Highly Promiscuous Nature of Prion Polymerization

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The primary structure of the prion protein (PrP) is believed to be the key factor in regulating the species barrier of prion transmission. Because the strength of the species barrier was found to be affected by the prion strain, the extent to which the barrier can indeed be attributed to differences in the PrP primary structures of either donor and acceptor species remains unclear. In this study, we exploited the intrinsic property of PrP to polymerize spontaneously into disease-related amyloid conformations in the absence of a strain-specified template and analyzed polymerization of mouse and hamster full-length recombinant PrPs. Unexpectedly, we found no evidence of species specificity in cross-seeding polymerization assays. Even when both recombinant PrP variants were present in mixtures, preformed mouse or hamster fibrils displayed no selectivity in elongation reactions and consumed equally well both homologous and heterologous substrates. Analysis of individual fibrils revealed that fibrils can elongate in a bidirectional or unidirectional manner. Our work revealed that, in the absence of a cellular environment, post-translational modifications, or strain-specified conformational constraints, PrP fibrils are intrinsically promiscuous and capable of utilizing heterologous PrP variants as a substrate in a highly efficient manner. This study suggests that amyloid structures are capable of accommodating local perturbations arising because of a mismatch in amino acid sequences and highlights the promiscuous nature of the self-propagating activity of amyloid fibrils.

Previous studies revealed that the transmission of mammalian prions between different species is limited because of a phenomenon referred to as the “species barrier” (1). The species barrier is typically seen as an increase in mean incubation time to disease, an increase in the range of incubation periods within a given host species, an abnormal neuropathological profile, and a drop in the percentage of animals succumbing to disease. The magnitude of the species barrier is thought to depend primarily on the differences in the amino acid sequence of the two PrP isoforms, PrPSc of the donor species and PrPC of the host (2, 3). The important role of PrP primary structure for prion transmission was indirectly supported by numerous reports where allelic heterogeneity in PrP sequence was shown to affect the prion transmission even within a single species (4–7). The species barrier is believed to be mediated through the physical interaction of PrPSc and PrPC, specifically via sequence-specific packing of the amino acid side chains within self-propagating cross-β-structures (8, 9). Formation of amyloid cross-β-structures was shown to be a common property of a polypeptide backbone regardless of its specific amino acid sequence (10). As such, fibril formation by proteins and peptides, even those not involved in the prion phenomenon, was found to exhibit strong species specificity (11). In vitro, the species specificity of amyloid formation was studied using cross-seeding reactions, where polymerization of a specific protein or peptide was seeded by fibrils made up of homologous or heterologous proteins or peptides. The efficacy of cross-seeding reactions was found to depend strongly on the extent to which the primary structure of seeds was similar to that of a substrate (12). Whereas the studies of the cross-seeded amyloid formation supported the idea that sequence differences account for the species specificity of prion propagation, the similar studies on mammalian prions were mostly limited to short peptide or unstructured protein fragments (13, 14). Furthermore, the efficiency of cross-seeding in the polymerization reactions that utilized PrP-derived peptides was found to be opposite to the efficacy of prion transmission observed in animals (14). Such a result is not surprising, considering that the amyloid cross-β-sheet structure formed by short PrP-derived peptides is likely to be fundamentally different from that of PrPSc. These findings, however, raise important questions as to whether the differences in PrP primary structures do indeed regulate the species barrier and, if so, whether the magnitude of the species barrier can be predicted solely based on such differences.

In this work, we used full-length PrP to investigate the role of PrP primary structure in regulating the efficiency of prion transmission between different species. Using an in vitro polymerization reaction that utilizes recombinant prion proteins (rPrP), we tested whether the polymerization of full-

The abbreviations used are: PrP, prion protein; PrPSc, normal cellular isoform of the prion protein; PrPSc, abnormal, disease-associated isoform of the prion protein; rPrP, recombinant PrP; Mo, mouse; Ha, hamster; AFTM, atomic force fluorescence microscopy; PK, proteinase K; ThT, thioflavine T; CJD, Creutzfeldt-Jakob disease; MES, 4-morpholineethanesulfonic acid; GdnHCl, guanidine hydrochloride; Ab, antibody; AFTM, atomic force microscopy; TSE, transmissible spongiform encephalopathy.

This work was supported by National Institutes of Health Grant NS045585 (to I. V. B.) and by the Program in Prion Diseases at the Medical Biotechnology Center, University of Maryland Biotechnology Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1 and 2.

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length rPrP mimics the species specificity of prion replication in the absence of a cellular environment, PrP glycosylation, or strain-specific conformational constraints. Surprisingly, we found no evidence of species specificity in cross-seeding polymerization assays that utilized mouse (Mo) and Syrian hamster (Ha) rPrPs. Even when Mo and Ha PrPs were present together in mixtures, rPrPs showed no selectivity in supporting elongation of fibrils made up of homologous or heterologous seeds. Our data strongly indicate that amyloid structures are intrinsically promiscuous, that the differences in PrP primary structure are not sufficient to block fibril growth in cross-seeded reactions, and that other factors such as strain-specific conformational constraints, species-specific glycosylation status, or host-encoded factors should be involved in regulating the species barrier.

