Visualization of Aggregation of the Rnq1 Prion Domain and Cross-seeding Interactions with Sup35NM*†

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Factors triggering the de novo appearance of prions are still poorly understood. In yeast, the appearance of one prion, [PSI+], is enhanced by the presence of another prion, [PIN+]. The [PSI+] and [PIN+] prion-forming proteins are, respectively, the translational termination factor Sup35 and the yet poorly characterized Rnq1 protein that is rich in glutamines and asparagines. The prion domain of Rnq1 (RnqPD) polymerizes more readily in vitro than the full-length protein. As is typical for amyloidogenic proteins, the reaction begins with a lag phase, followed by exponential growth. Seeding with pre-formed aggregates significantly shortens the lag. A generic antibody against pre-amyloid oligomer inhibits the unseeded but not the self-seeded reaction. As revealed by electron microscopy, RnqPD polymerizes predominantly into spherical species that eventually agglomerate. We observed infrequent fiber-like structures in samples taken at 4 h of polymerization, but in overnight samples SDS treatment was required to reveal fibers among agglomerates. Polymerization reactions in which RnqPD and the prion domain of Sup35 (Sup35NM) cross-seed each other proceeded with a shortened lag that only depends weakly on the protein concentration. Cross-seeded Sup35NM fibers appear to sprout from globular RnqPD aggregates as seen by electron microscopy. RnqPD spherical aggregates appear to associate with and, later occlude, Sup35NM seed fibers. Our kinetic and morphological analyses suggest that, upon cross-seeding, the aggregate provides the surface on which oligomers of the heterologous protein nucleate their subsequent amyloid formation.

Prions are currently viewed as proteinaceous genetic elements because of their potential to encode, propagate, and discretely modify phenotypes produced by an infectious alternative conformational state of the prion protein (1). In this state, proteins form amyloid aggregates characterized by a rigid structure that is stabilized by inter β-sheet bonds (2). These aggregates can convert molecules of the prion-forming protein from the normal conformation into the prion form. Propagation of the prion state in vivo is based upon fragmentation of already formed aggregates that results in multiplication of prion seeds. In mammals, the prion PrPSc is the causative agent of a series of neurodegenerative disorders, e.g. Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy, scrapie of sheep, etc. (for review see Ref. 3).

Several prions have been characterized in the yeast Saccharomyces cerevisiae (for review see Refs. 4 and 5), and a growing body of evidence suggests that there are interactions between them (for review see Ref. 6). These interactions range from the ability of one prion to facilitate the appearance of another to incompatibility between some (7–9).

Here we focus on two yeast prions, [PSI+] and [PIN+], as a model for prion–prion interactions. [PSI+] is composed of the alternatively folded translational termination factor Sup35 (10, 11) and is characterized by an increased level of readthrough of stop codons (nonsense suppression) because of the sequestration of Sup35 into prion aggregates (12). A Sup35 fragment spanning amino acids 1–253 (Sup35NM)3 forms fibers in vitro with sigmoidal kinetics in which rapid growth is preceded by a lag phase (13, 14). Such kinetics are typical of amyloidogenic proteins arguing that their polymerization is a nucleated process and that nucleation is the rate-limiting step (15). This model was developed further for Sup35NM by Serio et al. (16) who showed that the nucleus forms within the context of an oligomer, although the involvement of an oligomeric intermediate was not supported in another study (17). Unlike true amyloid fibers that appear after a lag, oligomers form soon after the resuspension of Sup35NM into an aqueous buffer (18). These in vitro made fibers (19) as well as fibers of a shorter Sup35 fragment (20) have been shown to be infectious as they convert the cells from [psi−] to [PSI+] upon transformation thus proving the protein-only hypothesis for the [PSI+] prion.

In vivo, the appearance of [PSI+] can be induced by overproduction of Sup35NM in the presence of another prion, [PIN+] (7). This prion is produced by a self-perpetuating aggregated form of Rnq1 (21), a protein whose function is not yet known. Rnq1 was discovered independently (22) in a genome-wide screen in silico for proteins with homology to the Q/N-rich prion domains of the previously characterized yeast prion proteins Sup35NM and Ure2 (23) and was shown to be able to form a prion in vivo.

