A Proposed Mechanism for the Promotion of Prion Conversion Involving a Strictly Conserved Tyrosine Residue in the $\beta_2$-$\alpha_2$ Loop of PrP$^{C*}$

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The transmission of infectious prions into different host species requires compatible prion protein (PrP) primary structures, and even one heterologous residue at a pivotal position can block prion infection. Mapping the key amino acid positions governed by cross-species prion conversion has not yet been possible, although certain residue positions have been identified as restrictive, including residues in the $\beta_2$-$\alpha_2$ loop region of PrP. To further define how $\beta_2$-$\alpha_2$ residues impact conversion, we investigated residue substitutions in PrP$^C$, using an in vitro prion conversion assay. Within the $\beta_2$-$\alpha_2$ loop, a tyrosine residue at position 169 is strictly conserved among mammals, and transgenic mice expressing mouse PrP having the Y169G, S170N, and N174T substitutions resist prion infection. To better understand the structural requirements of specific residues for conversion initiated by mouse prions, we substituted a diverse array of amino acids at position 169 of PrP. We found that the substitution of glycine, leucine, or glutamine at position 169 reduced conversion by $\sim$75%. In contrast, replacing tyrosine 169 with either of the bulky, aromatic residues, phenylalanine or tryptophan, supported efficient prion conversion. We propose a model based on a requirement for tightly interdigitating complementary amino acid side chains within specific domains of adjacent PrP molecules, known as “steric zippers,” to explain these results. Collectively, these studies suggest that an aromatic residue at position 169 supports efficient prion conversion.

Infectious prions can be transmitted to individuals expressing the same PrP$^{C^2}$ sequence, yet interspecies transmission to hosts expressing a different PrP$^{C^2}$ sequence requires overcoming a transmission barrier. Nevertheless, during the bovine spongiform encephalopathy epidemic, interspecies prion transmission led to infection of at least 15 species, including exotic bovids, felids, non-human primates, and humans (1-4). Chronic wasting disease prions from elk and deer readily cause infection of other cervid species (5-9), yet the risk of spread to humans, livestock, or other wildlife remains unclear. Identifying the molecular determinants of prion conversion is critical to predicting the susceptibility of a species to infection.

The mammalian prion protein consists of $\sim$210 amino acids arranged in a disordered amino-terminal tail and a globular C-terminal domain composed of three $\alpha$-helices and a short anti-parallel $\beta$-sheet (10, 11). Prion disease develops when a $\beta$-sheet-rich prion protein aggregate, known as PrP$^{\mathrm{Sc}}$, recruits and templates the conversion of the normal cellular prion protein, PrP$^C$, in an autocatalytic process (12, 13). The primary structure of host PrP$^C$ profoundly impacts cross-species prion transmission; for example, a polymorphic site in sheep PrP influences susceptibility to classical sheep scrapie infection, in that sheep expressing PrP with Gln-168 or Arg-168 (human numbering (14)) are susceptible or resistant to infection, respectively (15, 16). Additionally, nearly all patients with variant Creutzfeldt-Jakob disease, likely due to bovine spongiform encephalopathy transmission to humans, encode a methionine at polymorphic codon 129 of the PRNP gene (17). Single residue substitutions in mouse PrP$^C$ have also been shown to reduce or prevent prion conversion in vitro (e.g. I139M (18), N155Y (19), Q166R (20, 21), Q219E (20), Q172R (22), and N174S (23) (human numbering (14))). Interestingly, several substitutions that inhibit prion formation are located within the $\beta_2$-$\alpha_2$ loop of PrP (residues 165-175), suggesting that the amino acid sequence of this region may impact prion conversion.

Microcrystal structures of select hexapeptide segments from the prion protein have revealed a cross-$\beta$ fibril spine consisting of pairs of tightly packed $\beta$-sheets aligned parallel to the fibril axis. In each sheet, segments form backbone hydrogen bonds with segments above and below them along the fibril axis.
Between the two $\beta$-sheets, complementary side chains tightly interdigitate in a "steric zipper," forming a dry interface within the protofibril core (24, 25). Because this highly organized structure requires interdigitating side chains, heterologous PrP molecules with incompatible side chain interactions could sterically clash, which may explain the species barriers observed in prion disease (26, 27). For example, steric zipper segments composed of PrP residues 138–143 of hamster and human PrP crystalize into different space groups, with variation in the arrangement of $\beta$-strands and $\beta$-sheets (27). These differences in the preferred packing arrangements of the side chains, particularly at positions 138 and 139 (methionine and isoleucine) would probably lead to a steric clash for interacting segments of hamster and human PrP (27), in agreement with the poor fibrilization of a mixture of PrP segments (residues 23–144) having substitutions at positions 138 and 139 (28). The $\beta$-2-$\alpha$ loop of PrP has also been crystallized and forms parallel $\beta$-sheets with side chains arranged in a steric zipper (24).

