Multiple Amino Acid Residues within the Rabbit Prion Protein Inhibit Formation of Its Abnormal Isoform

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Transmissible spongiform encephalopathies (TSEs) are neurological diseases that are associated with the conversion of the normal host-encoded prion protein (PrP-sen) to an abnormal protease-resistant form, PrP-res. Transmission of the TSE agent from one species to another is usually inefficient and accompanied by a prolonged incubation time. Species barriers to infection by the TSE agent are of particular importance given the apparent transmission of bovine spongiform encephalopathy to humans. Among the few animal species that appear to be resistant to infection by the TSE agent are rabbits. They survive challenge with the human kuru and Creutzfeldt-Jakob agents as well as with scrapie agent isolated from sheep or mice. Species barriers to the TSE agent are strongly influenced by the PrP amino acid sequence of both the donor and recipient animals. Here we show that rabbit PrP-sen does not form PrP-res in murine tissue culture cells persistently infected with the mouse-adapted scrapie agent. Unlike other TSE species barriers that have been studied, critical amino acid residues that inhibit PrP-res formation are located throughout the rabbit PrP sequence. Our results suggest that the resistance of rabbits to infection by the TSE agent is due to multiple rabbit PrP-specific amino acid residues that result in a PrP structure that is unable to refold to the abnormal isoform associated with disease.

Transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders that include kuru and variant Creutzfeldt-Jakob disease (vCJD) in humans, chronic wasting disease (CWD) in deer and elk, scrapie in sheep and goats, and bovine spongiform encephalopathy (BSE). A critical event in TSE pathogenesis is the conversion of the normal, protease-sensitive host prion protein (PrP-sen) to an abnormal, partially protease-resistant form (PrP-res) that is closely associated with TSE. PrP-sen is a glycoprotein with an apparent molecular mass of 33 to 37 kDa, which is anchored to the cell membrane by a glycosylphosphatidylinositol anchor (3). No characteristic sequence differences or chemical modifications between PrP-sen and PrP-res have been identified (13), suggesting that the two isoforms differ mainly in their conformation (8, 23, 31, 33).

Initial transmission of the TSE agent from one species to another is usually associated with prolonged incubation times, which decrease as the TSE agent adapts over multiple passages through the new species. Understanding this so-called “species barrier” is of particular importance, because the BSE agent has crossed the human species barrier to cause vCJD and there are concerns that the CWD agent in North America could cross species barriers and pose a threat to human health. Transmission of TSE agents to laboratory animals such as mice, hamsters, and rats has greatly enhanced our understanding of the TSE species barrier. These animals are susceptible to a variety of TSE agents, including those from sheep, humans, and cattle. Rabbits are the only mammalian species reported to be resistant to TSE agents isolated from different species. Rabbits do not develop signs of TSE disease after inoculation with the CJD, kuru, or scrapie agent (10). Additionally, no clinical signs were observed when rabbits were challenged with scrapie agent that had been previously passaged in mice (1). These experiments suggest that resistance to infection by the TSE agent is a characteristic of the host species rather than the strain of TSE agent used for infection.

Previous studies with transgenic mice, scrapie-infected tissue cultures, or cell-free conversion assays for PrP-res formation have demonstrated that the PrP amino acid sequence strongly influences both PrP-res formation and interspecies transmission of the TSE agent (16, 25, 28, 34). The transmission of TSE agent from one species to another appears to be dependent on amino acid sequence homology between the host PrP-sen and the PrP-res in the inoculum. In several model systems, both species-specific formation of PrP-res and the transmission of the TSE agent across species barriers have been mapped to the central region of the PrP molecule comprising residues 108 to 171 (16, 25, 35). Recently, experiments with transgenic mice suggest that amino acid residues in the C terminus can also influence interspecies transmission of the TSE agent (36). Thus, the apparent resistance of rabbits to various TSE agents may reside in specific amino acid residues within the same regions of the rabbit PrP-sen molecule (1, 10, 19).

