases the proportion of [35 S]PrP-sen that ultimately becomes PK resistant.

Anti-PrP antibodies against the C-terminal part of PrP inhibited the conversion of PrP-sen to PrP-res

To investigate the sites of interaction between PrP-sen and PrP-res, we compared the abilities of antibodies

directed at five distinct PrP epitopes to inhibit the cell-free conversion. Figure 3A shows that each of the antibodies recognizes the two major 28 and 32 kDa bands of the GPI(-)PrP-sen, which represent the full-length unglycosylated and glycosylated molecules, respectively (Kocisko *et al.*, 1994). α 143–156 and α 218–232 also immunoprecipitated a 15 kDa fragment which appears to correspond to a previously recognized C-terminal product of cellular processing (Chen *et al.*, 1995).

Among the antibodies tested, only α 219–232 significantly inhibited the generation of PK-resistant [35 S]PrP in a dose-dependent manner, while other rabbit sera as well as monoclonal antibody (mAb) 3F4 showed no statistically significant inhibitory effect (Figure 3B). This inhibitory effect of α 219–232 was competed by adding the synthetic peptide hamster PrP218–232, although the peptide itself did not affect the generation of PK-resistant PrP (Figure 3C). This demonstrates the epitope specificity of the α 219–232 effect. When the PrP-res was treated with antibodies and washed before incubation with [35 S]PrP-sen, none of the antibodies inhibited the generation of PK-resistant PrP (Figure 3D). This indicated that the inhibitory effect of α 219–232 was due to the binding of antibody to its epitope on PrP-sen rather than PrP-res. Similar results were obtained in an experiment with more heavily glycosylated wild-type(GPI+) PrP-sen instead of GPI(-)PrP-sen, wherein the generation of PK-resistant PrP was inhibited by α 219–232 but not by the mAb 3F4 (data not shown). Thus, glycosylation of PrP-sen at residues 181 and 197 did not block its binding to PrP-res or eliminate the inhibitory effect of α 219–232.

Inhibition of conversion by α 219–232 was due to the inhibition of initial binding of PrP-sen to PrP-res

In order to address the question of whether α 219–232 inhibits the initial binding of PrP-sen to PrP-res, we performed binding analyses. When the [35 S]PrP-sen was pre-treated with negative control rabbit serum, 60% of the input [35 S]PrP was detected in the pellet with PrP-res (Figure 4A). As before (Figure 2A), this binding was a PrP-res-specific phenomenon, because little [35 S]PrP-sen was detected in the pellet without PrP-res. In contrast, only 10% of the input [35 S]PrP was detected in the pellet with PrP-res when [35 S]PrP-sen was pre-treated with α 219–232. Furthermore, the inhibition of binding by α 219–232 was competed by the synthetic peptide hamster PrP218–232, again confirming the epitope specificity of the inhibition (Figure 4B). Treatment of PrP-res with α 219–232 and washing before incubating with [35 S]PrP-sen did not inhibit the binding, indicating that the inhibition of the binding was due to the binding of α 219–232 to its epitope on PrP-sen (data not shown).

To eliminate the possibility that the inhibition by α 219–232 was due to an antibody-mediated cross-linking of [35 S]PrP-sen rather than blockage of the binding domain on the PrP-sen, we examined the effects of Fab fragments on binding. Only 27% of total [35 S]PrP-sen was detected in the pellet when [35 S]PrP-sen was pre-treated with Fab fragments of α 219–232, whereas >90% of the total [35 S]PrP-sen was detected in the pellet fraction in the presence of other Fab fragments (Figure 5A). Consistent with the inhibition of the binding, Fab fragments of α 219–