We previously demonstrated that residues 170 and 174 within the $\beta$-2-$\alpha$ loop act as a molecular switch in transgenic mice expressing mouse PrP with S170N and N174T substitutions (MoPrP$^{170,174}$). Tg(MoPrP$^{170,174}$) mice showed increased susceptibility to chronic wasting disease and hamster prions as compared with mice expressing wild type (WT) mouse PrP (MoPrP) (29). The secondary structure of the MoPrP$^{170,174}$ variant shows a well defined, "rigid" $\beta$-2-$\alpha$ loop, whereas the WT MoPrP loop is disordered by NMR spectroscopy (30). Thus, the altered susceptibility observed in Tg(MoPrP$^{170,174}$) mice could have been due to a difference in the primary structure or to the variant loop conformation. Interestingly, transgenic mice expressing mouse PrP with the D167S substitution (MoPrP$^{167}$), which also results in a well defined $\beta$-2-$\alpha$ loop by NMR (31), show no detectable change in species barriers (32), suggesting that the PrP primary sequence may override the secondary structure in promoting prion conversion.

Within the $\beta$-2-$\alpha$ loop (166–175), only 3 residues are strictly conserved, Tyr-169, Gln-172, and Asn-173 (33, 34). NMR structural studies have shown that a Y169G substitution modifies the loop structure from a 310-helix turn to a type-1 $\beta$-turn (35). We recently found that transgenic mice expressing MoPrP having the Y169G substitution together with the S170N and N174T substitutions completely resist infection with either mouse or deer prions, implicating tyrosine 169 as critical for prion conversion (36). We set out to test how amino acid side chains at position 169 influence conversion and to then consider our results in the context of atomic level models of PrP$^{\text{Sc}}$ structure. Here, we performed a series of in vitro prion conversion experiments in which diverse amino acids were substituted at position 169 of mouse PrP. We found robust differences in prion conversion among the PrP$^{\text{C}}$ variants, and we propose a structural model based on amino acid side chain interactions within a steric zipper comprising PrP residues 167–176 to explain these results.

**EXPERIMENTAL PROCEDURES**

**Prion Conversion Using PrP Mutants as Substrates**—Mouse Prnp cDNA containing the rigid loop mutations (corresponding to S170N and N174T) was subcloned into the pcDNA3.1C vector (Invitrogen). Site-directed mutagenesis was performed to obtain WT-pcDNA3.1C and to introduce the 3F4 tag (residues Met-109 and Met-112, human PrP numbering) into the PrP sequence. Further site-directed mutagenesis (Agilent) was performed to generate mouse Prnp encoding PrP with various amino acid substitutions. Mutations in the Prnp sequence were confirmed by DNA sequencing.

Confluent RK13 cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, cells were washed twice and harvested in 1 ml of PBS. Cells were then centrifuged at 1000 $\times$ g for 1 min, and the pellet was resuspended in PMCA buffer (PBS containing 1% Triton X-100 and 0.05% saponin) prior to being passed twice through a 27-gauge needle. The lysates were then centrifuged at 2000 $\times$ g for 1 min, the pellets were discarded, and protein concentrations of the supernatants were measured by a bicinchoninic acid assay.

To seed the lysates, 5 µl of a 10% prion-infected brain homogenate in PBS was added to 45 µl of RK13 cell lysates in 0.2 ml PCR tubes. Tubes were then positioned in a microplate sonicator (Qsonica S-4000) programmed to perform repeated cycles of 10-min incubation at 37 °C followed by a 5-s pulse of sonication at potency 50–60%. After 24 h, samples were digested with 100 µg/ml proteinase K for 30 min at 37 °C and analyzed by SDS-PAGE. Undigested samples for comparison of PrP$^{\text{C}}$ levels in different lysates consisted of 1.5 µl from unseeded samples.