We have studied the ability of rabbit PrP-sen to be converted to PrP-res in mouse neuroblastoma cells persistently infected with mouse-adapted scrapie (Sc*)-MNB cells. Here we show that rabbit PrP-sen was not converted to PrP-res in these cells. Multiple amino acid residues throughout the rabbit PrP sequence correlated with the inability of rabbit PrP-sen to form PrP-res. However, in one instance, the negative effect of a
specific amino acid residue on the formation of PrP-res could be overcome by altering an adjacent amino acid residue. Thus, the inability of rabbit PrP to be converted to PrP-res is likely due to the overall structural characteristics of the rabbit PrP molecule.

MATERIALS AND METHODS

Cell lines. Scrapie-infected mouse neuroblastoma cells (Sc″-MNB cells) are persistently infected with the mouse-adapted scrapie strain RML and have been described previously (29). These cells express mouse PrP-sen and accumulate mouse PrP-res. Cells were grown in Optimem (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). The retroviral packaging cell lines ø2 and PA317 (20, 21) used to produce retroviral supernatants were maintained in RPMI containing 10% FBS and 300 U of penicillin per ml.

Antibodies. The mouse monoclonal antibody L42 (R-biopharm, Darmstadt, Germany) was raised against a sheep PrP peptide and recognizes an epitope present in rabbit PrP, but not in mouse PrP (11). This epitope can be inserted into mouse PrP by an amino acid substitution from tryptophan to tyrosine at position 144 (40). R30 is a rabbit polyclonal antisera directed against a mouse PrP peptide spanning amino acids 89 to 103 (30). R30 reacts with mouse PrP, but does not cross-react with rabbit PrP. Since the exact epitopes for the antibodies present in the polyclonal serum have not been defined, it is possible that this serum reacts with PrP chimeras that contain mouse PrP-specific amino acid residues in a region spanning residues 89 to 103 (MoRa and mouse PrP constructs containing single- and double-amino-acid residue substitutions). Thus, when these constructs were expressed in Sc″-MNB cells, we refer to PrP-res detected by this antisera as “total PrP-res”.

Site-directed mutagenesis. The rabbit PrP open reading frame was amplified by PCR with rabbit chromosomal DNA. Chimeric mouse-rabbit (MoRa) PrP molecules were generated by utilizing the restriction sites Ndel and BstIII. Since the Ndel site is absent in the mouse and rabbit PrP sequence, it was inserted as a silent mutation at position 334 of the PrP coding region. The epitope for the mouse monoclonal antibody L42 was either present in the recombinant PrP molecules that contained rabbit sequence from 112 to 177 (constructs Ra and RaMo in Fig. 2A) or was introduced by site-directed mutagenesis (constructs MoRa, RaMoRa, and MoL42 in Fig. 2A and 4). Additional single amino acid residue changes within MoL42 PrP were introduced by PCR mutagenesis. The specific change is indicated in the name of the construct (e.g., MoV214I has a mouse PrP-specific valine at position 214 replaced by a rabbit PrP-specific isoleucine). Recombinant PrP molecules were cloned into the retroviral expression vector pSFF (4), and the sequence was verified by DNA sequencing.

