

Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation

(species barrier/Creutzfeldt–Jakob disease/dominant negative)

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ABSTRACT Studies on the transmission of human (Hu) prions to transgenic (Tg) mice suggested that another molecule provisionally designated protein X participates in the formation of nascent scrapie isoform of prion protein (PrP^{Sc}). We report the identification of the site at which protein X binds to the cellular isoform of PrP (PrP^C) using scrapie-infected mouse (Mo) neuroblastoma cells transfected with chimeric Hu/MoPrP genes even though protein X has not yet been isolated. Substitution of a Hu residue at position 214 or 218 prevented PrP^{Sc} formation. The side chains of these residues protrude from the same surface of the C-terminal α -helix and form a discontinuous epitope with residues 167 and 171 in an adjacent loop. Substitution of a basic residue at positions 167, 171, or 218 also prevented PrP^{Sc} formation: at a mechanistic level, these mutant PrPs appear to act as “dominant negatives” by binding protein X and rendering it unavailable for prion propagation. Our findings seem to explain the protective effects of basic polymorphic residues in PrP of humans and sheep and suggest therapeutic and prophylactic approaches to prion diseases.

Many lines of evidence have converged to argue persuasively that prions are composed largely, if not exclusively, of the scrapie isoform of prion protein (PrP^{Sc}) (1). The discovery that mutations in the PrP gene cause inherited prion disease in humans (2), which is transmissible to laboratory animals (3–5), and the generation of infectious prions in transgenic (Tg) mice expressing mutant PrP assert that prions are devoid of nucleic acid (6–8). Furthermore, the recent demonstration that prion diversity can be enciphered in the conformation of PrP^{Sc} no longer demands a scrapie-specific nucleic acid to explain the existence of strains of prions (9, 10).

That the cellular isoform of PrP (PrP^C) interacts with PrP^{Sc} during the formation of nascent PrP^{Sc} was surmised from Tg mouse studies where mice expressing a Syrian hamster (SHa) PrP transgene were susceptible to SHa prions (11). When similar Tg mice were produced expressing human (Hu) PrP, no transmission of Hu prions was found. However, mice expressing a chimeric Hu/MoPrP transgene denoted MHu2M were susceptible to Hu prions. In addition, we found that Tg mice expressing HuPrP did become susceptible to Hu prions when they were crossed with PrP-deficient (Prnp^{0/0}) mice. These data taken together argued that it is likely that a molecule other than PrP is involved in the formation of PrP^{Sc}. We assumed that this molecule is a protein and designated it “protein X” (5). Based on the results with the MHu2M transgene and earlier studies showing that the N terminus of PrP is not required for PrP^{Sc} formation (12), we surmised that the binding of PrP^C to protein X is likely to occur

through specific side chains of amino acids located at the C terminus of PrP^C.

Using scrapie-infected mouse neuroblastoma (ScN2a) cells transfected with a chimeric PrP gene in which the Mo C terminus was replaced by Hu residues, we identified the binding site for protein X. Substitution of a Hu residue at position 214 or 218 prevented mouse (Mo) PrP^C from being converted into PrP^{Sc}. The side chains of residues 214 and 218 protrude from the same surface of the C-terminal α -helix and form a discontinuous epitope with residues 167 and 171 in an adjacent loop. Substitution of a basic residue at position 167, 171, or 218 also prevented PrP^{Sc} formation: at a mechanistic level, these mutant PrPs appear to act as “dominant negatives” by binding to protein X and functionally sequestering it from the replication process. Our findings may explain the protective effects of polymorphic basic residues in PrP against Creutzfeldt–Jakob disease (CJD) and scrapie in humans and sheep, respectively.

MATERIALS AND METHODS

Cultured Cells and Antibodies. Mouse neuroblastoma (N2a) cells were obtained from American Tissue Culture Collection. ScN2a cells are the persistently infected clones as described (13). All the cells were grown and maintained at 37°C in minimal essential medium supplemented with 10% fetal bovine serum. α -PrP 3F4 is a mAb raised against SHa PrP27–30 (14). RO73 is an antiserum raised in a rabbit against SDS/PAGE-purified SHa PrP27–30 (15).

Recombinant Gene Construction. MHM2 PrP was constructed as described (16). MHM2, MH2M, or MoPrP in pSPOX was digested with *Bst*EII and *Hind*III; then the 1.4-kbp fragment was ligated into *Bst*EII–*Hind*III double-digested Hu or MHMHuA (Mo residues 214, 218, and 219 replaced with Hu) PrP in pSPOX, yielding MHMHu(A/B) (combined replacements), MH2HuA and M3HuA. MHMHu(A/B) in pSP72 was digested with *Xho*I and *Stu*I, then either the 2.4-kbp or the 880-bp fragment was ligated to MHM2 in pSP72, yielding MHMHuA or MHMHuB (Mo residues 226, 227, 228, and 230 replaced with Hu), which were then introduced into the expression plasmid pSPOX.