**Western Blot and Quantification of PrP Signal**—Immunoblotting was performed using the anti-PrP antibody 3F4 (37) followed by HRP-conjugated anti-mouse secondary antibody. Signals were captured and quantified using a Fujifilm LAS-4000 imager and Multi Gauge software. For quantification of conversion efficiency, the ratio of proteinase K-resistant PrP to total PrP was calculated and compared with WT samples according to the formula, $(\text{PrP}^{\text{Sc}}/\text{PrP}^{\text{C}})_{\text{mutant}}/(\text{PrP}^{\text{Sc}}/\text{PrP}^{\text{C}})_{\text{WT}}) \times 100$. The conversion products were easily distinguished from the PrP$^{\text{Sc}}$ by the 3F4 antibody because the 3F4 epitope is not present in mouse PrP$^{\text{Sc}}$. Unseeded controls were included in all experiments to evaluate the PrP$^{\text{C}}$ mutants for proteinase K-resistant PrP that may develop from self-aggregation. At least three experimental replicates were performed.

**Modeling $\beta$-2-$\alpha$ Loop Steric Zippers**—In order to explore structural models of prion conversion at the $\beta$-2-$\alpha$ loop, the zipper structure of PrP peptide 167–176 was built using Rosetta (38). The segment 167–176 was modeled as a parallel $\beta$-sheet. The pair of $\beta$-sheets was assembled by exploring all four possible arrangements (class 1–4). The zipper structure of PrP(167–176) was refined by simultaneously optimizing the rigid body degree of freedom between the $\beta$-sheets, side chain, and backbone torsions of each $\beta$-strand, guided by full-atom Rosetta energy functions (39). Taking advantage of the recently developed symmetry implementation in Rosetta (40), the fibril symmetry of each peptide subunit was restrained to assure that symmetrical geometry was satisfied during the whole optimization process. Finally, the models were inspected based on Rosetta energy and the packing between $\beta$-sheets, and the final zipper models with strong predicted zipper energy were selected. A class 4 zipper arrangement, highly similar to the
recently determined atomic structure of PrP(171–176) peptide fibrils, was most consistent with our experimental data.

RESULTS

To assess the effect of residue substitutions on PrPc to PrPsc conversion, we performed seeded conversion experiments using PrPsc derived from cell lysates as a substrate and PrPSc from prion-infected brain homogenate as a seed as described recently (22, 41, 42). PrPC-deficient RK13 cells (Fig. 1A, left) were transfected with a plasmid encoding mouse PrP with the 3F4 epitope tag, which is recognized by the 3F4 antibody (3F4, right), which recognizes various mammalian PrP structures (53). POM19 labeling shows that untransfected RK13 cells lack detectable PrPc. B, RK13 cell lysates expressing WT PrPc were seeded with RML or 22L prions and were either subjected to cycles of sonication at 37 °C (lanes 1 and 3) or were frozen (lanes 2 and 4). C, PrPc containing the Ser-167 substitution was efficiently converted by RML and 22L prions (RML, 127 ± 10%; 22L, 160 ± 42%) (mean ± S.E.) compared with WT mouse PrPc. D, conversion of PrPc containing the Arg-168 or Asn-170/Thr-174 substitutions was minimal compared with WT mouse PrPc (for Arg-168, RML was 10 ± 4% and 22L was 5 ± 1%; for Asn-170/Thr-174, RML was 4 ± 1% and 22L was 7 ± 2%). E, conversion of PrPc containing the Y169G and S170N/N174T substitutions was scarcely detectable as compared with WT mouse PrPc (RML, 6 ± 1%; 22L, 3 ± 0%). PrPc levels were uniform among the samples (–PK lanes). PK, proteinase K. Data are representative of at least three independent experiments. Quantification is an average of all experiments performed for each mutant PrP.

FIGURE 1. Prion conversion using mutant PrP-expressing cells replicates in vivo prion susceptibility. A, PrPc was only detected in RK13 cell lysates after transfection with Prnp plasmids. Anti-PrP monoclonal antibodies used were 3F4 (right), which recognizes the 3F4 epitope (residues 109–112), or POM19 (left), which recognizes various mammalian PrP structures (53). POM19 labeling shows that untransfected RK13 cells lack detectable PrPc. B, RK13 cell lysates expressing WT PrPc were seeded with RML or 22L prions and were either subjected to cycles of sonication at 37 °C (lanes 1 and 3) or were frozen (lanes 2 and 4). C, PrPc containing the Ser-167 substitution was efficiently converted by RML and 22L prions (RML, 127 ± 10%; 22L, 160 ± 42%) (mean ± S.E.) compared with WT mouse PrPc. D, conversion of PrPc containing the Arg-168 or Asn-170/Thr-174 substitutions was minimal compared with WT mouse PrPc (for Arg-168, RML was 10 ± 4% and 22L was 5 ± 1%; for Asn-170/Thr-174, RML was 4 ± 1% and 22L was 7 ± 2%). E, conversion of PrPc containing the Y169G and S170N/N174T substitutions was scarcely detectable as compared with WT mouse PrPc (RML, 6 ± 1%; 22L, 3 ± 0%). PrPc levels were uniform among the samples (–PK lanes). PK, proteinase K. Data are representative of at least three independent experiments. Quantification is an average of all experiments performed for each mutant PrP.