Fig. 3. Inhibition of the generation of PK-resistant [35 S]PrP by anti-PrP antibodies. (A) The reactivities of the antibodies in radio-immunoprecipitation. The culture supernatants of metabolically labeled GPI(-)PrP-sen-expressing cells were incubated with high a dose of antibodies in the presence of 1% sarkosyl (see Materials and methods) and then antigen-antibody complexes were precipitated with protein A-Sepharose. The antibodies used are indicated on the top. The lower and upper arrowheads indicate non-glycosylated and glycosylated GPI(-)PrP-sen, respectively. NRS, negative control rabbit serum. (B) Effect of anti-PrP antibodies. Relative percentage conversion was normalized to the percentage conversion of the samples that were treated with NRS. Filled and hatched columns indicate the results obtained by using low and high doses of antibodies, respectively (see Materials and methods). The bars indicate standard deviations ($n = 3$). (C) Competition of α 219-232 effect by synthetic peptide hamster PrP218-232. The results obtained by using a low dose of antibodies are shown. The first lane (left) shows the one-tenth equivalent of the PK-untreated [35 S]PrP-sen added to each reaction. The antibodies used are indicated in the row of 'Ab'. '+' in the row of 'PrP218-232' indicate that antibodies were pre-incubated with 25 mM peptide, while '-' means antibodies were not pre-incubated with the peptide. '+' and '-' in the row of 'PrP-res' indicate with and without PrP-res, respectively. (D) Pre-treatment of PrP-res with anti-PrP antibodies did not inhibit the conversion reactions. A 500 ng aliquot of PrP-res was incubated with 2 μ l of anti-PrP antibodies in the presence of 50 mM phosphate buffer (pH 7.0), 100 mM NaCl, 1% Sarkosyl, at 37°C for 4 h. Then PrP-res was recovered and washed twice by centrifugation to remove unbound antibodies. Finally, PrP-res in the pellet was resuspended and used for the analyses. The graph shows the percentage conversion ($n = 5$).

Fig. 4. Inhibition of the binding of PrP-sen to PrP-res by anti-PrP antibodies. (A) Binding analysis. The [35 S]PrP-sen was incubated with antibodies first (high dose), then incubated with PrP-res for 90 min. The columns indicate the percentage of [35 S]PrP detected in the pellet fraction. The antibodies used are indicated at the bottom. '+' and '-' in parentheses indicate in the presence and absence of PrP-res, respectively. The bars indicate standard deviations ($n = 5$). (B) Competition of the α 219-232 effect by synthetic peptide. α 219-232 was pre-incubated with the synthetic peptide of hamster PrP218-232 and then incubated with [35 S]PrP-sen. Finally, the antibody-pre-treated PrP-sen was incubated with PrP-res. Filled and hatched columns indicate in the presence and absence of 25 mM peptide, respectively. The bars indicate standard deviations ($n = 5$).

232 inhibited the generation of PK-resistant [35 S]PrP in the cell-free reaction, while other Fab fragments did not (Figure 5B). These results indicate that α 219-232 inhibited the initial binding of PrP-sen to PrP-res and, subsequently, PK-resistant [35 S]PrP formation by blocking the site on the PrP-sen molecule which is essential for the initial binding to PrP-res.

Removal of the α 219-232 epitope from PrP-sen did not affect its binding to PrP-res

To address the question of whether the region of the epitope for α 219-232 itself is essential for the binding of PrP-sen to PrP-res, we generated C-terminal truncations in [35 S]GPI(-)PrP-sen by limiting proteolysis using carboxypeptidases P and Y (Figure 6A). About 2 kDa of C-terminus was removed after 30 and 60 min treatment (indicated by arrowheads), although a small amount of intact GPI(-)PrP-sen still existed. Immunoblot analysis revealed that the smaller fragment lacked the epitope for α 219-232. The binding analysis using the same set of PrP-sen preparations revealed that not only intact [35 S]PrP-sen but also PrP-sen lacking the α 219-232 epitope were bound to PrP-res (Figure 6B). Furthermore, [35 S]PrP-sen lacking the α 219-232 epitope was still converted into the PK-resistant form, as can be seen by the PK-resistant [35 S]PrP bands with slightly lower molecular mass (marked with asterisks) compared with PK-resistant [35 S]PrP from the carboxypeptidase-untreated [35 S]PrP (Figure 6C). These results indicated that the C-terminal part of PrP-sen comprising the α 219-232 epitope is not essential for the binding of PrP-sen to PrP-res or the conversion of PrP-sen to PrP-res. Thus, α 219-232 appears to inhibit the binding of PrP-sen to PrP-res by affecting access to a binding site that is near the α 219-232 epitope but does not require it.

Binding between PrP-sen and PrP-res is highly selective

To investigate the selectivity of the binding between PrP-sen and PrP-res, we performed the binding analysis using culture supernatants and cell lysates of 35 S-labeled GPI(-)PrP-sen-expressing cells which contained many other 35 S-labeled proteins in addition to [35 S]PrP-sen.