**EXPERIMENTAL PROCEDURES**

Mo and Ha full-length recombinant PrPs encompassing residues 23–230 and 23–231, respectively, were expressed and purified as described earlier (15, 16). To form amyloid fibrils, stock solutions of rPrP were prepared immediately before use by resuspending lyophilized rPrP powder in 5 mM HEPES (pH 7.0). Solutions of rPrP were prepared immediately before use by resuspending lyophilized rPrP powder in 5 mM HEPES (pH 7.0).

**Formation of Amyloid Fibrils in 96-Well Plates**—Stock solutions of Mo or Ha rPrPs were diluted with MES (pH 6.0) and GdnHCl to final concentrations of 50 mM and 2 M, respectively, and a final protein concentration of 2 µM. ThT was added to the reaction mixture to a final concentration of 10 µM. The polymerization reactions were carried out in 96-well plates with a total reaction volume of 0.2 ml per well. To prepare fibrillar seeds for the seeded reactions, fibrils were formed in manual format as described below, then sonicated for 10 s using a Branson-2510 bath sonicator and added to the reaction mixtures to the final amount of 0.1% as calculated per total amount of rPrP substrate. 96-Well plates were incubated at 37 °C with constant shaking at 900 rpm in a Fluoroskan Ascent CF microplate reader (Thermo Labsystems) as described earlier (17). Multiple experiments were performed using Mo and Ha rPrPs purified in separate batches.

**Calculation of the Lag Phase of Polymerization Kinetics**—To estimate the lag phase of polymerization, the kinetic curves were fitted by quadratic and exponential functions that describe nucleation and elongation stages of fibril formation, respectively. These functions are limiting forms of hyperbolic cosine solution (Equation 1) of the first-order approximation of the reaction equation developed by Bishop and Ferrone (18) for analysis of the nucleation-controlled polymerization.

\[ F = A(\cosh Bt - 1), \quad F \to \frac{1}{2} B^t at Bt \to 0, \]

\[ F \to \frac{1}{2} A e^{Bt} at Bt >> 1 \quad (Eq. 1) \]

In Equation 1, \( F \) is the observable parameter (ThT fluorescence); \( t \) is polymerization time, and \( A \) and \( B \) are fitting coefficients. This approximation proved to be relevant as fitting coefficients for both stages corresponded to each other with less than 10% error. The lag phases were defined as time from the beginning of the polymerization reaction to the intersection of the fitting curves described by the quadratic and exponential functions. In the case of all seeded reactions, the fitting procedure gave the negative values for the intersection points, indicating the elimination of the nucleation stage.

**Formation of Fibrils in Manual Format**—Stock solutions of Mo or Ha rPrPs were diluted with MES (pH 6.0) and GdnHCl to final concentrations of 50 mM and 2 M, respectively, and to a final protein concentration of 0.25 mg/ml. The polymerization reaction was carried out in 1.5-ml conical plastic tubes (Fisher) at a total reaction volume of 0.42–0.6 ml at 37 °C with continuous rotation at 24 rpm using a Nutator (model 1105, Clay Adams). For the seeded reactions, preformed fibrils were sonicated for 10 s using Branson-2510 bath sonicator and added to the reaction mixture to a final amount of 1% as calculated per total amount of rPrP substrate. For the experiments where composition of individual fibrils was analyzed by immunofluorescence microscopy or atomic force fluorescence microscopy, the seeding reactions in (Ha)Mo and (Mo)Ha reaction formats were carried out in the presence of 30% preformed fibrillar seeds. However, seeds were not subjected to sonication prior to seeding.

**SDS-PAGE in Denaturing and Nondenaturing Conditions**—To estimate the yield of amyloid formation, the aliquots were taken at the end points of the polymerization reactions and treated with two sample buffers as follows: denaturing (the final 60 mM Tris, 2% SDS, and 5% β-mercaptoethanol, 2.25 mM ube, heating for 15 min at 90 °C), and nondenaturing (no SDS, β-mercaptoethanol or urea, no heating). 12% SDS-PAGE (precast NuPAGE gels, Invitrogen) were used for analysis of samples treated with both denaturing and nondenaturing sample buffers.