Transduction and analysis of Sc″-MNB cells expressing recombinant PrP. Production of retroviral particles coding for PrP molecules and transduction of Sc″-MNB cells were performed as previously described (25). Seven days after transduction with the retroviral particles, cell lysates were tested for PrP-sen and PrP-res. Expression of recombinant PrP-sen in Sc″-MNB cells was confirmed either by Western blot analysis of cell lysates or by immunoprecipitation of radiolabeled PrP (41) with the mouse monoclonal antibody L42. Following proteinase K (PK) treatment of cell lysates (20 μg of PK per ml, 37°C, 1 h), samples were subjected to centrifugation (260,000 × g, 4°C, 1 h), and pellets were sonicated in sample buffer (2.5% sodium dodecyl sulfate [SDS], 3 mM EDTA, 2% β-mercaptoethanol, 5% glycerol, 0.02% bromphenol blue, 63 mM Tris-HCl [pH 6.8]). Samples were electrophoresed on 14% NOVEX precast gels (Invitrogen) and transferred to polyvinylidene difluoride (PVDF) membrane, and blots were developed by using the Pharmacia ECL enhanced chemiluminescence kit according to the manufacturer’s instructions. Total PrP-res was analyzed with the rabbit polyclonal antisera R30. Recombinant PrP-res was detected with the mouse monoclonal antibody L42.

RESULTS

Rabbit PrP is not converted to protease resistance in Sc″-MNB cells. To determine if rabbit PrP-sen could be converted to protease resistance, rabbit PrP-sen was expressed in Sc″-MNB cells. These cells are persistently infected with the mouse-adapted scrapie strain RML, express mouse PrP-sen, and accumulate mouse PrP-res. Rabbit PrP-sen was transduced into Sc″-MNB cells as previously described, and the cells were analyzed for the expression of rabbit PrP by using the mouse monoclonal antibody L42, which reacts only with rabbit PrP. Rabbit PrP-sen was readily expressed in Sc″-MNB cells (Fig. 1A). However, no protease-resistant rabbit PrP was detected (Fig. 1B). Thus, the mouse PrP-res present in the Sc″-MNB cells was unable to convert rabbit PrP-sen to protease resistance.

The expression of some exogenous PrP-sen molecules has been shown to interfere with the formation of endogenous mouse PrP-res in Sc″-MNB cells (12, 25, 27, 41). It has been postulated that this interference is due to exogenous PrP molecules blocking the PrP-res binding site (14). Therefore, we studied the effect of rabbit PrP-sen expression on the accumulation of endogenous mouse PrP-res in Sc″-MNB cells. Sc″-MNB cells expressing rabbit PrP accumulated substantially lower levels of endogenous mouse PrP-res compared to untransduced cells (Fig. 1C). The fact that the expression of rabbit PrP-sen interfered with endogenous mouse PrP-res accumulation suggests that the inability of rabbit PrP-sen to form PrP-res was not due to an inability to bind to mouse PrP-res. Furthermore, these results suggest that the resistance of rabbit PrP to mouse scrapie challenge could be due to the inability of mouse PrP-res to convert the rabbit PrP-sen molecule to its abnormal, PK-resistant isofrom.

Regions in rabbit PrP that inhibit conversion to PrP-res. To identify the regions in rabbit PrP that inhibit conversion to PrP-res, several chimeric rabbit-mouse PrP constructs were generated. PrP molecules were constructed that encoded either rabbit- or mouse-specific amino acid sequences from residues 1 to 111 (hereafter defined as the N-terminal region), residues 112 to 177 (central region), or residues 178 to 254 (C-terminal region) (Fig. 2A). To detect the chimeric PrP molecules over the background of endogenous mouse PrP expressed in Sc″-MNB cells, all constructs contained the epitope for the mouse monoclonal antibody L42. Recombinant PrP molecules were expressed in Sc″-MNB cells and assayed for their ability to form PrP-res. Expression of L42-positive...
PrP-sen was detected in all cells except control cells (Fig. 2B, Mock) or cells transduced with the retroviral vector pSFF alone (Fig. 2B). As expected, MoL42 PrP-sen molecules were readily converted to PrP-res, demonstrating that the introduction of the L42 epitope did not significantly influence the formation of PrP-res (Fig. 2C) (42). Substitution of mouse PrP amino acid sequence with rabbit PrP amino acid sequence at the C terminus drastically decreased but did not abolish the formation of L42-positive PrP-res (Fig. 2C, MoRa PrP). Interestingly, additional substitution of mouse amino acid sequence with rabbit amino acid sequence at the N terminus totally prevented PrP-res formation (Fig. 2C, RaMoRa PrP). Finally, the construct RaMo PrP, which codes for rabbit amino acid residues in the N terminus and middle portion of PrP and mouse PrP residues in the C terminus, failed to be converted to prionase resistance. All of the mouse-rabbit chimeras also interfered with the accumulation of total PrP-res (Fig. 2A and D). These experiments suggest that amino acid residue differences within the N terminus, C terminus, and possibly the central portion of the rabbit PrP-sen molecule all negatively affected the formation of PrP-res.