Fig. 5. Effect of Fab fragments on binding and conversion reactions. (A) Binding analysis. Fab fragments used are shown on the top. One-quarter of the supernatant (sup) obtained after centrifugation and an equivalent amount of the corresponding pellet fraction (ppt) were loaded for each sample. Numbers below the figure show the percentage of [35 S]PrP detected in the pellet fraction (an average of two independent experiments). '+' and '-' indicate with and without PrP-res, respectively. (B) Generation of PK-resistant [35 S]PrP. Numbers below the figure show the relative percentage conversions that were normalized to the percentage conversion obtained in the presence of Fab fragments of normal rabbit serum (an average of two independent experiments).

Three major 35 S-labeled bands, which appeared identical to those obtained in the [35 S]PrP-sen immunopurified from the culture supernatants with α 219-232, were pelleted after PrP-res was added to untreated culture supernatants (data not shown) or to culture supernatants pre-treated with negative control rabbit serum and protein A-Sepharose (Figure 7A, lanes 2 and 3). Comparison of the [35 S]PrP-sen bands in the remaining supernatants between samples containing and lacking PrP-res revealed that more than half of PrP-sen was removed by pelleting with PrP-res (lane 9 versus 10, indicated by arrowheads). When the culture supernatants were pre-treated with α 90-104 or α 219-232 and subsequently with protein A-Sepharose in order to deplete PrP-sen, the intensities of two of the three bands (28 and 32 kDa) decreased. The small fragment of 15 kDa was detected in the pellet from the culture supernatants pre-treated with α 90-104 and protein A-Sepharose but not in the pellet from those pre-treated with α 219-232 and protein A-Sepharose (Figure 7A, lanes 4 and 6). Thus, these results showed that the major three bands pelleted with PrP-res were GPI(-) PrP-sen and that PrP-res selectively bound PrP-sen in complex mixtures of many proteins. Furthermore, antibody inhibition studies analogous to those done with immunopurified [35 S]GPI(-)PrP-sen (Figure 4) were performed

Fig. 6. Effect of removal of the α 219-232 epitope from PrP-sen on binding and conversion. (A) Carboxypeptidase treatment. Top: PhosphorImager analysis of [35 S]GPI(-)PrP-sen treated with carboxypeptidases P and Y. Incubation periods with the carboxypeptidases are shown on the top (min). The sample treated under the same conditions without carboxypeptidases for 60 min [CP(-)] is also shown. Bottom: immunoblot analysis. The same set of samples as in the top panel were analyzed by immunoblot using α 219-232. (B) Binding analysis. The same set of [35 S]PrP-sen as in (A) was used. One-quarter of the supernatant obtained after centrifugation (sup) and an equivalent amount of the corresponding pellet fraction (ppt) were loaded. Top and bottom panels correspond to experiments in the absence and presence of PrP-res, respectively. (C) Generation of PK-resistant [35 S]PrP. 'PK(+)' and 'PK(-)' indicate PK-treated and untreated samples, respectively, at a reaction equivalent ratio of 9:1. Numbers below the figure show the relative percentage conversions that were normalized to the percentage conversion obtained from PrP-sen of 0 min treatment with carboxypeptidases (an average of two independent experiments).

with α 23-37, α 90-104, α 143-156, α 218-232 and mAb 3F4 by using the culture supernatants. While each of the antibodies bound at least 70% of the [35 S]PrP-sen in the culture supernatants, only α 219-232 again showed significant inhibition (>80%) of the binding of PrP-sen to PrP-res (data not shown).

In order to demonstrate the selectivity under alternative conditions, we performed binding analysis using detergent lysates of cells (Figure 7B). Two major bands of the same

Fig. 7. Selectivity of the binding between PrP-sen and PrP-res. (A) Binding analysis using culture supernatants of metabolically [^{35}S]methionine-labeled GPI(-)PrP-sen-expressing cells. The culture supernatants were pre-treated with negative control rabbit serum and protein A-Sepharose (lanes 2, 3, 9 and 10), $\alpha 219$ -232 and protein A-Sepharose (lanes 4, 5, 11 and 12) or $\alpha 90$ -104 and protein A-Sepharose (lanes 6, 7, 13 and 14). After the centrifugation to remove the protein-antibody-protein A-Sepharose complexes, the resulting supernatant was incubated with PrP-res. Lanes 2-7 show the pellet fraction (ppt), while lanes 9-14 show one-twentieth the volume of the corresponding supernatant (sup). '+' and '-' indicate with and without PrP-res, respectively. Lanes 1 and 8 show GPI(-)PrP-sen immunopurified from culture supernatants with $\alpha 219$ -232. (B) Binding analysis using cell lysates. The cell lysates were pre-treated with protein A-Sepharose (lanes 2, 3, 7 and 8) or mAb 3F4 plus protein A-Sepharose (lanes 4, 5, 9 and 10). After the centrifugation, the resulting supernatant was incubated with PrP-res. Lanes 2-5 show the pellet fraction, while lanes 7-10 show one-twentieth the volume of the corresponding supernatant. Lanes 1 and 6 show the GPI(-)PrP-sen immunopurified from cell lysates with mAb 3F4.