**Maturation of Amyloid Fibrils and PK Digestion Assay**—In previous studies, we described that rPrP fibrils acquired 16-kDa PK-resistant core similar to that of PrPSc upon brief heating at 80 °C in the presence of 0.1% Triton X-100, the procedure referred to as maturation (19). For maturation, rPrP fibrils were dialyzed against 10 mM sodium acetate buffer (pH 5.0), and then Tris–HCl buffer (pH 7.5) and Triton X-100 were added to final concentrations of 100 mM and 0.1%, respectively. Aliquots (20–40 µl, 0.1 mg/ml of rPrP) were placed into 0.5-ml conical plastic tubes, incubated at 80 °C for 5 min, and cooled down. Fibrils were treated with PK for 1 h at 37 °C at a PK to rPrP ratio of 1:100. Digestion was stopped by adding denaturing sample buffer. Samples were heated for 15 min at 90 °C and analyzed by 12% SDS-PAGE (precast NuPAGE gels, Invitrogen) followed by silver staining or Western blotting.

**Immunostaining and Fluorescence Microscopy**—rPrP fibrils (1 µg/ml) were deposited onto Permanox 8-well Lab-Teks chamber slides and stained with antibody as described previously (20) with minor modifications. Formaldehyde fixation was omitted, and the staining was performed in the following order: 1) anti-PrP human Ab D13 (1:6000, recognizes epitope 96–104); 2) mouse Ab 3F4 (1:3000, hamster-specific and recognizes epitope 109–112); 3) the mixture of secondary Abs, goat anti-human and goat anti-mouse labeled with Alexa 488 and Alexa 546, respectively (Invitrogen, 1:1000 for both Abs). Fluorescence microscopy was carried out on an inverted microscope (Nikon Eclipse TE2000-U) using 1.3 aperture Plan Fluor ×100 numerical aperture objective. The exposure times were 300 ms for Fab D13 and 900 ms for Ab 3F4. Collected images
were washed with 50 mM Tris saline buffer (TBS). The cover slips was analyzed using immunofluorescence microscopy as ing heterologous substrate. The composition of individual cross-seeding experiments, where 30% of freshly formed Mo or involved in elongation of preformed fibrils, we performed Fibrils seeds were used for cell-free conversion. similar regardless of whether homologous or heterologous 1). This experiment illustrates that the efficiency of seeding was produced from heterologous rPrPs were used for seeding (Fig. 1). Unexpectedly, the lag phase was also eliminated when fibrils (see “Experimental Procedures”). As expected, the lag phase trolled polymerization described by Bishop and Ferrone (18) fitting procedure developed for analysis of nucleation-con- of full-length Mo and Ha rPrP. The spontaneous polymeriza- tion of Ha or Mo rPrPs showed a lag phase of 16 and 18 h, respectively (Fig. 1). The lag phases were calculated using a fitter procedure developed for analysis of nucleation-controlled polymerization described by Bishop and Ferrone (18) (see “Experimental Procedures”). As expected, the lag phase was eliminated upon seeding with homologous rPrP seeds (Fig. 1). Unexpectedly, the lag phase was also eliminated when fibrils produced from heterologous rPrPs were used for seeding (Fig. 1). This experiment illustrates that the efficiency of seeding was similar regardless of whether homologous or heterologous seeds were used for cell-free conversion.

Cross-seeding Resulted in Formation of “Hybrid” Mo-Ha Fibrils—To test whether the heterologous substrate was involved in elongation of preformed fibrils, we performed cross-seeding experiments, where 30% of freshly formed Mo or Ha fibrils were added to the polymerization reactions containing heterologous substrate. The composition of individual fibrils was analyzed using immunofluorescence microscopy as described previously (20). To determine the species of rPrP within individual fibrils, we used double staining with Ab 3F4, which detects Ha but not Mo rPrP, and Fab D13, which detects both Ha and Mo rPrP. Because the secondary antibody to Ab 3F4 was labeled with Alexa 546 (red), and the secondary antibody to Fab D13 with Alexa 488 (green), fibrillar parts composed of Mo rPrP were expected to be green, whereas parts made of Ha rPrP were expected to be orange. We found that fibrils formed in both cross-seeded reactions as follows: Mo-seeded Ha (referred to as (Ha)Mo) or Ha-seeded Mo (referred to as (Mo)Ha) consisted of heterogeneous (green and orange) fragments containing Mo or Ha rPrPs (Fig. 2, A and B). As expected, the fibrils formed in nonseeded reactions from Mo rPrP were entirely green (Fig. 2C), whereas the fibrils formed from Ha rPrP were orange confirming that both Ab 3F4 and Fab D13 bind to Ha rPrP (Fig. 2D). Using an alternative staining procedure, in which Ab 3F4 was utilized in combination with the amyloid-specific dye ThT, we confirmed that the individual fibrils formed in the cross-seeded reactions consisted of both Mo and Ha parts (supplemental Fig. S1). Fluorescence intensity profiles recorded in both the green and red channels along individual