3 The abbreviations used are: Sup35NM, region encompassing the N-terminal and middle domains of the yeast Sup35 protein (amino acids 1–254); TEM, transmission electron microscopy; ThT, thioflavin T; RnqPD, prion domain of the yeast Rnq1 protein (amino acids 132–405).
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The first studies of Rnq1 polymerization were done using a construct accidentally lacking the NNGNQN sequence at the C terminus (22), which we refer to as altered Rnq1. It polymerized in vitro with a lag that could be shortened by self-seeding, and the presence of these Rnq1 aggregates enhanced polymerization of Sup35NM in vitro (24). This finding is one of the major arguments in favor of a cross-seeding mechanism postulated for the enhanced induction of [PSI⁺] in the presence of [PIN⁺]. Once formed, Sup35 and Rnq1 prion aggregates constitute separate structures in [PSI⁺][PIN⁺] yeast (25), suggesting that cross-seeding is a transient event required only for the initiation of Sup35 polymerization which then proceeds via a more efficient self-templating mechanism.

However, the relationships between [PSI⁺] and [PIN⁺] are complex; certain variants of the two destabilize each other (9). In addition, the formation of Sup35 nonheritable amyloid polymers is dependent upon Rnq1 aggregates (26). Thus, interactions between Sup35 and Rnq1 occur at different levels of aggregation and may represent a regulatory component in yeast prion metabolism.

Cross-seeded fibrillization has also been documented for other amyloidogenic proteins, i.e. hen lysozyme (27), islet amyloid polypeptide, and AB (1–40) (28). In all cases of cross-seeding, a reduction of the lag in polymerization kinetics was observed and is proposed to be due to the templating effect of the heterologous seed at the initial rate-limiting step of the amyloid formation.

Here we analyze the morphology of in vitro made aggregates formed by the prion domain of RnqPD in conjunction with the kinetics of their formation. Sup35NM and RnqPD aggregates can cross-seed each other, and our kinetic analyses of the lag phases in both reactions suggest that nucleation is the rate-limiting step in cross-seeded polymerization. Visualization of hybrid aggregates by electron microscopy argues that cross-seeding involves a direct interaction of Sup35NM and RnqPD, and this interaction is on-pathway, providing further support for the cross-seeding model of de novo prion appearance.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Recombinant His₁₀-tagged Sup35NM, RnqPD, and full-length Rnq1 were overexpressed in Escherichia coli (BL21AI; Invitrogen) from the pJC45 expression vector (29) harboring the following inserts: SUP35-NM (1–762 bp; kind gift of S. Lindquist), RNQ-PD (395–1215 bp), and full-length RNQ1 (30). Bacterial lysis was performed in 20 mM Tris-HCl, RNQ1 (BL21AI; Invitrogen) from the pJC45 expression vector (29) and full-length Rnq1 were overexpressed in Escherichia coli (BL21AI; Invitrogen), pre-cleared by centrifugation at 4000 rpm (30). Bacterial lysis was performed in 20 mM Tris-HCl, pH 8.0, 8 M urea for 20 min at room temperature. Lysates were made aggregates by electron microscopy argues that cross-seeding involves a direct interaction of Sup35NM and RnqPD, and this interaction is on-pathway, providing further support for the cross-seeding model of de novo prion appearance.

**SDS-Agarose Gel Electrophoresis and Immunoblotting**—The assay was performed as described earlier (31). Briefly, samples containing 0.7–10 µg of protein were treated with 2% SDS for 7 min, separated on 1.5% agarose gels supplemented with 0.1% SDS, and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Membranes were probed with 1:50,000 rabbit anti-Rnq antibody (type II (22)) with the use of a Western-STAR™ immunodetection kit (Applied Biosystems). For molecular weight standards, we used chicken pectoralis extract that, upon Coomassie staining, reveals several abundant muscular proteins as follows: titin (∼3000 kDa), nebulin (∼750 kDa), and myosin heavy chain (∼200 kDa). Although this ladder cannot be used for a precise determination of molecular mass, it does provide an estimate.