To create MHM2 encoding specific amino acid changes, *Bst*EII and *Stu*I or *Kpn*I restriction sites were introduced using mismatched oligonucleotides. After PCR, amplified fragments were digested with restriction enzymes and then ligated into the MHM2 PrP in the pSP72 vector. Subsequently the mutated MHM2 PrPs were introduced into the expression plasmid pSPOX.

Transfection and Western Blot Analysis. ScN2a cells were transiently transfected with each construct using a DNA

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Abbreviations: Hu, human; Tg, transgenic; PrP, prion protein; PrP^{Sc}, scrapie isoform of PrP; PrP^C, cellular isoform of PrP; Mo, mouse; CJD, Creutzfeldt–Jakob disease; SHa, Syrian hamster; Prnp^{0/0}, PrP-deficient; N2a, mouse neuroblastoma; ScN2a, scrapie-infected mouse neuroblastoma; wt, wild type.

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transfection kit (DOTAP; Boehringer Mannheim). Cell lysis and Western blot analyses were performed as described (16).

Cotransfection in which ScN2a cells were exposed simultaneously to two different genes showed that transfected cells co-expressed the two genes at an extremely high frequency (>85%) in 10–15% of total ScN2a cells in which the transfection was successfully proceeded (data not shown) (17). When equal amounts of two different DNAs cloned into pSPOX were applied for the transfection as in the current experiments, the expression levels of each molecule were equal by Western blot analysis (data not shown).

RESULTS

Seven amino acids distinguish HuPrP from MoPrP at the C terminus (residues 168–231). Four of these residues are close to the glycosylphosphatidylinositol anchor attached to Ser-231 whereas the remaining three residues lie within the C-terminal α -helix (18–20). We reasoned that replacement of Mo residues with Hu counterparts at these positions would inhibit PrP^{Sc} formation given the previous evidence for the species specific preferences for protein X (5). To distinguish the recombinant PrP^{Sc} from endogenous wild-type (wt) MoPrP^{Sc}, we used the SHa/Mo chimeric PrP designated MHM2 that contains an epitope for the anti-SHaPrP 3F4 mAb (16). In one assay, we measured the conversion of mutated MHM2 PrP into PrP^{Sc} which allowed us to assess the influence of amino acid substitutions on PrP^{Sc} formation. In a second assay, we measured the ability of mutated MHM2 PrP to inhibit the conversion of wt MHM2 PrP into PrP^{Sc}. With this second assay, we were able to measure the relative affinities of two PrPs for protein X.

Mutant PrP Conversion into PrP^{Sc}. Three chimeric constructs, denoted as MHMHuA, MHMHuB, and MHMHu(A/B), were transfected transiently into ScN2a cells. Neither MHMHu(A/B) nor MHMHuA was converted into PrP^{Sc} as judged by the absence of protease resistance (Fig. 1*B*). In contrast, MHMHuB was

converted into PrP^{Sc} as efficiently as the control MHM2. We interpreted these results as indicating that Mo protein X did not bind to MHMHu(A/B) or MHMHuA but did recognize MHMHuB and MHM2, both of which were converted into PrP^{Sc}. The mutant PrP molecules were all expressed at about the same level (Fig. 1*A* and *D*) and no inhibition of wt MoPrP^{Sc} formation could be detected (Fig. 1*C* and *F*).

Having identified the HuA region that prevents conversion of modified PrP^C into PrP^{Sc}, we produced additional constructs with Mo residues 214, 218, and 219 replaced by their Hu counterparts. To test the replacement of these residues either alone or in combination, we generated five constructs and expressed them in ScN2a cells. Substitution of Hu residue 218 abolished PrP^{Sc} (Fig. 1*E*, lanes 8, 9 and 11) whereas substitution of Hu residue 219 was not inhibitory (Fig. 1*E*, lane 10). Substitution of Hu residue 214 was partially inhibitory (Fig. 1*E*, lanes 7). Studies of chimeric PrP^C release from the cell surface with phosphatidylinositolphospholipase C digestion revealed no topological changes (data not shown).

Specificity of PrP Mutations at Residues 214 and 218. In humans, position 219 corresponding to Mo 218 is polymorphic: in Caucasians only Glu at this residue has been reported, whereas about 12% of the Japanese population have the Lys allele (21). The substitution of Lys at Mo residue 218 abolished PrP^{Sc} formation (Fig. 2, lane 4). To examine the specificity of amino acid substitutions at position 218, we introduced seven artificial mutations: Ile, Ala, Trp, Pro, Phe, Arg, or His (Table 1). The constructs expressing Ala, Pro, Phe, Arg, or His at position 218 were not converted into PrP^{Sc}, whereas, low amounts of PrP^{Sc} were made with Ile or Trp at residue 218 (Fig. 2*B*, lanes 5–8; data not shown).