RESULT

Lack of Species Specificity in Mo and Ha Fibril Formation—The species barrier for prion transmission between Syrian hamster and mouse has been well established in previous studies. The species specificity was primarily attributed to differences in the primary structure of Ha and Mo PrP variants (Mo and Ha PrPs are different at eight residues within the amyloidogenic region 90–231). To test whether the species specificity of prion conversion persists in the absence of strain-specified conformational constraints, a cellular environment, or PrP glycosylation, we examined the seeding specificity for fibrillation of full-length Mo and Ha rPrP. The spontaneous polymerization of Ha or Mo rPrPs showed a lag phase of 16 and 18 h, respectively (Fig. 1). The lag phases were calculated using a fitter procedure developed for analysis of nucleation-controlled polymerization described by Bishop and Ferrone (18) (see “Experimental Procedures”). As expected, the lag phase was eliminated upon seeding with homologous rPrP seeds (Fig. 1). Unexpectedly, the lag phase was also eliminated when fibrils produced from heterologous rPrPs were used for seeding (Fig. 1). This experiment illustrates that the efficiency of seeding was similar regardless of whether homologous or heterologous seeds were used for cell-free conversion.

Figure 1. Kinetics of cross-seeded polymerization. A, kinetics of polymerization reactions of Mo rPrP (2 μM) seeded with 0.1% of Mo fibrils (solid lines, (Mo)Mo), 0.1% of Ha fibrils (dashed lines, (Mo)Ha), or without seeding (circles, Mo). B, kinetics of polymerization reactions of Ha rPrP (2 μM) seeded with 0.1% of Ha fibrils (solid lines, (Ha)Ha), 0.1% of Mo fibrils (dashed lines, (Ha)Mo), or without seeding (circles, Ha). The kinetic curves are shown in duplicate. The kinetics were monitored in 96-well plate as described under “Experimental Procedures.” In case of all seeded reactions, fitting procedure gave negative value for the intersection of fitting curves described by quadratic and exponential functions that indicated elimination of the nucleation stage.
FIGURE 2. Fluorescence microscopy images of fibrils produced in cross-seeded elongation reactions. A, images of Mo rPrP fibrils produced in the reactions seeded with 30% Ha fibrils, (Mo)Ha. B, images of Ha rPrP fibrils produced in the reactions seeded with 30% Mo fibrils, (Ha)Mo. C and D show images of fibrils produced from Mo rPrP or Ha rPrP, respectively, in nonseeded reactions. Fibrils in A–D were double-stained with Fab D13 (green) and Ab 3F4 (red). Right panels show the fluorescence intensity profiles measured along individual fibrils and recorded in both red and green channels. Fluorescence intensity profiles of fibrils produced as a result of cross-seeding showed heterogeneous composition, whereas the fluorescence profiles of fibrils formed in nonseeded reactions were homogeneously colored. Arrows show presumed directionality of fibril assembly that occurred in either a monodirectional or bidirectional manner. Fibrils were produced in manual format as described under “Experimental Procedures.” Scale bars = 3 μm.
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FIGURE 3. Atomic force fluorescence microscopy imaging of individual (Ha)Mo fibrils. Fibrils were formed from Ha rPrP in the presence of 30% Mo rPrP seeds, stained with Ab 3F4 (red fluorescence) and ThT (green fluorescence), and observed by atomic force fluorescence microscopy. A, fluorescence microscopy images of individual (Ha)Mo fibrils show that fibrillar assembly proceeds either in a monodirectional or bidirectional manner. B, fluorescence microscopy (inset) and atomic force microscopy image of a single (Ha)Mo fibril. This fibril consists of two sections as follows: the section made of Mo rPrP binds ThT only and displays green fluorescence, the section made of Ha rPrP is decorated by Ab 3F4 and is therefore thicker and displays a red fluorescence. C, fluorescence microscopy and atomic force microscopy image of a single (Ha)Mo fibril. This fibril consists of three sections: the section made of Mo rPrP binds ThT only (green) and the sections made of Ha rPrP are decorated by Ab 3F4 (red).

fibrils indicated that fibrils can grow in a bidirectional or unidirectional manner (Fig. 2 A and B, and Fig. 3A).

To make sure that hybrid Mo-Ha fibrils were indeed produced as a result of elongation and not because of lateral association of preformed individual Mo and Ha fibrils or filaments, we implemented a technique referred to as AFFM. AFFM consisted of simultaneous measurement of the topological profile of individual fibrils by AFM and fibril composition by dual color staining with Ab 3F4 and ThT. As judged by AFFM, individual fibrils consisted of red and green sections, where the red sections were substantially thicker than the green sections (Fig. 3B). The differences in thickness observed within individual fibrils were because of the binding of Ab 3F4 and secondary Alexa 546-labeled Ab to the fibrillar parts made of Ha rPrP. Because only ThT bound to the parts made up of Mo rPrP, they were substantially thinner. AFFM confirmed that the hybrid Mo-Ha fibrils were not artifacts of lateral aggregation of individual Mo and Ha fibrils. Taken together, our data provide direct evidence that monomeric rPrP supported equally well the elongation of fibrils produced from both homologous and heterologous rPrP seeds.