**Transmission Electron Microscopy**—Sup35NM and RnqPD were polymerized in 1.7-ml polypropylene tubes in 300–600-µl PBS (see figure legends for protein concentrations). Agitation was ensured by rotation of the tube at about 50 rpm. Because of the different conditions used to make fibers when we analyzed the kinetics of polymerization (performed in a microplate) and those used to make larger amounts suitable for TEM samples (performed in Eppendorf tubes), we cannot definitively correlate the TEM time points with the kinetics monitored by ThT fluorescence. Steady-state aggregates made in a microplate looked the same as overnight aggregates made in a tube (Fig. 2E).

For the morphological TEM analysis, 2 µl of reaction mixture were applied to copper Formvar carbon-coated slot grids (Electron Microscopy Sciences) for 90 s. Grids were washed four times with water, negatively stained in aqueous 2.0–2.5% uranyl acetate for 1 min, and again washed in water four times.

For analyses of SDS-treated samples, 100 µl of polymerization reaction were diluted 10 times and incubated with 0.5% SDS for 7 min (10 × dilution was to prevent precipitation), pelleted for 10 min at 10,000 × g, and resuspended in 75 µl of PBS. The SDS was then removed on a Microspin chromatography column (Bio-Rad). As the eluate volume was about 100 µl, fibers were pelleted again and resuspended in 12 µl of PBS yielding enough fibers to make six grids. Grids were washed four times with water, negatively stained in aqueous 2.0–2.5% uranyl acetate for 1 min, and again washed in water four times.

Immunostaining was performed in silicone staining plates (Electron Microscopy Sciences), and the volume of the drop on which the grid floated during all incubations and washes was 50–100 µl. Grids with immobilized fibers were incubated in...
0.5% glycine in PBS for 15 min and normal goat serum (Electron Microscopy Sciences) for 30 min, stained with anti-Rnq antibody (type II (22)) diluted 1:1000 in PBS with 0.001% Tween 20 for 1 h, washed with PBS six times, stained with secondary 10 nm gold-conjugated antibody (Electron Microscopy Sciences) diluted 1:325 in PBS with 0.001% Tween 20 for 1 h, washed in PBS nine times, fixed in 2% glutaraldehyde in PBS for 5 min, washed with PBS once, washed with water once, negatively stained in aqueous 2.0–2.5% uranyl acetate for 1 min, and washed with water four times. Images were obtained on JEOL JEM-1220 transmission electron microscope, operating at 80 kV at a magnification of 25,000–100,000 and collected using a Gatan digital camera.

RESULTS

RnqPD and Full-length Rnq1 Form Prion-like Aggregates in Vitro—To study the details of Rnq1 aggregation with respect to its role in prion-prion interactions in yeast, we focused on the QN-rich domain of unaltered Rnq1 (RnqPD; amino acids 132–405). RnqPD formed amyloid aggregates with sigmoidal kinetics as assayed by ThT fluorescence (Fig. 1, A and B) consistent with another study that used different conditions (30). The threshold concentration of unseeded RnqPD polymerization lies between 10 and 20 μM (Fig. 1A). However, the addition of preformed RnqPD aggregates that serve as homologous seeds enables polymerization even below the threshold. The lag was shortened in proportion to the amount of seed added (Fig. 1B) and was eliminated only at very high concentrations of both RnqPD and seed (e.g. 35 μM and 8%). The lag duration in unseeded reactions exhibited a weak concentration dependence (as will be analyzed later in Fig. 4B) consistent with the nucleated polymerization model for amyloids (15). A close-up of the initial portion of the kinetics of RnqPD polymerization reveals an immediate slight, but reproducible, enhancement in ThT fluorescence in both unseeded (Fig. 1C) and self-seeded (data not shown) reactions. This initial increase was followed by a plateau throughout the lag.