Substitution of Hu Ile at residue 214 diminished but did not completely abolish PrP^{Sc} formation (Fig. 2*B*, lane 11); similarly, modest PrP^{Sc} formation was observed with Ala. No PrP^{Sc} was observed when Lys, Glu, Trp, or Pro were substituted at position 214 (Fig. 2*B*, lanes 12–16). The mutant PrP molecules were all expressed at about the same level (Fig. 2*A*) except when Pro was

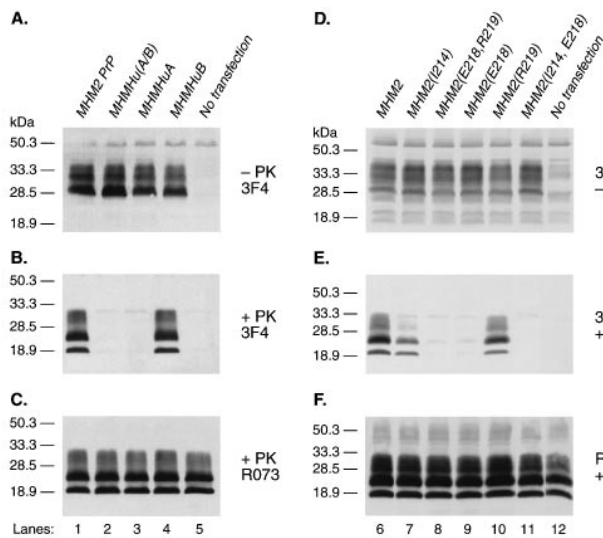


FIG. 1. Characterization of the binding site for protein X. Western blot analysis of each MHM2 chimeric construct expressed in ScN2a cells is shown. (*A–C*) Lanes: 1, MHM2 PrP; 2, MHMHu(A/B); 3, MHMHuA; 4, MHMHuB; and 5, untransfected control ScN2a cells. (*D–F*) Lanes: 6, MHM2 PrP; 7, MHM2(I214); 8, MHM2(E218,R219); 9, MHM2(E218); 10, MHM2(R219); 11, MHM2(I214,E218); and 12, untransfected control ScN2a cells. *A* and *D* demonstrate the expression of each chimeric MHM2 PrP construct: 40 μ l of undigested cell lysates was applied to each lane and MHM2 PrP was detected by staining with α -PrP 3F4 mAb. *B* and *E* demonstrate the conversion of chimeric MHM2 PrP^C into PrP^{Sc} and were stained with α -PrP 3F4 mAb. *C* and *F* show endogenous MoPrP^{Sc} as well as chimeric constructs detected with α -PrP R073 rabbit antiserum. In *B–C* and *E–F*, 500 μ l of cell lysate was digested with proteinase K (20 μ g/ml) at 37°C for 1 h followed by centrifugation at 100,000 \times g for 1 h and the loading of the resuspended pellet onto the gel.

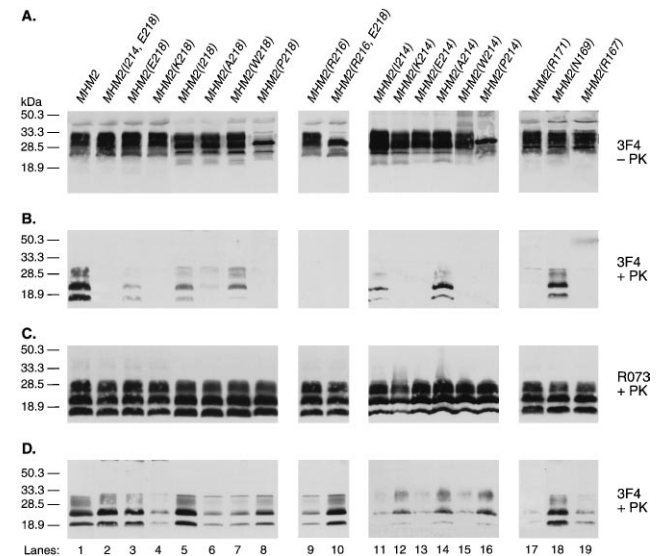


FIG. 2. Mutations at codons 214, 216, and 218 affect PrP^{Sc} formation. Western blot analysis of mutated MHM2 PrP constructs expressed in ScN2a cells. Films were exposed longer than other figures to detect faint signals. (*A–C*) Lane: 1, MHM2 PrP; 2, MHM2(I214,E218); 3, MHM2(E218); 4, MHM2(K218); 5, MHM2(I218); 6, MHM2(A218); 7, MHM2(W218); 8, MHM2(P218); 9, MHM2(R216); 10, MHM2(R216,E218); 11, MHM2(I214); 12, MHM2(K214); 13, MHM2(E214); 14, MHM2(A214); 15, MHM2(W214); 16, MHM2(P214); 17, MHM2(R171); 18, MHM2(N169); and 19, MHM2(R167). (*D*) Coexpression with MHM2 in the same orientation as in *A–C*. Samples were prepared and processed as described in the Fig. 1 legend.