sizes as [^{35}S]PrP-sen immunopurified with mAb 3F4 from cell lysates were bound specifically to PrP-res (Figure 7B, lanes 2 and 3). The amount of the two bands decreased to ~10% of that in cell lysates treated with protein A-Sepharose when [^{35}S]PrP-sen-depleted cell lysates was used (Figure 7B, lanes 4). This demonstrated that the bands pelleted with PrP-res from cell lysates were [^{35}S]GPI(-)PrP-sen. Thus, although the cell lysates contain even more proteins than the culture supernatants, highly selective binding of PrP-sen to PrP-res was still observed.

Discussion

Adaptation of the cell-free conversion reactions to more physiological conditions

Since conversion of PrP-sen into PrP-res is one of the key events in TSE pathogenesis, it is important to understand the mechanistic details of the reaction. The cell-free model has allowed the conversion reaction to be studied under conditions that are much simpler and more defined than those existing *in vivo*. However, in its previous manifestations, the cell-free conversion was enhanced greatly by the presence of chaotropic salts (Kocisko *et al.*, 1994) and/or non-mammalian chaperone proteins (DebBurman *et al.*, 1997) that clearly were not involved in the *in vivo* reaction. To improve the relevance of this model to the *in vivo* situation, we sought to eliminate such non-physiological factors and replace them with more physiological ones. Our present observation indicated that appropriate concentrations of physiological salts (NaCl and KCl) can, to a large extent, replace GdnHCl and chaperone proteins to drive the reactions under conditions that better approximate the *in vivo* situation.

The acquisition of PK resistance by PrP-sen is not explained simply by its binding to PrP-res

Kinetic studies (Figure 2; DebBurman *et al.*, 1997) revealed that the binding of PrP-sen to PrP-res can be segregated temporally from the acquisition of PK resistance by PrP-sen, especially in the absence of KCl. This provides evidence that the acquisition of PK resistance in cell-free reactions is not explained simply by the binding of PrP-sen to a stoichiometric excess of PrP-res aggregates; other event(s) must occur in the conversion reaction which may include time-dependent conformational changes, interactions involving other parts of the PrP-sen and PrP-res molecules, relocations to other binding sites and/or further polymerization.

Sites of interaction between PrP-sen and PrP-res

The analysis of the effects of various anti-PrP antibodies on the binding of PrP-sen to PrP-res provides insight into the contacts between PrP-sen and PrP-res that initiate the conversion reaction. The fact that these contacts are blocked by the binding of PrP-sen by $\alpha 219$ -232 but not the antibodies to residues 23-37, 90-104, 106-115 (mAb 3F4) and 143-156, or the attachment of glycans at residues 181 and 197, provides evidence that PrP-sen and PrP-res interact initially through a localized domain. This result suggested to us initially that the C-terminus of PrP-sen comprising the epitope for $\alpha 219$ -232 might be essential for the initial binding of PrP-sen to PrP-res; however, the analyses using PrP-sen lacking the $\alpha 219$ -232 epitope revealed that this was not the case (Figure 6). The most probable explanation for this is that the residues that comprise the epitope for $\alpha 219$ -232 do not form a critical part of the binding site but are close enough in space to