As shown previously for Sup35 (17), there was a significant effect of shaking on the lag time of the RnqPD polymerization reaction. Indeed, no RnqPD polymerization was detected in the unshaken 10 μM reaction (data not shown) or in the 10 μM reaction shaken occasionally (Fig. 1A). More frequent shaking lowered the threshold concentration required for aggregation, e.g. 10 μM polymerized with a 3-h lag (Fig. 1D). Full-length wild-type Rnq1 also polymerized with a lag that could be shortened by seeding (supplemental Fig. S1A). However, a higher concentration of Rnq1 was needed to detect amyloid formation reliably, and the reaction had to proceed in the presence of 1.2 M NaCl.
Many amyloidogenic proteins and peptides aggregate via oligomeric intermediates that can be recognized by a generic antibody (32). This antibody is reactive to the oligomeric structure, rather than to any motif in the amino acid sequence, and was shown previously to inhibit nonseeded, but not self-seeded, polymerization of Sup35NM (33). We examined its effect on RnqPD, and the result was similar to that obtained for Sup35NM; only unseeded polymerization was inhibited (Fig. 1D).

Another characteristic of prions is the SDS resistance of prion particles demonstrated for in vivo made prions of PrP (34), Sup35 (35), and Rnq1 (25). In vitro made Sup35NM fibers were also shown previously to break, in 2% SDS at room temperature, into subparticles migrating slower than the monomer on agarose gels (31). Thus, we examined the effect of SDS treatment on our in vitro made RnqPD aggregates. Indeed, SDS-resistant RnqPD subparticles were revealed when polymerized RnqPD treated with 2% SDS was examined. In contrast, freshly dissolved RnqPD treated with 2% SDS migrated as a monomer and some low oligomeric forms (Fig. 1E). SDS-resistant subparticles were also observed when in vitro made aggregates of full-length Rnq1 were examined (supplemental Fig. S1B).

**Morphology of RnqPD Aggregates**—To analyze the morphology of aggregated RnqPD, we used TEM. RnqPD aggregates were made in bulk in a vigorously agitated reaction mixture (see “Experimental Procedures”). Spherical species could be seen in the 0-min samples (Fig. 2A) that later co-associate in chains and networks abundant in the 90-min samples (Fig. 2B). This architecture of RnqPD aggregates remains essentially the same overnight (magnified image, Fig. 2E). The RnqPD content of these aggregates was confirmed by immuno-EM analysis using anti-RnqPD antibody and a 10-nm gold-conjugated secondary antibody (Fig. 2E). We could also see fibrous structures lying separately (Fig. 2C) or associated with spherical species (Fig. 2D) in the 90-min samples but with a lower frequency. Note that the electron density of these fibers was very low, and significant contrasting of images was needed to reveal them. Interestingly, no fibrous structures were found in the overnight samples even after exhaustive screening. To ask whether they completely dis-

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**FIGURE 2.** RnqPD aggregates and fibers visualized by TEM. Samples were taken from reactions of unseeded polymerization of 20 μM RnqPD agitated in Eppendorf tubes at 0 min (A), 90 min (B–D), and overnight (E). E, samples were immunostained with anti-Rnq antibody. Some gold particles are obscured in the very dense portions of the aggregates. A few gold particles that can be seen on the edges are indicated by arrows. F–I, overnight samples were treated with 0.5% SDS. Scale bars: A–D and G–I, 800 nm; E and F, 200 nm.

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appeared by that late time point or whether they remained but were hidden among the large agglomerates, we treated overnight RnqPD aggregates with 0.5% SDS prior to TEM analysis. In these samples, fiber-like structures were observed in addition to the large agglomerates. These fibers were quite heterogeneous in morphology; they could be smooth (Fig. 2, F–H) or rough (Fig. 2I) and were composed of several filaments (Fig. 2, F and G) or one filament (Fig. 2H). The length of these fibers was in excess of 1 μm, which is larger than the diameter of nontreated RnqPD aggregates (ranging from 200 to 300 nm), suggesting that at least some of the aggregates resulted from supercoiling and/or co-association of fibers that was reversed by the SDS treatment.