Multiple amino acid residues in rabbit PrP inhibit PrP-res formation. The amino acid sequences of rabbit and mouse PrP differ at 22 residues within the mature protein (Fig. 3A). Several of these differences reside in regions of the PrP molecule known to be important in PrP-res formation. To further map the amino acid residues within rabbit PrP-sen that prevent PrP-res formation, we chose to concentrate on several amino acid candidates. In vivo and in vitro studies have demonstrated that residues 1 to 94 within the N-terminal region of the PrP molecule are not crucial for the formation of PrP-res (9, 18, 32). However, three amino acid differences between rabbit and mouse PrP reside within the remaining N-terminal region (positions 95 to 111) (Fig. 3A): amino acid residues 99, 107, and 108. One of these, a serine at position 107, appears to be unique to the rabbit PrP sequence.

Rabbit-specific amino acid residues were introduced into MoL42 PrP at positions 99, 107, and 108 and the constructs expressed in Scv-MNB cells (Fig. 4A). The expression of MoN107S led to the formation L42-positive PrP-res and did not affect the accumulation of total PrP-res (Fig. 4B and C). In contrast, MoN99G and MoL108M were not converted to PrP-res and interfered with the accumulation of total PrP-res (Fig. 4B and C). Interestingly, MoN107S/L108M was readily converted to prionase resistance and did not interfere with total PrP-res accumulation (Fig. 4B and C). Thus, at least two different rabbit-specific amino acid residues in the N-terminal portion of PrP negatively affected PrP-res formation. In some
instances, however, the negative effect of a specific residue could be compensated for by another amino acid substitution.

We next examined the effect of amino acid substitutions within the middle portion of the PrP molecule. Three amino acid differences between rabbit and mouse PrP are present in the central region of the molecule (residues 112 to 177) (Fig. 3A and B). Replacement of the mouse-specific tryptophan by tyrosine at position 144 introduces the epitope for the mouse monoclonal antibody L42 and does not significantly affect the formation of PrP-res (Fig. 2) (42). Similarly, MoM137L was readily converted to PrP-res (Fig. 4B) and had no influence on total PrP-res accumulation (Fig. 4C). In contrast, expression of MoN173S abolished formation of L42-positive and total PrP-res (Fig. 4B and C).

Amino acid residues within the C terminus of Ra PrP also appeared to influence the formation of PrP-res in Sc57-MNB cells (Fig. 2). Ten amino acid differences reside within the flexible tail of PrP-sen. Since the N-terminal flexible tail of PrP-sen has few or no stable structural elements, it is not depicted in the nuclear magnetic resonance structure shown. This figure was prepared with the program MOLMOL (17).