Fig. 8. Antibody epitopes and glycans on the three-dimensional NMR structures of a C-terminal fragment (residues 90–231) of hamster PrP-sen (James *et al.*, 1997; 1b10 update in Protein Data Bank). Backbone secondary structures (upper) and spacefilling models (lower) are shown in front and back view. The regular open or colored circles represent residues in this fragment that are known to be highly flexible and without discernible secondary structure (Riek *et al.*, 1996, 1997; Donne *et al.*, 1997; James *et al.*, 1997). The Asn residues to which *N*-linked glycans can be attached (upper models) or representative glycans (lower models) are marked in yellow. The *N*-linked glycan structures [*N*-acetylglucosamine-*N*-acetylglucosamine-mannose-(mannose)₂] that are shown in the spacefilling models are minimal generic *N*-linked glycan structures; they are included to indicate their relative size (although most *N*-linked glycans are bigger than these structures) and are not meant to represent actual PrP-sen structures. In considering the effects of antibodies binding to these epitopes, it is useful to bear in mind that antibody molecules themselves are much larger than PrP-sen itself and, therefore, would probably block access to more of the PrP-sen surface than the epitope residues themselves. The approximate size of a typical Fab fragment is indicated by the yellow cross-section on the lower right. Cysteine residues are shown in orange. Surfaces in the vicinity of the C-terminus that might be blocked by α 219–232 and not by the antibodies to residues 90–104, 106–115, 143–156 or the presence of *N*-linked glycans at residues 181 and 197 are encircled with dashed lines. The potential binding surface in the left hand models is drawn specifically to include residues 119 and 120 because of their importance in peptide inhibition of the conversion reaction (Chabry *et al.*, 1998); however, their importance in the initial binding reaction remains to be determined.

the binding site on the PrP-sen molecule to inhibit the binding by steric hindrance, i.e. the bound antibody may block access to a region on PrP-sen which is required for binding to PrP-res.

The NMR structures of recombinant PrP-sen (Riek *et al.*, 1996; James *et al.*, 1997) helped us to consider potential binding surfaces that might be blocked by α 219–232 and not the other antibodies or *N*-linked glycans (Figure 8). One potentially important surface is an area which is formed by near-C-terminal α -helical residues from ~206 to ~223 and the loop of residues 165–174 linking the second β -strand and second α -helix (marked with a dashed line in Figure 8, right-hand models). This area presumably can be hindered by binding of α 219–232 to its epitope because of its proximity to the C-terminus

in space (Billeter *et al.*, 1997; James *et al.*, 1997), or the binding of antibody to the α 219–232 epitope might result in the conformational rearrangement of the region proximal to the epitope on PrP-sen which is involved in the initial binding (Colman *et al.*, 1987). However, PK-resistant PrP has been generated from the PrP lacking residues 141–176 in scrapie-infected neuroblastoma cells (Muramoto *et al.*, 1996), suggesting that the loop region might not be critical for the initial binding. Some of the residues in this area were also suggested to be involved in recognition of 'protein X', a hypothetical factor that might facilitate the conversion of PrP-sen to PrP-res (Kaneko *et al.*, 1997b). However, the cell-free system here is comprised largely of purified PrP-res and PrP-sen, so that it seems unlikely that the inhibition of the binding between PrP-sen and

PrP-res by α 219–232 *in vitro* was due to the inhibition of the binding of an unknown protein to PrP-sen.

Another potential binding surface on the other side of PrP-sen that would be consistent with the current data is formed mostly by residues from ~119 to ~138 which include the first β -strand and the loop preceding the first α -helix (marked with a dashed line in Figure 8, left-hand models). Access to this surface might also be blocked by binding of α 219–232, but perhaps not by the other non-inhibitory antibodies or the presence of glycans at Asn181 and Asn197. In support of an important role for these residues in the conversion reaction is the observation that a synthetic peptide comprised of residues 119–141 is a potent inhibitor of the overall conversion reaction (Chabry *et al.*, 1998). It remains to be determined whether this peptide also blocks the initial binding of PrP-sen to PrP-res and, if so, whether it is substituting for PrP-sen or PrP-res in the process.

Of particular interest is the lack of inhibition by the antibodies recognizing residues 90–104 and 106–115 (mAb 3F4) because residues in these regions have been shown to influence PrP-res formation (Muramoto *et al.*, 1996; Kaneko *et al.*, 1997a; Chabry *et al.*, 1998; Hölscher *et al.*, 1998). Indeed, biochemical and spectroscopic studies have suggested that residues 90–124 undergo a major conformational change during the formation of PrP-res (Oesch *et al.*, 1985; Caughey *et al.*, 1991a; Pan *et al.*, 1993; Kocisko *et al.*, 1996; Donne *et al.*, 1997; Riek *et al.*, 1997). One explanation for these counter-intuitive results might be the following. After the initial binding via the localized site near the C-terminus, further interactions and conformational rearrangements occurring in the process of conversion might allow dissociation of antibodies that are bound to other parts of the PrP-sen molecule and/or prevent their reassociation once dissociation occurs. So, even though antibodies α 90–104 and mAb 3F4 did not inhibit the generation of PK-resistant PrP, it is conceivable that the epitopes for these antibodies might be involved in the conversion process after the initial binding.