**RnqPD Cross-seeds Sup35NM Polymerization**—Previously, aggregates of altered full-length Rnq1 were shown to increase the initial rate of Sup35NM polymerization in a concentration-dependent manner as monitored by ThT fluorescence measured at dispersed time points (24). Here we report a detailed kinetic analysis of cross-seeding using His-tagged wild-type RnqPD and a continuous ThT fluorescence assay. We now show that the addition of RnqPD aggregates is sufficient to shorten the lag phase of Sup35NM polymerization in a concentration-dependent manner but never eliminates it (Fig. 3A). Sup35NM was polymerized in an 8–12 μM reaction mixture using the conditions optimized earlier (see “Experimental Procedures”) (36).

Next we analyzed the dependence of the lag phase on the concentration of Sup35NM at a fixed RnqPD:Sup35NM ratio in comparison to the unseeded reaction. Consistent with previous reports (16, 17), the duration of the lag of unseeded polymerization of Sup35NM weakly depended on the protein concentration (Fig. 3B). This was explained earlier by the nucleated conformational conversion model, according to which an oligomeric intermediate has to take on the amyloidogenic conformation prior to efficient fibrillization (16). This conformational change is the rate-limiting step in the polymerization reaction (18) resulting in a lag weakly dependent on concentration (less than first order). We show here that the lag phase in the cross-seeded polymerization of Sup35NM is shortened upon increasing the Sup35NM concentration but also by less than a first order dependence (Fig. 3B). This implies that conformational conversion of a nucleus is required even in the cross-seeded reaction. In this case, the conformational conversion could be templated by the heterologous seed. The templating effect of RnqPD aggregates on Sup35NM polymerization was further demonstrated by the dependence of the lag duration on the extent of seed fragmentation; cross-seeding was efficient.
only if RnqPD aggregates were sonicated before they were used as seed. This effect depended on the level of sonication (Fig. 3C).

**Sup35NM Cross-seeds RnqPD Polymerization**—Because aggregated RnqPD cross-seeds Sup35NM polymerization, we investigated the reciprocal reaction, i.e. the effect of Sup35NM fibers on RnqPD polymerization. Indeed, when RnqPD was polymerized in the presence of Sup35 fibers, the lag was shortened by increased levels of seed, and the plateau was higher than in the unseeded reaction (Fig. 4A). However, the lag in the cross-seeded reaction was never as short as in the self-seeded reaction, even at levels of up to 50% seed (data not shown). Fig. 4B describes the results of the kinetic analysis of polymerization of RnqPD similar to the one described above for Sup35NM polymerization (Fig. 3B). The lag phase in both unseeded and cross-seeded RnqPD reactions decreased weakly (less than first order) with increasing RnqPD concentrations (Fig. 4B; note that the cross-seeded reaction was performed at a fixed seed:monomer ratio). Cross-seeding was facilitated by sonication of Sup35NM seed (Fig. 4C).

Thus, both cross-seeded reactions had similar kinetic characteristics. However, the effect of the heterologous seed was more evident in cross-seeded polymerization of RnqPD, as a lower amount of Sup35 seed was needed to achieve a significant effect.

**Cross-seeding Occurs via Formation of Hybrid Aggregates Revealed by TEM**—To gain direct evidence of physical contact between Sup35NM and RnqPD upon cross-seeding, we analyzed cross-seeded Sup35NM fibers by TEM. Cross-seeded Sup35NM fibers appeared to sprout from unsonicated (Fig. 5, A–C, F, and G) and sonicated (data not shown) RnqPD seed. These fibers did not differ morphologically from self-seeded Sup35NM fibers (Fig. 5D) but tended to associate laterally more frequently (Fig. 5E). We identified the highly electron-dense amorphous structures on the TEM images as RnqPD seed based on a morphological comparison with the "RnqPD only" sample (Fig. 2E) and immunostaining by anti-Rnq antibody (Fig. 5, F and G). Anti-Rnq antibody had an extremely low level of background immunostaining (Fig. 2E and Fig. 5, F and G) and negligible affinity to Sup35NM fibers (data not shown). Since after overnight polymerization RnqPD alone failed to produce any fiber-like structures (Fig. 2E), the presence of frequent fibers in the cross-seeded reactions suggests a direct contact between Sup35NM fibers and RnqPD seed.
In the reciprocal cross-seeding reaction, rapidly formed RnqPD spherical intermediates appeared to be associated with sonicated Sup35NM fibers at 0 min (Fig. 6A). In samples taken at 4 h, almost all Sup35NM fibers were covered with aggregated RnqPD (Fig. 6B) that often formed chains and networks similar to those shown in Fig. 2. Frequent fibers did not appear in 4-h samples when Sup35NM seed was not present (data not shown). Remarkably, RnqPD appeared to coat individual Sup35NM fibers along their length rather than continue their growth at the tip. SDS treatment of the 4-h samples resulted in rare fiber-like structures (Fig. 6C) similar to those in the unseeded reaction (Fig. 2F) (in addition to globular RnqPD aggregates). Interestingly, no fibers that look like Sup35NM seed (Fig. 6A) were observed in the SDS-treated samples.