**FIG. 3.** Comparison of mouse and rabbit PrP. (A) Amino acid sequence comparison of mouse and rabbit prion proteins. The numbering of the amino acid residues is according to the mouse PrP sequence. The overall sequence homology between mouse and rabbit PrP is 87% (33 total amino acid residue differences). During posttranslational processing, a signal peptide and a glycosylphosphatidylinositol (GPI) anchor signal peptide are cleaved off, decreasing the number of amino acid differences in the mature PrP molecules to 22. The secondary structure elements of mouse PrP-sen are indicated by gray boxes. Boxed residues indicate the residues tested. Dashes indicate identical amino acid residues, and deletions are symbolized by dots. (B) Nuclear magnetic resonance spectroscopy structure of mouse PrP amino acid residues 120 to 230 (31). The positions of the amino acid residues 137, 144, 173, and 214 are indicated. These residues were altered in mouse PrP, and their influence on the formation of PrP-res was studied. Additional amino acid residue substitutions included residues 99, 107, and 108, which reside within the flexible tail of the mouse PrP molecule. Since the N-terminal flexible tail of PrP-sen has few or no stable structural elements, it is not depicted in the nuclear magnetic resonance structure shown. This figure was prepared with the program MOLMOL (17).
FIG. 4. Amino acid residue substitutions at codons 99, 108, 173, and 214 in MoL42 PrP inhibit its conversion into PrP-res. (A) Sc/H11001-MNB cells expressing mutant MoL42 PrP were labeled with a [35S]methionine-[35S]cysteine mixture, and PrP molecules were immunoprecipitated from the cell lysates with the monoclonal antibody L42. The autoradiogram demonstrates the expression of each construct. (B) Detection of L42-positive PrP-res. Constructs MoN99G, MoL108M, and MoN173S were not converted to PrP-res. (C) Analysis of total PrP-res by using the polyclonal antiserum R30. The expression of constructs MoN99G, MoL108M, and MoN173S drastically decreased total PrP-res levels. (D) MoV214I PrP-sen expression was analyzed by Western blotting (left panel). The expression of MoV214I PrP-sen led to the formation of multiple bands (right panel). The expression of MoV214I PrP-sen was detected with antibodies to the conversion of PrP-res (upper panel) and with antibodies to the expression of PrP-sen (middle panel). (E) Summary of the results in panels B, C, and D.
previous studies have demonstrated the importance of prion protein codon 214 (15) for PrP-res formation, we chose this position as a candidate residue within the C terminus that could potentially influence the conversion of PrP-sen to PrP-res. When MoV214I PrP was expressed in Sc + -MNB cells (Fig. 4D, left panel), only a small amount of this mutant was converted to PrP-res (Fig. 4D, middle panel), while the amount of total PrP-res decreased (Fig. 4D, right panel). Thus, at least one amino acid residue that decreased PrP-res formation was also present in the C-terminal region of rabbit PrP. In conclusion, amino acid residues in the N terminus, the central region, and the C terminus of rabbit PrP can all inhibit the formation of PrP-res in Sc + -MNB cells (Fig. 4E).

**DISCUSSION**

Previous studies have demonstrated that amino acid residues that can affect the interspecies induction of PrP-res are located in the central region (16, 25, 35) or C terminus (36) of the PrP molecule. Here we show that amino acid residues that interfered with the formation of PrP-res in scrapie-infected mouse neuroblastoma cells were present throughout the rabbit PrP-sen molecule. Thus, these results suggest that PrP amino acid differences in the N terminus, the C terminus, and the central part can all potentially influence the TSE species barrier.

We have shown that rabbit PrP-sen is not converted to PrP-res in tissue culture cells persistently infected with mouse-adapted scrapie. Since conversion of PrP-sen to PrP-res is believed to be the fundamental process underlying TSE agent pathogenesis, the resistance of rabbits to mouse TSE agent could be due to the inability of mouse PrP-res to convert rabbit PrP-sen to rabbit PrP-res. Recent studies suggest that the amino acid residues critical for species-specific PrP-res formation can differ, depending upon the species from which PrP-sen and PrP-res originate. For example, in the case of the species barrier between mice and hamsters, sequence homology at either amino acid residue 138 or 154 is important, depending upon whether or not PrP-res has been isolated from mice or hamsters, respectively (26). Thus, it is possible that within the rabbit PrP sequence, amino acid residues other than those identified here could influence the TSE species barriers that exist between rabbits and other mammals besides mice (1, 10). However, the fact that amino acid residues negatively affecting PrP-res formation in scrapie-infected mouse neuroblastoma cells are located throughout the rabbit PrP molecule suggests that the three-dimensional structure of rabbit PrP either cannot easily refold to PrP-res or is converted to PrP-res only at an extremely low efficiency by PrP-res molecules originating from different species. This may explain why rabbits are among the few mammalian species resistant to challenge with several different TSE agents.