Fibrils Displayed Poor Selectivity in Converting Homologous Versus Heterologous rPrP—Next we were interested in knowing whether the preformed fibrils displayed any species selectivity with respect to binding and converting homologous versus heterologous monomeric rPrP. To test the selectivity, Mo or Ha seeds were added to the reactions containing mixtures of Mo and Ha rPrPs (these reaction formats are abbreviated as (Mo+Ha)Mo and (Mo+Ha)Ha, respectively). In such a format, the cell-free conversion assay mimicked the experiments where the replication of Mo and Ha prions was studied using transgenic mice that co-expressed Mo and Ha PrPC (2).

First, we examined the amount of monomeric rPrP that remained soluble at the very late stages of the reactions by comparing the amounts of rPrP that enter PAGE under denaturing or non-denaturing conditions. In our preliminary studies, we found that the fibrillar rPrP enters PAGE only after being denatured, whereas monomeric rPrP enters PAGE regardless of its denaturation status. Surprisingly, we found that monomeric rPrPs were consumed almost entirely in both (Mo+Ha)Mo and (Mo+Ha)Ha conversion reactions (Fig. 4A, lanes 7 and 8, respectively). When the conversion reactions were carried out in the Mo+Ha mixtures in the absence of seeds, the yield of amyloid formation was very poor, as judged by large amounts of nonconverted monomeric rPrP seen in the nondenaturing gel (Fig. 4A, lanes 2–4 and 6). Therefore, seeding substantially improved the yield of fibrillation in Mo+Ha mixtures. The almost complete depletion of substrate in seeded reactions suggested that both homologous and heterologous monomeric rPrP supported elongation of preformed fibrils.

Second, to investigate the selectivity of elongation in more detail, we examined the composition of the fibrils using two assays. Double staining fluorescence microscopy was employed for testing the composition of individual fibrils (Fig. 5), whereas a PK digestion assay followed by Western blotting examined the relative level of newly converted Mo and Ha rPrP across the entire fibrillar population (Fig. 4B). Fluorescence microscopy revealed that Ha rPrP participated in the elongation of Mo fibrils in the (Mo+Ha)Mo reaction format (Fig. 5A). Vice versa, Mo rPrP was recruited by Ha seeds in the (Mo+Ha)Ha reaction format (Fig. 5B). Fluorescence intensity profiles indicated that individual fibrils were composed of both Mo and Ha rPrPs. Because of limitations in spatial resolution of the light microscopy technique, it was difficult to determine without doubt whether both Mo and Ha rPrPs were uniformly recruited into growing fibrils or were incorporated as alternating segments. Analysis of the fluorescence microscopy profiles did not exclude the second scenario. However, this question needs to be examined in future experiments using a more advanced approach.

The PK digestion assay followed by Western blotting with Ab 3F4 (recognizes Ha rPrP) and Ab P (recognizes both Ha and Mo rPrP) showed that both (Mo+Ha)Mo and (Mo+Ha)Ha reactions produced the same amount of PK-resistant material and that the portion of PK-resistant Ha rPrP was similar regardless of whether Ha or Mo seeds were used (Fig. 4B). Vice versa, Mo rPrP was recruited equally well in both reaction formats, as
judged from the comparison of the relative band intensities in the blots developed with Ab 3F4 and Ab P, and from comparison of band intensities in the (Mo+Ha)Mo and (Mo+Ha)Ha reaction formats (Fig. 4B). Taken together, these results demonstrated that, when used in a mixture, both Mo and Ha rPrPs were recruited with similar efficiency by Mo or Ha seeds.

Mixing of Ha and Mo rPrP Interferes with Spontaneous Polymerization—Although seeds appeared to show no preferences in recruiting homologous versus heterologous substrate, we were next interested in determining whether Mo and Ha rPrPs were capable of polymerizing in a mixture in the absence of seeds (this reaction format is referred to as Mo+Ha). Surprisingly, we found that Mo+Ha mixtures displayed longer lag phase than that observed for individual conversion reactions of Mo or Ha rPrPs, a sign of mutual interference (Fig. 6A). As judged by the amounts of rPrP in PAGE carried out under non-denaturing versus denaturing conditions, the yield of non-seeded polymerization was very low in Mo+Ha reactions (Fig. 3A).

Electron microscopy confirmed the presence of amyloid fibrils; however, the morphology of fibrils produced in the Mo+Ha mixtures was strikingly different from that observed in Mo or Ha reactions or in (Mo+Ha)Mo or (Mo+Ha)Ha reactions. Mo+Ha fibrils displayed dichotomous branching, an indication of a deficiency in lateral assembly of filaments into high order fibrils (supplemental Fig. S2). The double-staining fluorescence microscopy revealed that the fibrils formed from Mo+Ha mixtures were relatively homogeneous in color (supplemental Fig. S2), arguing against the presence of two individual populations of Mo and Ha fibrils, and suggesting the presence of hybrid Mo-Ha fibrils. These results illustrated that in the mixtures Mo and Ha rPrPs were engaged in a physical interaction that resulted in an unproductive complex that seems to be relatively inefficient for further polymerization.