Studies using deletion mutants of PrP have shown that the region from the N-terminus to amino acid 88 is not essential for the PrP-sen–PrP-res interaction that results in conversion (Rogers *et al.*, 1993; Fisher *et al.*, 1996). Consistent with these observations, α 23–37 did not inhibit the conversion reaction (Figure 3) and PrP-sen bound to PK-treated PrP-res which lacked the N-terminus to approximately residue 90 of the molecule (data not shown).

Our observation that mAb 3F4 did not substantially inhibit the interaction between PrP-sen and PrP-res is inconsistent with the observation reported by Kaneko *et al.* (1995) in which mAb 3F4 inhibited the interaction between PrP-sen and PrP-res. Perhaps different experimental conditions may explain this discrepancy. Not only have we found a lack of mAb 3F4 inhibition of the conversion of both GPI(–)PrP-sen and wild-type PrP-sen, but the C-terminal PrP-sen fragment which lacks the 3F4 epitope still binds to PrP-res (Figure 7). Our results, therefore, suggest that the residues in the mAb 3F4 epitope are not critical in the initial binding of PrP-sen to PrP-res.

The binding of PrP-sen to PrP-res is highly selective

Many studies have suggested that interaction between PrP-sen and PrP-res occurs during TSE pathogenesis and

PrP-res propagation (Prusiner *et al.*, 1990; Büeler *et al.*, 1993, 1994; Brandner *et al.*, 1996), and cell-free reactions have demonstrated the direct association between the two PrP isoforms in the generation of PK-resistant PrP (Kocisko *et al.*, 1994). However, the selectivity of the initial binding between the two PrP isoforms was uncertain. Our present results show that the binding of PrP-sen with PrP-res was as selective as the immunopurification of PrP-sen with anti-PrP antibodies (Figure 7). This high selectivity of the binding and the localized nature of the binding site provide direct evidence that the binding between PrP-sen and PrP-res is like a ligand–receptor interaction.

Glycosaminoglycans appear to bind both PrP-sen and PrP-res (Snow *et al.*, 1989, 1990; Gabizon *et al.*, 1993; Caughey *et al.*, 1994), and sphingolipids also appear to bind PrP-res (Klein *et al.*, 1998). Furthermore, several proteins have been suggested to interact with PrP-sen and/or PrP-res (Oesch *et al.*, 1990; Martins *et al.*, 1997; Rieger *et al.*, 1997; Yehiely *et al.*, 1997). Although it remains to be determined whether those molecules modulate or facilitate the binding between PrP-sen and PrP-res, our observation of highly selective binding between PrP-sen and PrP-res suggests that cellular PrP-sen itself may act as the receptor for incoming PrP-res in the course of TSE infection.

The culture supernatants used for the binding studies were not treated with denaturants (Figure 7). Furthermore, GPI(–)PrP-sen purified from such supernatants without denaturation was found to have a circular dichroism spectrum (B.Caughey, unpublished observation) similar to that of brain-derived PrP-sen (Pan *et al.*, 1993), suggesting that GPI(–)PrP-sen in the culture supernatants was in its native conformation. Collectively, the selective binding of PrP-sen to PrP-res under non-denatured conditions suggests that denaturation of PrP-sen is not essential for the initial binding between PrP-sen and PrP-res.

Applications of the new cell-free reactions

The primary merit of the cell-free reactions described here is that the reaction can be performed under near physiological conditions in terms of salt concentration and pH. This advantage allowed us to analyze the effect of anti-PrP antibodies on the interaction between PrP-sen and PrP-res. If the conversion process is modulated by other cellular factors, the cell-free reaction described here may aid in the search for such factors because they may be inactivated under non-physiological conditions. In addition, near physiological reaction conditions will be an advantage in the screening for potentially therapeutic anti-TSE compounds which interfere with the binding and/or conversion reactions.

Materials and methods

Antibodies

Monoclonal antibody 3F4 (Kascsak *et al.*, 1987), recognizing the region from residues 106 to 115 containing Met109 and Met112 (Bolton *et al.*, 1991), and rabbit sera raised against mouse PrP synthetic peptides corresponding to residues 23–37, 89–103, 142–155 and 218–232 were used (Caughey *et al.*, 1991b). The hamster PrP equivalents of these synthetic peptides are as follows; residues 23–37, 90–104, 143–156 and 219–232, respectively. To avoid confusion in this text, we refer to those rabbit sera as α 23–37, α 90–104, α 143–156 and α 219–232.