**DISCUSSION**

This work is focused on the QN-rich prion domain of Rnq1 (amino acids 132–405), which is sufficient to maintain a heritable aggregated state in vivo (22), and its interaction with Sup35NM. Kinetic analysis of polymerization and visualization of the resulting products by TEM showed that RnqPD efficiently forms self-templated aggregates in vitro and interacts with Sup35NM by a cross-seeding mechanism.

The sigmoidal kinetics of the RnqPD polymerization assayed with the amyloid-specific fluorescent dye ThT (Fig. 1, A–D) places it in a growing class of amyloidogenic proteins that aggregate by a nucleated mechanism. Several results suggest that RnqPD oligomerizes immediately after dilution in reaction buffer as follows. (i) A solution of freshly dissolved RnqPD turns opalescent immediately (data not shown). (ii) There is an initial slight enhancement of thioflavin T fluorescence (Fig. 1C). (iii) Unseeded polymerization is inhibited by the anti-oligomer antibody. (iv) Low n oligomers are detected by SDS-agarose gel electrophoresis (Fig. 1E). The inhibitory effect of the anti-oligomer antibody is the most direct evidence that the amyloid formation occurs via an oligomeric species. Although we cannot directly correlate the oligomers detected by the different techniques, nor can we tell if they are all on-pathway intermediates, oligomeric species were previously shown to be direct intermediates in aggregation of two other yeast prionogenic proteins, Sup35NM (33) and Ure2 (38). However, reports for other amyloidogenic proteins, e.g. β-oligomer of PrP (39), the variable domain of an immunoglobulin light chain (40), β-amyloid (41), and others, show the formation of nonfibrilligenic (off-pathway) isoforms in addition to self-propagated fibers.

RnqPD polymerization generally proceeds with a lag even when the reaction is self-seeded (Fig. 1B). A considerable lag was also reported in self-seeded recombinant PrP polymerization. It was proposed that conformational rearrangement following binding of PrP to fibers determines the rate-limiting step (42). Based on this idea, we speculate that the lag in self-
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seeded RnqPD polymerization is the time required for the establishment of fiber-forming centers associated with the spherical seed species. The newly formed fiber itself might bind to spherical aggregates laterally, concomitantly with its growth. This, and the propensity of the spherical aggregates to agglomerate, may inactivate fiber-forming centers and explains why spherical RnqPD aggregates, not fibers, were the major structure observed in TEM samples taken at different polymerization time points (Fig. 2). Because SDS treatment greatly facilitated the visualization of fibers (Fig. 2, F–I), at least some of the spherical aggregates are probably composed of compacted fibers. Moreover, the rare fibers observed in TEM images of non-SDS-treated samples mostly colocalize with the spherical species (Fig. 2D). Therefore, amyloidogenic fibrillation of RnqPD initiated by the fiber-forming centers on the spherical species proceeds efficiently as long as the supply of unconverted RnqPD lasts but is always limited by high order agglomeration. The latter process could be utilized as a negative feedback loop to maintain RnqPD prionization in the cell at the appropriate level. However, Rnq1 aggregates constituting the $\text{PIN}^{+}$ prion in vivo may have a different morphology from those studied in vitro.