Aromatic Residue at PrP 169 Promotes Prion Conversion

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In Vitro Prion Conversion Assay Correlates with in Vivo Susceptibility—To assess whether the RK13 cell-derived PrP conversion assay reproduced known transmission barriers, we measured the conversion of mutant PrPc from in vivo models. In transgenic mouse models, the D167S substitution did not impact the susceptibility of mice to RML prions (32). Consistent with in vivo findings, here we found that the D167S substitution also had no impact on in vitro conversion by RML or 22L prions (Fig. 1C). In contrast, transgenic mice expressing the Q168R substitution are protected from prion infection (21), and similarly, the Q168R substitution blocked in vitro conversion by RML or 22L prions in the prion conversion assay (Fig. 1D). Furthermore, conversion of PrPc containing the S170N and N174T substitutions was reduced to only 4–7% (Figs. 1D and 2A), analogous to

3 H. MacFarlane and D. Eisenberg, unpublished data.
Aromatic Residue at PrP 169 Promotes Prion Conversion

Recruitment of monomeric PrP\textsuperscript{C} into \(\beta\)-sheet-rich PrP\textsuperscript{Sc} requires sequence similarity, and certain residues obstruct conversion (20, 22, 48). Adhesive segments of the prion protein may contain key residues that contribute to the stability of a \(\beta\)-sheet (24). One such segment in PrP was identified as the \(\beta_2\)-2 loop, which is rich in asparagine, glutamine, and tyrosine residues that could provide increased hydrogen bonding between \(\beta\)-strands (24, 25). Single substitutions within the \(\beta_2\)-2 loop can inhibit prion conversion (20, 22, 48, 49), yet the molecular mechanism underlying the inhibition is unclear. Previous work indicates that transgenic mice expressing WT PrP\textsuperscript{C} with substitutions at loop positions 169, 170, and 174 resist infection with prions from mice and deer (36). Using the \textit{in vitro} cell-free conversion assay to test the individual 169, 170, and 174 substitutions, we found that Y169G had the most pronounced effect on prion conversion.

### TABLE 1

\textit{In vitro} prion conversion assay correlates with \textit{in vivo} susceptibility

| PrP\textsuperscript{C} sequence | Inoculum | Attack rate | In vitro conversion relative to WT PrP\textsuperscript{C} (Mean ± S.E.) |
|---------------------------------|----------|-------------|---------------------------------------------------------------|
| MoPrP\textsuperscript{D167F} | RML      | 100% (32)   | 127 ± 10                                                     |
| MoPrP\textsuperscript{D167F} | RML      | 0% (21)     | 10 ± 4                                                       |
| MoPrP\textsuperscript{D167F} | E4        | 0% (36a)    | 4 ± 1                                                        |
| MoPrP\textsuperscript{D167F} | H9251     | 0% (36)     | 6 ± 1                                                        |

\(a\) Although the attack rate is 100%, Tg(MoPrP\textsuperscript{D167F}) mice express PrP\textsuperscript{C} at approximately 2–3-fold WT levels and have incubation periods more than 2-fold longer than WT mice, suggesting a significant barrier to infection with RML prions.

Tg(MoPrP\textsuperscript{170,174}) mice that show a barrier to infection with mouse prions.

We recently showed that mice expressing three substitutions in the \(\beta_2\)-2 loop at positions 169, 170, and 174 completely resist infection with mouse prions (RML and 22L) and deer prions (chronic wasting disease) (36). Here we found that the same substitutions (Y169G, S170N, and N174T) reduced \textit{in vitro} conversion by RML and 22L to only 3–6% of the levels observed with WT mouse PrP\textsuperscript{C} (Fig. 1E). These data indicate that the prion conversion assay accurately reflects the \textit{in vivo} susceptibility of transgenic mice to prion infection (Table 1).