Preparation of Fab fragments

Rabbit sera and ascites of mAb 3F4 were incubated with protein A-Sepharose overnight. The antibody-protein A-Sepharose complex was washed three times with phosphate-buffered saline (PBS), and then washed once with buffer for papain digestion (20 mM phosphate buffer, 10 mM EDTA, 10 mM cysteine-HCl, pH 7.0). Immobilized papain (Pierce) pre-treated with the same buffer was mixed with the antibody-protein A-Sepharose complex, and incubated at 37°C for 4 h. Supernatant was collected after centrifugation, and Fab fragments in the supernatant were concentrated and the buffer was exchanged for PBS by using Microcon 10 ultrafiltration devices (Amicon). The preparations of Fab fragments were analyzed by protein staining of gels after SDS-PAGE and found to have virtually no detectable intact IgG and F(ab')₂ molecules. The reactivities of Fab fragments to PrP molecules were confirmed by enzyme-linked immunosorbent assay (ELISA) prior to use.

Cells and purification of ³⁵S-labeled PrP-sen

The mutant PrP-sen which lacks the GPI anchor expressed in PA317 and ψ2 mouse fibroblasts was used as a source of PrP-sen (Kocisko *et al.*, 1994). The mutant PrP gene carries a stop codon at codon 231 and its translation product lacks the GPI anchor because the GPI attachment site (Ser231) and the C-terminal GPI addition sequence are missing. We refer to this mutant PrP-sen as GPI(-)PrP-sen. Metabolic labeling of the cells with [³⁵S]methionine and purification of ³⁵S-labeled PrP-sen were performed as described previously (Caughey *et al.*, 1995) except for the elution of ³⁵S-labeled PrP-sen from protein A-Sepharose immunocomplex; PrP-sen was eluted with 0.1 M acetic acid (pH 2.8) and kept at 4°C until use. Although 3F4 antibody molecules were also present in the acetic acid eluates, attempts to dilute and neutralize the acid and re-immunoprecipitate the [³⁵S]PrP-sen with fresh protein A-Sepharose failed; this indicated that the 3F4 antibody did not recover activity after denaturation and storage in 0.1 M acetic acid. In some experiments, culture supernatants from [³⁵S]methionine-labeled cells were used.

Pre-treatment of purified [³⁵S]PrP-sen with antibodies or Fab fragments

Purified [³⁵S]GPI(-)PrP-sen (100 000 c.p.m.) was incubated in 4 µl of solution containing 1% Sarkosyl, 100 mM NaCl and 125 mM phosphate buffer (pH 7.0) at room temperature for 15 min. Then antibodies (0.2 µl/reaction as a low dose and 1 µl/reaction as a high dose) or Fab fragments (6 µg/reaction) were added and the mixture was incubated in 10 µl of solution containing 1% Sarkosyl, 100 mM NaCl and 75 mM phosphate buffer (pH 7.0) at room temperature for 90 min. The 'high dose' of each antibody corresponded to that which was capable of binding and immunoprecipitating at least 500 000 c.p.m. of [³⁵S]GPI(-) PrP-sen. For peptide competition analysis, antibodies were incubated with synthetic peptide in the presence of 1% Sarkosyl, 100 mM NaCl and 42 mM phosphate buffer (pH 7.0) at room temperature for 60 min. The antibody-PrP-sen mixture containing 20 000 c.p.m. of PrP-sen was used for each cell-free reaction or binding analysis. Quantitative comparison of the binding of these antibodies (at 'high dose') indicate that each bound and immunoprecipitated (with protein A-Sepharose) at least 60% as much [³⁵S]PrP-sen as the others under the explicit conversion reaction condition (see below) and that a further 2.5-fold increase in the amount of each antibody did not significantly increase these binding efficiencies (data not shown). This indicated that the 'high dose', at least, was sufficient for maximal binding of [³⁵S]PrP-sen by each antibody in the conversion reaction buffer.

Carboxypeptidase P and Y treatment

Immunopurified [³⁵S]GPI(-)PrP-sen (1 500 000 c.p.m.) was treated with 1.4 mM carboxypeptidase P and Y (Boehringer Mannheim) in 50 ml of solution containing citrate (pH 6.0), 0.2% zwittergent 3-14, for 0-60 min. Aliquots of the reaction were stopped by adding phenylmethylsulfonyl fluoride (PMSF) to 1 mM, leupeptin to 0.5 µg/ml, and aprotinin to 1 µg/ml.