Species-specific Interaction Regulates Maturation of rPrP Fibrils into the PrPSc-like Isoform—Our previous studies revealed that fibrils produced from rPrP in the absence of a cellular environment displayed unusually short PK-resistant cores that encompassed residues 138–230, 152–230, and 162–230 (PK-resistant bands of ~12, 10, and 8 kDa, respectively) (21). Subsequently, we found that a brief incubation of rPrP

![FIGURE 4. SDS-PAGE analysis of fibril composition.](image)

![FIGURE 5. Fluorescence microscopy images of fibrils produced in (Mo+Ha)Mo and (Mo+Ha)Ha reactions.](image)
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fibrils at high temperatures in the presence of brain homogenate or at low concentrations of Triton X-100 leads to a substantial extension of the PK-resistant core; this procedure was referred to as annealing or maturation (19). Upon maturation, the PK-resistant core of rPrP fibrils became similar to that of "classical" PrPSc (19). Because maturation consisted of conformational rearrangements that followed conversion into fibrillar form, we wanted to test whether the composition of individual fibrils dictates the ability of fibrils to acquire a PrPSc-like PK-resistant conformation: (i) was not determined by the composition of individual fibrils, but rather by their origin or history; and (ii) the ability to undergo maturation was transferred to the daughter fibrils upon seeding regardless of whether the daughter fibrils were made from homologous, heterologous, or mixtures of homologous and heterologous rPrPs. These data also suggest that the substructure of Mo + Ha fibrils was fundamentally different from that of (Mo + Ha)Mo and (Mo + Ha)Ha fibrils.

**DISCUSSION**

The primary structure of the prion protein is thought to be a critical factor in determining the species barrier of prion transmission. Moreover, the degree of similarity between PrP sequences of the donor and acceptor species is believed to regulate the magnitude of the species barrier. In this study, we showed that, in the absence of a cellular environment, PrP glycosylation, or strain-dependent conformational constraints, fibrillation of full-length Mo and Ha rPrPs displayed no species specificity despite substantial differences in their primary structures. Ha and Mo fibrils showed a similar efficiency of seeding in the polymerization reaction with homologous or heterologous substrates. Both Mo and Ha monomeric rPrPs were competent in elongating preformed fibrils produced from heterologous rPrP variants. Furthermore, fibrils displayed no species selectivity in experiments, when both homologous and heterologous substrates were provided in the mixtures. Our studies support the hypothesis that the cross-β self-propagating structures are intrinsically promiscuous and can seed polymerization of homologous and heterologous polypeptides.

In contrast to the present studies, Surewicz and co-workers (14, 22) observed strong species specificities in their in vitro fibrillation reaction that utilized recombinant PrP peptide 23–144. The two polymerization assays are different in several key aspects. The reaction described in our work utilized a biologically more relevant range of protein concentrations (2–10 versus 400 µM) and a more relevant rPrP construct (full-length rPrP versus rPrP-(23–144)) than those reported in the work by Jones and Surewicz (22). Because the prion-forming domain is missing in rPrP-(23–144), it is likely that rPrP-(23–144) fibrils had a substructure substantially different from that of full-length rPrP fibrils. In fact, our previous studies established that, in fibrils made of full-length rPrP, the PK-resistant cross-β-sheet self-propagating core was formed by segment 152/162–230 (19–21, 23), a region that was entirely missing in rPrP-(23–144). Therefore, a distinct fibrillar substructure may account, at least in part, for the differences in species specificity observed for polymerization of full-length and rPrP-(23–144).

The fibrillation reaction in our studies, however, was carried out in a physiologically less relevant environment (2 M GdnHCl). The presence of GdnHCl was required for fibrillation of full-length PrP to occur within a reasonable time frame. It is unclear whether partially denaturing conditions abolished the species specificity of this reaction. Previous experiments on
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PrPSc-dependent conversion of PrPSc into the PrPSc-like PK-resistant form showed strong species-specific differences in the conversion efficacy regardless of whether a partially denaturing concentration of GdnHCl (2 M GdnHCl) was present in the conversion reactions or not (24, 25). Furthermore, our former fibrillation reactions that were performed under partially denaturing conditions using full-length PrP and mini-prion protein PrP106 showed strong specificity in cross-seeding (15). Both studies indicated that the seeding specificity could be preserved even in the presence of GdnHCl.