Two rabbit-specific amino acid residues (99G and 108M) that inhibited the formation of PrP-res in scrapie infected mouse neuroblastoma cells reside within the N-terminal domain of the PrP-sen molecule. While this polypeptide segment appears to be relatively flexible and unstructured, it may be involved in intra- or intermolecular interactions with the protein core, the cell surface, or with a natural ligand (5). Amino acid residues 90 to 120 within this region have also been demonstrated to be crucial for both PrP-res formation (12, 18, 32) and interspecies transmission of TSE agent (9, 39). During PrP-res formation, the N-terminal domain appears to undergo major structural changes to a more structured conformation and is thus partially protected from proteolytic cleavage within the PrP-res aggregate (24, 37). Therefore, one explanation for the detrimental effect of residues 99G and 108M on PrP-res formation is that an N-terminal region containing these amino acid residues would be unable to undergo this conformational change.

An unexpected finding of this study was that within the N terminus, a rabbit-specific methionine at position 108 in Mol42 PrP prevented PrP-res formation, while Mol42 PrP molecules with rabbit-specific residues at positions 108 and 107 were readily converted to PrP-res. This demonstrates that the negative effect of one amino acid residue can be compensated for by an additional amino acid residue substitution. Again, these data suggest that it is the overall tertiary structure of PrP-sen rather than sequence homology that is the critical determinant in the induction of PrP-res formation. A compensatory effect could also explain why PrP molecules lacking amino acid residues 23 to 88 and 141 to 176 are conversion competent, although they lack regions shown to be important in PrP-res formation (38, 41).

We have also identified amino acid residues that inhibit the conversion of rabbit PrP-sen to its abnormal isoform within the central region and the C-terminal portion of the PrP molecule (residues 173S and 214I). Residue 173 is located in the turn leading into the second α-helix (Fig. 3B), an area that has been implicated in the species barrier between mink and ferrets (2). Amino acid mismatches in this region appear to also critically affect the interspecies transmission of scrapie between different breeds of sheep or mice (22, 43, 44) and the formation of sheep and mouse PrP-res (6, 7, 30). Both amino acid residues 173 and 214 are part of a surface region of PrP formed by the loop between the second β-strand and the second α-helix and part of the C terminus (Fig. 3B) that could potentially function as an initial interaction site between PrP-sen and PrP-res (residues 164 to 173 and 205 to 222) (14). Substitutions at positions 173 (N→S) and 214 (V→I) are not expected to alter the global structure of this interaction site. However, they could possibly modify its specificity for both long-range electrostatic interactions and short-range hydrogen bonding with other proteins (5).

Interestingly, amino acid residues within this surface region (residues 167, 171, 214, and 218) have also been proposed to be involved in the binding of an as-yet-unidentified factor, protein X, hypothesized to be involved in the conversion process (15). The identification of the protein X binding site was based on tissue culture experiments demonstrating that the expression of mouse PrP mutated at these residues could interfere with PrP-res formation. However, as shown here and by others, amino acid residues other than the proposed protein X binding residues also interfere with PrP-res formation (12, 25, 27, 41). Additionally, cell-free conversion assays using purified PrP-res and PrP-sen have demonstrated that heterologous PrP-sen molecules bind to PrP-res in regions containing the putative protein X site, thus blocking PrP-res formation (14). Therefore, the effect of changes at residue 214 on PrP-res formation is most likely due to direct competition between different PrP-sen molecules for a common binding site on PrP-res.
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