Table 1. Mutations in epitope tagged MHM2 PrP inhibit PrP^{Sc} formation in ScN2a cells

Mo codon number	PrP residue*				Mutant MHM2	Type of inhibition of PrP ^{Sc} formation
	Mouse	Human†	Syrian hamster†	Sheep‡		
167	Q	E	Q	Q/R	R	2
					E	1
169	S	S	N	S	N	None
170	N	N/S	N	N	S	None
171	Q	Q	Q	Q	R	2
209	V	V	V	V	K	1
210	E	E	E	E	K	None
211	Q	Q	Q	Q	K	None
214	V	I	T	I	I	2
					K	1
					E	2
					A	1
					W	2
					P	1
					Q	None
215	T	T	T	T	Q	None
216	Q	Q	Q	Q	R	3
218	Q	E/K	Q	Q	E	1
					K	2
					I	1
					A	2
					W	2
					P	1
					F	1
					R	2
H	2					
219	K	R	K	R	R	None
221	S	S	S	S	A	None
222	Q	Q	Q	Q	K	None

*Multiple residues at a particular position indicate naturally occurring polymorphisms.

†The corresponding codon number is that of Mo increased by one.

‡The corresponding codon number is that of Mo increased by four.

substituted at position 218, Arg at 216, or Pro at 214 (Table 1). Substitutions of Pro in the C-terminal α -helix are expected to destabilize this secondary structure and may result in the increased lability of the protein. No inhibition of wt MoPrP^{Sc} formation by the mutant PrP molecules could be detected (Fig. 2C).

Mutant PrPs and Conversion of wt PrP into PrP^{Sc}. Because only a minority of the ScN2a cells express the mutant PrPs in these transient transfection experiments, we could not assess the effect of expressing mutant PrP on conversion of wt MoPrP into PrP^{Sc} (Figs. 1C and F and 2C). To measure the influence of mutant PrP on the conversion of wt PrP into PrP^{Sc}, we performed cotransfection studies. Several studies have established that the transfecting DNAs are generally taken up together and coexpressed.

Substitution of Glu, Ile, Pro, or Phe at residue 218 did not inhibit conversion of epitope tagged wt MHM2 PrP^C into PrP^{Sc} (Fig. 2D, lanes 2, 3, 5, and 8; data not shown). In contrast, Lys, Ala, Trp, Arg, or His at position 218 inhibited wt PrP^{Sc} formation (Fig. 2D, lanes 4, 6, and 7; data not shown). These results argue that MHM2 PrP with Lys, Ala, Trp, Arg, or His at residue 218 binds to protein X with a greater affinity than does wt MHM2 with Glu at 218 (Table 1). These findings also contend that the two polymorphic Hu residues Glu and Lys interact very differently with Mo protein X. Mutant MHM2 PrP(E218) binds more weakly to Mo protein X than does wt MHM2 PrP(Q218), which results in MHM2 PrP(E218) not being converted into PrP^{Sc} and no inhibition of the conversion of wt MHM2 PrP^C into PrP^{Sc}. In contrast, mutant MHM2 PrP(K218) binds more tightly to Mo protein X than does wt MHM2 PrP(Q218), which results in both MHM2 PrP(K218) not being converted into PrP^{Sc} and inhibition of the conversion of wt MHM2 PrP^C into PrP^{Sc}.

The substitution of Lys, Ala, or Pro at residue 214 did not inhibit conversion of epitope tagged wt MHM2 PrP^C into PrP^{Sc}

(Fig. 2D, lanes 12, 14, and 16; data not shown). In contrast, Ile, Glu, or Trp at position 214 inhibited wt PrP^{Sc} formation (Fig. 2D, lanes 11, 13, and 15; data not shown). These results argue that the MHM2 PrP carrying Ile, Glu, or Trp at position 214 binds to protein X with a greater affinity than does wt MHM2 with Val at position 214.

Mutation at Residue 216. The side chain of MoPrP Gln at residue 216 protrudes from the opposite face of the C-terminal α -helix relative to residues 214 and 218. Further, a mutation of the corresponding HuPrP Gln at residue 217 causes inherited prion disease; a Swedish family with Gerstmann-Sträussler-Scheinker disease has been reported with a Q \rightarrow R mutation (22). Although brain sections showed PrP amyloid plaques, extracts showed neither infectivity nor protease resistant PrP^{Sc} on Western blots (23, 24).

We introduced Arg at residue 216 in MHM2 PrP and MHM2 PrP(E218) which contains the Hu Glu residue at 218. Neither of these mutant PrPs acquired protease resistance when expressed in ScN2a cells (Fig. 2B, lanes 9 and 10). The Arg substitution at residue 216 inhibited conversion of epitope tagged wt MHM2 PrP^C into PrP^{Sc} (Fig. 2D, lane 9), whereas no inhibition was observed if both residues 216 and 218 were mutated (Fig. 2D, lane 10). We interpret these findings as showing that the Q \rightarrow R mutation destabilizes the structure of PrP^C leading to inherited prion disease but prevents folding PrP^{Sc} into a protease resistant form. The Mo Glu residue at 218 allows MHM2 PrP(R216) to compete with wt MHM2 PrP for binding to protein X, whereas the Hu Glu residue decreases the affinity of this protein for protein X. With the Hu Glu residue, no inhibition of the conversion of wt MHM2 PrP into PrP^{Sc} was observed (Fig. 2D, lane 10).