Single \(\beta_2\)-2 Loop Residue Substitutions Differ in Their Impact on Prion Conversion—The Y169G, S170N, and N174T substitutions readily supported conversion by mouse prions at position 169. We modeled other amino acid substitutions at position 169, including histidine, isoleucine, and valine. The zipper models formed by these residues contain a cavity, suggesting that they would not support efficient prion conversion. Calculations of the predicted zipper energies suggest that formation of a zipper containing His, Ile, Val, Leu, Gly, or Gln at position 169 would be less energetically favorable than Tyr. In contrast, the predicted energy for formation of a zipper containing Trp or Phe at position 169 is comparable with that of Tyr. Taken together, our experimental results on prion conversion are compatible with the steric zipper model.

DISCUSSION

Recruitment of monomeric PrP\textsuperscript{C} into \(\beta\)-sheet-rich PrP\textsuperscript{Sc} requires sequence similarity, and certain residues obstruct conversion (20, 22, 48). Adhesive segments of the prion protein may contain key residues that contribute to the stability of a \(\beta\)-sheet (24). One such segment in PrP was identified as the \(\beta_2\)-2 loop, which is rich in asparagine, glutamine, and tyrosine residues that could provide increased hydrogen bonding between \(\beta\)-strands (24, 25). Single substitutions within the \(\beta_2\)-2 loop can inhibit prion conversion (20, 22, 48, 49), yet the molecular mechanism underlying the inhibition is unclear. Previous work indicates that transgenic mice expressing WT PrP\textsuperscript{C} with substitutions at loop positions 169, 170, and 174 resist infection with prions from mice and deer (36). Using the \textit{in vitro} cell-free conversion assay to test the individual 169, 170, and 174 substitutions, we found that Y169G had the most pronounced effect on prion conversion.
How does the Y169G substitution inhibit prion conversion? The strictly conserved Tyr-169 has a key role in maintaining the α-helical turn in the PrPC loop, and the Y169G substitution results in the loss of a β-stacking interaction with 175F and a switch to a type I β-turn, forming a well-defined loop (35, 50, 51). The new loop orientation may obstruct PrPSc-PrPC interactions, preventing efficient binding and conversion. However, we have found that two MoPrPC variants, MoPrP^D167S and MoPrP^S170N/N174T, both transform the disordered β2-α2 loop into a well-defined “rigid loop” structure yet have opposing effects on species barriers; the MoPrP^D167S causes no detectable impact on species barriers, whereas the MoPrP^S170N/N174T substitutions have a major impact on species barriers. We also report here that the MoPrP^D167S variant, which shows a well-defined β2-α2 loop by NMR (52), was efficiently converted by mouse prions (Fig. 5). Thus, prion conversion may require certain compatible features in the primary structures of PrP that override secondary structural differences, and further studies will aid in distinguishing between these possibilities.

A second possible structural explanation for the strong impact of the Y169G and other non-aromatic amino acid substitutions on prion conversion is the altered side chain interactions of residues 165–175 predicted to occur within a β-sheet. The atomic structure of crystallized PrP peptide fibrils encompassing amino acids 170–175 has been well characterized (27) and forms the basis of the models proposed here (Fig. 4). The models are consistent with our experimental data, in which the size of the side chains at position 169 contributes to prion conversion efficiency. Bulky residues (Phe, Tyr, and Trp) at position 169 could facilitate prion conversion by providing maximum hydrophobic contacts and tighter shape complementarity at zipper interfaces, whereas residues with either hydrophobic or hydrophilic side chains (such as Leu or Gln) could not support conversion because they offer fewer residue contacts and...
result in poor packing at zipper interfaces. Whether other transmission barriers can be explained by the steric zipper at 170–175 remains to be seen.

Although the seeded prion conversion assay does not report the absolute susceptibility of a species to prion disease, the assay enables a comparison of how specific residue substitutions singly or in combination impact conversion relative to the WT sequence using post-translationally modified PrP. This work together with previous findings by our laboratory and others demonstrates that residues within the β2-α2 loop may modulate susceptibility to prion disease. Because multiple steric zipper segments have been identified in PrP, including the β2-α2 loop (24, 26, 27), different PrPSc aggregates may have different zipper segments exposed, and thus the PrPc-PrPSc interacting segments may shift depending on the PrPSc conformation. Additionally, the packing arrangement within a single zipper may alter the side chains that interact (25). It will be of great interest to further investigate how the exposed segments and the side chain packing arrangements in distinct PrPSc conformations impact species barriers in prion disease.

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