We showed that RnqPD and Sup35NM cross-seed each other’s polymerization in both directions. Possibly, because the high order aggregation of fibers is not as significant for Sup35NM as for RnqPD, Sup35NM makes a more efficient seed than RnqPD. There appears to be a competition between cross-seeded and spontaneous Sup35NM polymerization as the latter can mask the effect of heterologous seed under certain conditions, e.g. if the concentration of seed is low or the reaction proceeds upon agitation, the cross-seeded polymerization of Sup35NM becomes kinetically indistinguishable from the unseeded reaction (Fig. 3 and data not shown).

Unseeded Sup35NM (Fig. 3B) (16) and RnqPD (Fig. 4B) polymerize with a lag suggesting a nucleated process, and the lag time exhibits a weaker-than-first order concentration dependence. To explain such a dependence, amyloidogenic conversion was proposed to occur within the context of unstructured oligomers (16), because if the conversion occurred at the monomeric level the concentration dependence would be stronger. We observe virtually the same weaker-than-first order dependency in our cross-seeded reactions (Figs. 3B and 4B) and likewise hypothesize that nucleation within the oligomeric species, not the binding of unconverted or pre-converted protein to the seed, is the rate-limiting step in cross-seeded polymerization. We further speculate that the formation of the nucleus is kinetically more favorable on a template as the heterologous seed shortens the lag. Such a template (RnqPD or Sup35NM aggregates, in our case) may stabilize a group of monomers (or a pre-existing oligomer) and limit the number of accessible conformations, increasing the probability of finding the amyloidogenic one.

The concept of templating is further supported by the visualization of hybrid aggregates resulting from cross-seeding (Figs. 5 and 6). Non-SDS-treated steady-state RnqPD aggregates used as seed do not appear to expose fiber ends (Fig. 2E) even after sonication (data not shown), yet they abundantly sprout off Sup35NM fibers upon cross-seeding (Fig. 5, A–C).

Thus we have no evidence attributing the cross-seeding event to fiber ends. Rather, it may be the aggregate surface that actually templates nucleation. The physical and chemical properties that make a surface an efficient nucleator require further study. In the reciprocal reaction, cross-seeded RnqPD pre-amyloid species were observed to bind to Sup35NM fibers (Fig. 6). If we assume that these aggregates are on-pathway, then the concept of stabilization of an intermediate by the seed surface is applicable here as well. In addition, Sup35 seed may retard agglomeration of immobilized RnqPD spherical aggregates further facilitating nucleation and keeping the newly formed fibers from rapid lateral association. This could result not only in a reduced lag but also in a higher rate of cross-seed RnqPD polymerization (Fig. 4A).

The direct interaction between Sup35NM and RnqPD upon cross-seeding demonstrated here can be used as a model of the in vivo situation. There may be differences, however, because prionization in vivo is under the tight control of chaperones (43) and proceeds in an environment packed with highly ordered structures that could provide a templating surface for nucleation (for example, a link between the cytoskeleton and Sup35 NMH11001prion formation was reported recently (44)). These factors could alter the rate constants of different levels of aggregation in vivo and, consequently, modify properties such as inducibility, stability, infectivity, and the penetrance of the associated phenotype. Also the difference between the cross-seeded and unseeded prion formation could be either increased or alleviated by intracellular factors. Overproduced Sup35 co-purifies with Rnq1 from $\text{PIN}^{+}$ cells even before the establishment of $\text{PSI}^{+}$ (26), which correlates with our in vitro data showing an affinity of Sup35NM to aggregated RnqPD. However, the frequency of the $\text{PIN}^{+}$-dependent appearance of $\text{PSI}^{+}$ is only $10^{-3}$ with and $10^{-6}$ without overproduction of Sup35 (7). This could be explained by assuming that the Sup35–Rnq1 interaction is required but not sufficient to establish $\text{PSI}^{+}$. Also, the newly seeded Sup35 aggregates could be destabilized by unknown in vivo factors resulting in the observed low frequency of $\text{PSI}^{+}$ induction. In general, it becomes apparent that the de novo formation of a prion should be studied while considering potential seeding templates. This offers a new dimension of therapeutic approaches targeting the cross-seeding events as a triggering point in prionogenesis.

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