Mutations at Residues 215, 221, and 222. Substitution of Glu for Thr at MoPrP residue 215, Ala for Ser at Mo residue 221, or

Lys for Gln at Mo residue 222 did not inhibit conversion of these epitope-tagged MHM2 PrP molecules into PrP^{Sc} when expressed in ScN2a cells (Table 1). Coexpression of these mutant MHM2 PrPs with wt MHM2 PrP did not inhibit wt PrP^{Sc} formation. When the V214D mutation was introduced into MHM2 PrP(S221A), the protein was not converted into PrP^{Sc}, suggesting that the V214D mutation prevented binding to protein X.

Mutations at Residues 167, 169, 170, and 171. The NMR structure of SHa rPrP90–231 shows a loop composed of residues 165–171 immediately adjacent to the protein X binding site on the C-terminal helix raising the possibility that one or more of these residues also participate in the binding to protein X. To explore this possibility, we constructed mutants MHM2 PrP(Q167R), MHM2 (Q167E), MHM2 PrP(S169N), MHM2 PrP(N170S), and MHM2 PrP(Q171R) and transfected the DNAs into ScN2a cells. MHM2 PrP(N170S) is equivalent to human polymorphism N171S (25). MHM2 PrP(S169N) and MHM2 PrP(N170S) were converted into PrP^{Sc} (Fig. 2B, lane 18; data not shown), whereas MHM2 PrP(Q167R) and MHM2 PrP(Q171R) were not (Fig. 2B, lanes 17 and 19). MHM2(Q167E) exhibited less efficient conversion with no inhibition of wt MHM2 PrP^{Sc} formation (data not shown). The Asn or Ser substitutions at residues 169 or 170 did not inhibit conversion of epitope-tagged wt MHM2 PrP^C into PrP^{Sc} (Fig. 2D, lane 18; data not shown), whereas the Arg substitution at residues 167 or 171 inhibited PrP^{Sc} formation (Fig. 2D, lanes 17 and 19). These findings argue that Q167 and Q171 in MoPrP form a discontinuous epitope with V214 and Q218 to which protein X binds.

SHaPrP Inhibits Conversion of MHM2 into PrP^{Sc}. The level of SHaPrP^C expression in Tg(SHaPrP)Prnp^{+/+} mice was directly proportional to the length of the incubation time after inoculation with Mo prions (11). To simulate these conditions in ScN2a cells, epitope-tagged MHM2 PrP was coexpressed with SHaPrP or chimeric SHa/MoPrP. SHaPrP and chimeric MH3 PrP (16) inhibited conversion of MHM2 PrP into PrP^{Sc}, but this inhibition was relieved by substitution of Hu residues at positions 214, 218, and 219, designated HuA (data not shown).

The foregoing findings help define the order of addition during formation of the protein X/PrP^C/PrP^{Sc} complex as well as the limits of the central domain of PrP where PrP^C and PrP^{Sc} interact. When Hu residues at 214, 218, and 219 (HuA) were introduced into MH2M or MH3 PrP, these chimeric PrPs no longer inhibited the conversion of MHM2 PrP into PrP^{Sc} (data not shown). Because the HuA substitutions relieve inhibition by preventing the binding of the chimeric MH3HuA PrP, we argue that the lack of conversion of SHaPrP or MH3 PrP into PrP^{Sc} is not due to a low affinity for protein X. Instead, MoPrP^{Sc} does not stimulate conversion of SHaPrP or MH3 PrP into PrP^{Sc} even though these molecules are probably bound to protein X. This contention is supported by the ability of SHaPrP^{Sc} to stimulate conversion of SHaPrP^C into PrP^{Sc} in Tg(SHaPrP)Prnp^{+/+} mice when MoPrP^C is coexpressed (11). From these data, it seems most likely that PrP^C binds first to protein X and the protein X/PrP^C complex then binds to PrP^{Sc}.

Because introduction of the HuA sequences into MH3 relieved the inhibition of MHM2 PrP conversion into PrP^{Sc}, we conclude that the protein X binding site does not include SHa residues 203 and 205 (data not shown). Instead, these residues seem to be part of the central domain where PrP^C and PrP^{Sc} interact because lack of conversion of MH3 into PrP^{Sc} by MoPrP^{Sc} in ScN2a cells could be partially overcome by changing these two SHa residues into Mo as found in MH2M PrP (data not shown). Because SHaPrP and MH3 PrP are not converted into PrP^{Sc}, they are not released from protein X, which in turn prevents MHM2 PrP from binding and being converted.