How can the results of this study, illustrating the apparent lack of seeding specificity, be reconciled with previous studies? It seems that when fibrils are produced from large polypeptides such as full-length PrP, mismatches at several positions are not sufficient to block fibril growth. Energetically favorable interactions within the cross-β core appear to be sufficient to overcome local perturbations that might be caused by variations in the primary structures between Mo and Ha variants. However, the outcome of cross-seeded polymerization could be different for fibrillation of small peptides or for variants of different length such as full-length PrP and mini PrP 106 (15). If a peptide acquiring cross-β-structures is relatively short and converts into highly ordered crystal-like structures, mismatches at even a single residue could counteract otherwise energetically favorable intermolecular interactions that stabilize the fibrillar core creating an artificial species barrier. Such artificially generated species barriers, however, might not mimic the natural species barriers observed in transmission of prions in animals (14).

Our current findings regarding efficient cross-seeding between Mo and Ha fibrils seem to contradict previous studies where the species barrier of prion transmission between mouse and Syrian hamster was well demonstrated (26–29). Mo or Ha prions were shown to produce disease quickly only when inoculated into animals of the same species, whereas cross-species passage either did not cause disease or induced pathological changes only after a prolonged incubation time (2, 26, 29, 30). Cross-species transmission was also found to be asymmetric, i.e. Mo PrPSc strains were more efficient in transmitting the disease to hamsters than Ha strains in passing the disease to mice (31). Cell-free conversion experiments with partially purified components revealed that the relative efficiency of Mo and Ha PrPSc to convert heterologous PrPSc into PK-resistant forms was similar to the relative transmissibility of Mo and Ha prions to the opposite species in vivo (25, 32).

Co-expression of Ha PrPSc with Mo PrPSc in transgenic mice seemed to impede replication of Mo PrPSc (2, 26). Consistent with animal studies, expression of Ha PrPSc in mouse neuroblastoma cells infected with mouse scrapie was found to abolish the replication of prions (33). Experiments with partially purified PrPSc and PrPSc showed that, in the mixture of Mo and Ha PrPSc, heterologous PrPSc bound to PrPSc but failed to acquire a PK-resistant conformation and interfered with the conversion of homologous PrPSc (24). Remarkably, upon inoculation of Mo prions into transgenic mice co-expressing Mo and Ha PrPSc, low levels of Ha PrPSc were detected in their brains (26). This result argues that Mo PrPSc catalyzes the conversion of Ha PrPSc, although at very slow rate. Subsequent studies showed that cross-species transmission of prions between mice and hamsters resulted in slow or “silent” replication of prions that often does not cause clinical disease within the lifetime of an animal. However, it could be seen in subsequent passages (27, 28). Therefore, the species barrier between mice and hamsters is not absolute, and prion transmission could be achieved under an appropriate experimental setup.

How can the lack of the seeding specificity observed in this study be reconciled with the mouse-hamster species barrier that appeared to be well established in animal studies? Although differences in PrP sequence between host and donor species are believed to be the primary factor in regulating the species barrier, a number of studies demonstrated that it is actually impossible to predict the transmissibility of prions based on sequence homology between PrPSc and PrPSc. Despite identity in the amino acid sequence between donor and host PrP, a longer incubation time of disease was observed in transgenic mice expressing bovine PrP inoculated with bovine spongiform encephalopathy than in wild type mice (34, 35). Likewise, an increase in incubation time was seen upon inoculation of transgenic mice expressing human PrP with new variant CJD when compared with that observed in wild type mice inoculated with the same prion strain (34, 35). Remarkably, a number of sporadic CJD strains were shown to transmit the disease more efficiently to the same humanized transgenic mice than to wild type mice. These studies argued that the sequence identity between PrPSc and PrPSc does not always abolish the species barrier and that strain-specific properties appear to be more important in regulating the species barrier than sequence homology. In other studies, transmission of CJD isolates to bank voles showed little or no evidence of a transmission barrier despite a low sequence homology between human and bovine PrP (36). In contrast, despite almost identical PrP primary structure, a striking species barrier was observed during transmission of prions from mouse or hamster to voles (36). Again, an increase in sequence homology between PrPSc and PrPSc changed the incubation time in a manner opposite to that one would predict based on sequence homology.

Considering that different TSE strains from the same host species exhibit different efficiencies in overcoming the species barrier when transmitted to a new species, strain-specific properties seem to determine to a large extent the strength of the species barrier (36, 37). When transmitted to a new host, TSE strains often change producing new or modified strains. In fact, interspecies transmission is known to be a major source of strain diversity (for a review of this subject see the article by Soto and co-workers (38)). All mouse and hamster strains that are currently used by prion researchers were generated by transmission of the TSE agent isolated from other species such as sheep, mink, bovine, or human and did not arise from sporadic disease in mice or hamsters. Therefore, all mouse and hamster TSE strains have been generated artificially in a sense that the properties of these strains are determined by properties of original TSE isolate, the history of passages through intermediate species, and PrP sequence of the final host.