Cell-free conversion reactions

PrP-res was purified without PK treatment from the brains of hamsters infected with the 263K strain as described previously (Caughey *et al.*, 1991a). The purity of the preparation was shown elsewhere (Caughey *et al.*, 1995). Cell-free reactions in the presence of GdnHCl were performed as described elsewhere (Kocisko *et al.*, 1994; Caughey *et al.*, 1995). In brief, PrP-res was incubated with 2.5 M GdnHCl at 37°C for 1 h, and then 100 ng of GdnHCl-treated PrP-res was mixed with [³⁵S]GPI(-)PrP-sen (20 000 c.p.m.) in the presence of 1 M GdnHCl,

1.25% Sarkosyl, 5 mM cetyl pyridinium chloride and 50 mM citrate buffer (pH 6.0). Cell-free reactions without GdnHCl were performed as follows: PrP-res was diluted with deionized water and sonicated for 15 s, and then 100 ng of PrP-res was mixed with [³⁵S]GPI(-)PrP-sen (20 000 c.p.m.) in 20 µl of reaction mixture containing 0.625% Sarkosyl, 5 mM MgCl₂ and 50 mM citrate buffer (pH 6.0), and various concentrations of KCl or NaCl. In both conditions, the reaction mixtures were incubated at 37°C for 2 days. Nine-tenths of the reaction mixtures were treated with 20 µg/ml of PK [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, in 100 µl] at 37°C for 45 min. Digestion by PK was stopped by adding Pefabloc (Boehringer Mannheim) to 2 mM, 20 µg of thyroglobulin as a carrier protein and then 4 vols of methanol. The remaining one-tenth of the reaction mixture was analyzed without PK treatment. The protein samples collected by centrifugation were subjected to SDS-PAGE using Novax pre-cast acrylamide gels (Novax), and radioactive proteins were visualized and quantified with a PhosphorImager (Molecular Dynamics).

Binding analyses

With purified PrP-sen. A 100 ng aliquot of PrP-res and 20 000 c.p.m. of purified [³⁵S]GPI(-)PrP-sen were mixed in 20 µl of binding buffer [200 mM KCl, 5 mM MgCl₂, 1.25% Sarkosyl and 50 mM citrate buffer (pH 6.0)] and incubated at 37°C for 24-48 h. The reaction mixture was centrifuged at 10 000 g for 15 min. The supernatant was saved as the unbound fraction. The resulting pellet was washed once with 150 µl of the binding buffer and centrifuged again. The final pellet was dissolved with 20 µl of 1% SDS. The unbound fraction and dissolved pellet were analyzed by SDS-PAGE.

With culture supernatants. The culture supernatants from [³⁵S] methionine-labeled cells were centrifuged at 10 000 g for 10 min before use. A 10 µl aliquot of the supernatants was mixed with 100 ng of PrP-res in 20 µl of solution containing 1.25% Sarkosyl, 200 mM KCl and 50 mM citrate buffer (pH 6.0) and incubated at 37°C for 24 h. Centrifugation was done in the same manner as above. The final pellet was dissolved with 1× sample buffer [4 M urea, 5% SDS, 3 mM EDTA, 4% β-mercaptoethanol, 5% glycerol, 0.02% Bromophenol Blue and 62.5 mM Tris-HCl (pH 6.8)].

With cell lysates. Metabolically [³⁵S]methionine-labeled cells were lysed with lysis buffer [1% NP-40, 1% sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl and protease inhibitor cocktails] and centrifuged at 10 000 g for 10 min at 4°C. A 4 µl aliquot of cell lysate was mixed with 100 ng of PrP-res in 20 µl of solution containing 1.25% Sarkosyl, 200 mM KCl and 50 mM citrate buffer (pH 6.0), and incubated at 37°C for 24 h. Centrifugation was done in the same manner as above, except that the pellet was washed three times.

Immunoblotting

Transfer of the proteins from acrylamide gels to Immobilon-P membranes (Millipore) was performed as described elsewhere (Caughey *et al.*, 1991b). PrP on the membrane was visualized by using anti-PrP antibodies and an ECF Western blotting reagent pack (Amersham), and quantified with a PhosphorImager (Molecular Dynamics).

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