DISCUSSION

To explain the results on the transmission of Hu prions from the brains of CJD patients to Tg mice, we suggested that a macro-

molecule provisionally designated protein X participates in the conversion of PrP^C into PrP^{Sc} (5). In those studies, Hu prions did not transmit disease to Tg(HuPrP)Prnp^{+/+} mice coexpressing Hu and MoPrP^C but did transmit to Tg(MHu2M)Prnp^{+/+} mice coexpressing MHu2M PrP^C and MoPrP^C (26). Subsequently, transmission of Hu prions to Tg mice expressing HuPrP^C was achieved when the mice were crossed into a Prnp^{0/0} background. These findings were interpreted in terms of MoPrP^C binding to Mo protein X more avidly than HuPrP^C, thus inhibiting the conversion of HuPrP^C into PrP^{Sc} (5). MoPrP^C binding to Mo protein X was similar to that of MHu2M PrP^C; thus, MoPrP^C did not inhibit appreciably the conversion of MHu2M PrP^C into PrP^{Sc}. An alternative interpretation of these results was that the C terminus of MoPrP^C bound to HuPrP^{Sc} more avidly than HuPrP^C. In this scenario, heterologous PrP^C binds to PrP^{Sc} more avidly than does homologous PrP^C; yet homotypic interactions seem to govern conversion of PrP^C into PrP^{Sc} whenever this has been studied (11, 27).

In the studies reported here, the results seemed most readily interpreted in terms of the binding of PrP^C to protein X. If we try to explain the results in terms of PrP^C binding to PrP^{Sc}, then we must postulate that the C terminus of MoPrP^C binds more avidly to MoPrP^{Sc} than does that of MHu2M PrP^C. In other words, homologous PrP^C binds to PrP^{Sc} more avidly than does heterologous PrP^C; however, this is antithetical to the alternative interpretation offered above where heterologous PrP^C binds to PrP^{Sc} more avidly than does homologous PrP^C. On this basis, we argue that the data presented here in concert with the earlier results build a convincing edifice for the existence of protein X.

Structure of the Protein X Binding Site on PrP. Determination of the NMR structure of a recombinant fragment of SHaPrP corresponding to the residues in PrP 27–30 greatly facilitated our studies of the protein X binding site on PrP^C. Once we determined that MoPrP residues 214 and 218 were pivotal in the binding of PrP^C to protein X, we examined the orientation of their side chains on the surface of the C-terminal α -helix denoted helix C. MoPrP residues 214 and 218 correspond to SHaPrP residues 215 and 219. The side chains of these two residues protrude onto the surface of helix C away from the Asn-linked oligosaccharides and the interface that is formed when PrP^{Sc} binds to PrP^C (Fig. 3A) (20).

The loop consisting of residues 165–171 lies immediately adjacent to the region of helix C that contains residues that bind to protein X. Although no structure could be assigned to this loop in a study of a recombinant MoPrP fragment consisting of 111 amino acids (19), the proximity of the loop to the C-terminal helix and its potential role in the passage of prions from one species to another were appreciated (28). To assess the possible role of amino acids in this loop, substitutions at MoPrP residues 167, 169, 170, and 171 were made. The replacement of Gln with Arg at 167 or 171 completely abolished PrP^{Sc} formation. These substitutions also inhibited the conversion of wt MHM2 PrP into PrP^{Sc} arguing that the mutants MHM2 PrP(Q167R) and MHM2 PrP(Q171R) bind to protein X more avidly than does wt MHM2 PrP.

Mechanism of PrP^{Sc} Formation. Studies on the inhibition of PrP^{Sc} formation reported here provide considerable insight into the mechanism by which PrP^C is converted into PrP^{Sc}. Our results argue that PrP^C forms a complex with protein X and that PrP^{Sc} then binds to PrP^C resulting in a ternary complex. Whether PrP^C and PrP^{Sc} are monomers or dimers in this scheme is unknown. The ionizing radiation target size of prions is \approx 55 kDa suggesting that PrP^{Sc} in its infectious form is a dimer. Molecular modeling studies suggested that the N-terminal region (residues 90–140) of PrP^{Sc} might participate in the dimer interface. The stoichiometry of the protein X/PrP^C complex is unknown since the number of binding sites for PrP^C on protein X remains to be established. We presume that the protein X/PrP^C complex may play a role in the function of PrP^C which remains unknown; it is doubtful that the protein