It is important to emphasize that only one strain of Ha PrPSc referred to as Sc237 or 263K was used in all the aforementioned studies on the species barrier between mouse and hamster. 263K originated from the natural pool of TSE that was origi
nally isolated from the Cheviot breed and two hybrids of Cheviot and Border sheep in 1945 (39). Since then, the ancestor of 263K was transmitted through sheep of different breeds, then inoculated to goats, to Swiss mice, to goats again, to rats, and finally transmitted to Syrian hamsters, where it was stabilized after four consecutive passages (30, 31, 40–43). Considering such a complex history of interspecies passages, it is likely that 263K accumulated a number of peculiar features that may not be intrinsic for a strain that would originate in a hamster. Therefore, the extent to which the species barrier can be attributed to the differences between primary structures of mouse and hamster PrP or to the peculiar properties of Sc237 strain is unclear. Unique conformational features of Sc237 rather than primary structure may, in fact, be the key factors in limiting transmission of this Ha strain to mice.

This study demonstrates that spontaneous fibrillation conducted in the mixtures of Mo and Ha rPrPs displayed low yield and prolonged lag phase. An increase in the lag phase suggested that Mo and Ha rPrPs interacted at the very early stage of fibrillation, presumably producing hybrid Mo-Ha nuclei. If hybrid nuclei were formed, they should impose a profound imprint on the physical properties of the resulting fibrils. Indeed, we found that filaments produced in the Mo + Ha mixtures were deficient in their lateral assembly into high order fibrils (supplemental Fig. S2) and failed to acquire a 16-kDa PK-resistant core (Fig. 6B). These results suggest that Mo and Ha PrPs were not as mutually compatible at the nucleation step as they were found to be in the cross-seeded elongation or competition reactions. These findings were not unprecedented. Previous studies revealed remarkable differences in behavior of small β-oligomeric species formed separately from two variants of human rPrP, 129V, and 129M and from their mixture (44). Taken together, our studies indicate that species specificity could be manifested at the nucleation step.

In contrast to hybrid fibrils formed in nonseeded reactions, Mo + Ha fibrils produced in seeded assays were able to form PrPSc-like PK-resistant conformations. When the original seeds were produced from a single rPrP variant, the daughter fibrils were able to inherit the ability to form mature PrPSc-like structures regardless of fibrillar composition. These results re-emphasize the fundamental differences in the substructures of Mo + Ha fibrils produced in nonseeded versus seeded reactions. They also illustrate that physical properties of fibrils are controlled by seeds or by nuclei rather than by fibrillar composition.

The hypothesis that cross-β amyloid structures are intrinsically promiscuous might have important implications for a number of human and animal maladies. A growing amount of evidence indicates that in vivo amyloidosis of one protein can be stimulated by fibrils of an unrelated protein in a manner similar to cross-seeded polymerization (45, 46). Pathological studies revealed that amyloid fibrils produced from two different proteins or peptides, including PrP, Aβ, α-synuclein, immunoglobulin light chain λ, and β2-microglobulin, often co-localize within the same amyloid plaques in a variety of organs or tissues (47–51). Although one might consider that amyloids of two proteins could co-deposit by coincidence, recent studies provide direct evidence in support of a cross-seeding mechanism for amyloid deposition in vivo. Reactive protein A amyloidosis and senile apolipoprotein A-II amyloidosis were found to develop in mice as a result of cross-seeding by fibrils of apolipoprotein A-II or protein A, respectively (46). Cross-talk between several yeast prion proteins provides other examples of how direct interactions between newly forming and pre-existing heterologous fibrils might take place in a cell (52–54). These studies illustrate that, in the complex environment of a cell, prions can arise even without a homologous prion template via seeding with heterologous fibrils. The possibility that cross-seeded amyloidosis might develop in vivo raises a question as to whether sporadic CJD is indeed sporadic. The idea that sporadic prion disease is initiated by amyloidosis of unrelated protein might seem to be too radical. It is not inconceivable, however, that CJD could be initiated by slow progressing amyloidosis of a nonrelated protein, which is difficult to observe first because of the fast progression of CJD.

Our studies raise several important implications for conformational diseases and prions. The promiscuous nature of the self-propagating activity of amyloid structures can lead to devastating consequences for cellular health. If amyloidosis of a polypeptide could indeed be stimulated by amyloid fibrils made of heterologous or nonrelated proteins, the cross-seeding mechanism may offer a possible explanation for development of the conformational disorders that are considered to be sporadic. Considering that the differences in primary structure do not always guarantee a strong species barrier (36), the emergence of a novel prion strain that is well adapted to different mammalian species or exhibits minimal species specificity is not completely inconceivable. The strength of the species barrier cannot be predicted solely from the differences in PrP primary structure or from modeling prion replication using PrP-derived peptides. Factors other than the PrP sequence appear to have a greater impact in regulating prion transmission between mammalian species.

Acknowledgments—We thank Robert Rohwer for help in tracking the history of 263K strain and Pamela Wright for editing the manuscript.

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