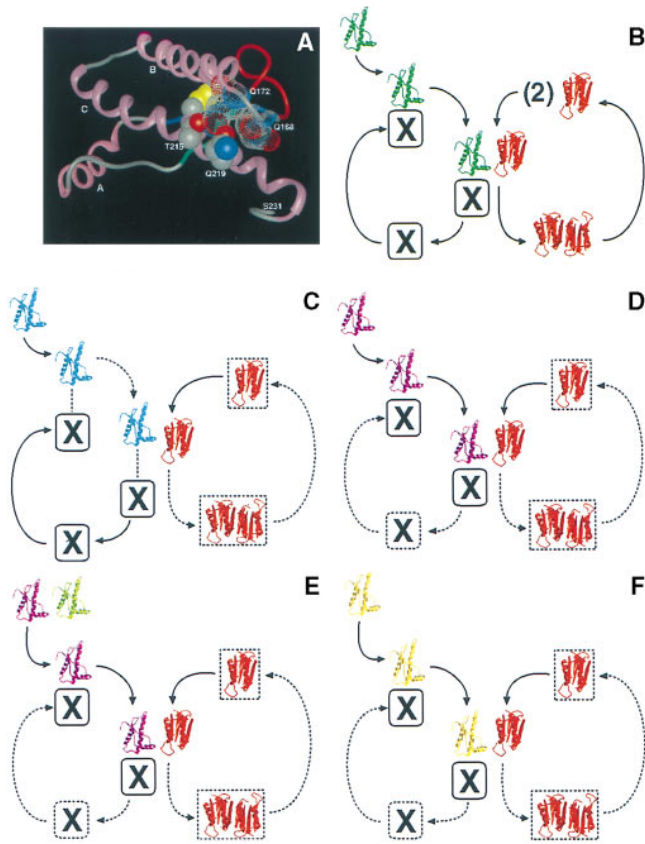


FIG. 3. The role of protein X in PrP^{Sc} formation and the influence of mutations in PrP^C on the prion replication cycle. (A) NMR structure of SHa rPrP90–231. The color scheme is as follows: α -helices A (residues 144–157), B (172–193), and C (200–227) in pink; disulfide between Cys-179 and Cys-214 in yellow; hydrophobic cluster composed of residues 113–126 in red; loops in gray; residues 129–134 in green encompassing strand S1 and residues 159–165 in blue encompassing strand S2; the arrows span residues 129–131 and 161–163, as these show a closer resemblance to β -sheet (20). Structure of protein X binding site of SHa rPrP90–231 illustrating the proximity of the 165–171 loop, where residues Q168 and Q172 are depicted with a low density van der Waals rendering and helix C residues T215 and Q219 depicted with a high density van der Waals rendering. SHaPrP residues Q168, Q172, T215, and Q219 correspond to MoPrP residues Q167, Q171, T214, and Q218, respectively. The illustration was generated with the program UCSF MIDASPLUS. (B) Ordering experiments demonstrate that PrP^C interacts with protein X prior to the creation of the PrP^C/PrP^{Sc} complex. Two cycles are required for PrP^{Sc} formation. The left hand cycle shows protein X binding to PrP^C (green) resulting in a heterologous complex that is then competent to interact with PrP^{Sc} (red). Upon conversion of PrP^C to nascent PrP^{Sc}, protein X dissociates from the complex owing to its relative lack of affinity for PrP^{Sc}. Protein X is subsequently recycled. The right hand cycle depicts the interaction of PrP^{Sc} with the PrP^C/protein X complex and the conversion of PrP^C into nascent PrP^{Sc}. With time, the result is an exponential increase in PrP^{Sc} concentration as the template for conversion is recycled. (C) Type 1 inhibition (Table 2): mutant PrP^C (blue) containing an amino acid substitution in the PrP^C/protein X interface (e.g., E218 in MoPrP) interacts weakly with protein X. Dotted lines depict the failure of the mutant PrP^C to interact with protein X and the subsequent inability to form the protein X/PrP^C/PrP^{Sc} complex. Under these circumstances PrP^{Sc} formation either does not occur or proceeds slowly. (D) Type 2 inhibition: mutant PrP^C (purple) containing an amino acid substitution in the protein X/PrP^C interface (e.g., K218 in MoPrP) forms a very tight complex. PrP^{Sc} is able to bind to this protein X/PrP^C complex but conversion of PrP^C to PrP^{Sc} is prevented owing to the failure of the protein X/PrP^C/PrP^{Sc} complex to release protein X. Dotted lines are shown for the steps in the replication cycle that are blocked. (E) Dominant-negative effect of tight binding mutants of PrP^C. Mutant PrP^C [e.g., K218 (purple)] successfully competes with wt PrP^C (green) for binding to protein X. The protein X/PrP^C(K218)/PrP^{Sc} complex is formed but conversion is inhibited as in D. (F) Type 3 inhibition:

X/PrP^C complex exists for the production of PrP^{Sc} unless PrP^{Sc} at low levels has a cellular function that has eluded us, to date.

In our studies we were able to distinguish three classes of inhibition of PrP^{Sc} formation, designated as types 1, 2, and 3 (Table 2, Fig. 3 B–F). Type 1 inhibition is illustrated by the competition between MoPrP^C and HuPrP^C(E219) for binding to Mo protein X. In the absence of MoPrP^C, HuPrP^C(E219) is converted into PrP^{Sc} (5). The mutant MHM2 PrP(E218) was not converted into PrP^{Sc} in ScN2a cells and did not prevent conversion of wt MHM2 PrP^C into PrP^{Sc}. Type 2 inhibition appears to be noncompetitive and is depicted by MHM2 PrP(K218) which binds to protein X in ScN2a cells and prevents conversion of wt MHM2 PrP^C into PrP^{Sc}. The binding is sufficiently tight that MHM2 PrP(K218) is also not converted into PrP^{Sc}. Type 3 inhibition is also noncompetitive with respect to protein X but occurs through a different mechanism. This case is demonstrated by SHaPrP^C which binds to Mo protein X but is not released by interacting with MoPrP^{Sc}. In Tg(SHaPrP) mice, SHaPrP^C is converted into PrP^{Sc} in the presence or absence of MoPrP^C when the animals are inoculated with SHa prions (11, 29, 30).

Human Polymorphism at Codon 219. A HuPrP polymorphism at codon 219, which corresponds to MoPrP codon 218, has been reported in the Japanese population (21); about 12% of the people carry the Lys allele instead of Glu. To date, the Lys allele has not been found in 50 autopsied CJD cases in Japan (T. Kitamoto, personal communication). This finding is highly significant (Fisher's exact test, $P = 0.00005$), which suggests that HuPrP^C(K219) acts as a dominant negative in preventing CJD. In view of the results presented here with MHM2 PrP(K219), it seems likely that the K219 allele prevents CJD through the high avidity of HuPrP^C(K219) for protein X. The high affinity binding of HuPrP^C(K219) to protein X prevents HuPrP^C(K219) from being converted into PrP^{Sc}, and it prevents HuPrP^C(E219) from interacting with protein X. The latter mode of action of HuPrP^C(K219) in patients heterozygous for the polymorphism would explain the dominant negative effect of the K219 substitution (Fig. 3E). When we introduced the K218 mutation into MHM2 PrP expressed in ScN2a cells, the recombinant protein was not converted into PrP^{Sc} and it inhibited the conversion of wt MHM2 PrP into PrP^{Sc}.

Sheep Polymorphism at Codon 171. In sheep, the substitution of a basic residue at position 171 probably prevents scrapie through a dominant-negative mechanism similar to that postulated for a basic residue at 219 protecting humans from CJD. With few exceptions, only sheep that are Q/Q at 171 develop scrapie; sheep that are Q/R or R/R are resistant (31–39). These findings suggest that R171 creates a PrP^C molecule in sheep that acts as a dominant negative in preventing PrP^{Sc} formation (Fig. 3E). When we introduced the Q167R or Q171R mutation into MHM2 PrP expressed in ScN2a cells, the recombinant protein was not converted into PrP^{Sc} and it inhibited the conversion of wt MHM2 PrP into PrP^{Sc}. Q167R and Q171 in MoPrP correspond to Q171 and Q175 in sheep PrP, respectively.

New Approaches to Preventing and Treating Prion Diseases. As our understanding of prion propagation increases, it should be possible to design effective therapeutics. Because people at risk for inherited prion diseases can now be identified decades before neurologic dysfunction is evident, the development of an effective therapy for these fully penetrant disorders is imperative (40, 41). Although we have no way of predicting the number of individuals who may develop neurologic dysfunction from bovine prions in the future (42), seeking an effective therapy now seems most prudent. Interfering with the conversion of PrP^C into PrP^{Sc} seems to be the

PrP^C from a distinct species [e.g., SHa (gold)] is able to bind Mo protein X, but the Mo protein X/SHaPrP^C/MoPrP^{Sc} complex is not competent for conversion. The result is that protein X is sequestered and scrapie prions are not replicated.

Table 2. Protein X-mediated mechanisms of inhibition of PrP^{Sc} formation

Type of inhibition	Example	Putative mechanism	Relative affinity for protein X*
1	HuPrP ^C (E219) binding to Mo protein X inhibited by MoPrP ^C	Competitive	Low
2	HuPrP ^C (K219) prevents MoPrP ^C binding to protein X	Noncompetitive	High
3	SHaPrP ^C binds to protein X and is not released by MoPrP ^{Sc}	Noncompetitive	Similar

*Affinity is relative to that of MoPrP^C for Mo protein X.

most attractive therapeutic target (43). Either stabilizing the structure of PrP^C by binding a drug or modifying the action of protein X that might function as a molecular chaperone are reasonable strategies. Whether it is more efficacious to design a drug that binds to PrP^C at the protein X binding site or one that mimics the structure of PrP^C with basic polymorphic residues that seem to prevent scrapie and CJD remains to be determined (Fig. 3A). Because PrP^{Sc} formation seems limited to caveolae-like domains, drugs designed to inhibit this process need not penetrate the cytosol of cells but they do need to enter the central nervous system. Alternatively, drugs that destabilize the structure of PrP^{Sc} might also be possible to construct.

The production of domestic animals that do not replicate prions may also prove to be important with respect to preventing prion disease. Sheep encoding the R/R polymorphism at position 171 seem resistant to scrapie (31–39); presumably, this was the genetic basis of Parry's scrapie eradication program in Great Britain 30 years ago (44, 45). A more effective approach using dominant negatives for producing prion resistant domestic animals including sheep and cattle is probably the expression of PrP transgenes encoding K219 or R171, or possibly both basic residues (Fig. 3E). Such an approach can be readily evaluated in Tg mice and once shown to be effective, it can be instituted by artificial insemination of sperm from males homozygous for the transgene.

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