Strong Growth Polarity of Yeast Prion Fiber Revealed by Single Fiber Imaging*

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Using the yeast prion as a model, we have developed a novel system to observe the growth of individual prion fibers directly. NM fragments, the prion-determining region of the yeast protein Sup35p, were labeled by either red or green fluorescent dyes, and the fiber growth was observed under a fluorescence microscope. When green Sup35NM was added to the preformed fibers made of red Sup35NM, 70–97% of green fibers grew unidirectionally, from only one end of individual red fibers, whereas the remainder grew from both ends. Similarly, the majority of red fibers grew from only one end of green fibers when the order of addition was reversed. Sonication of preformed fibers to expose fresh ends did not change the results, excluding a possibility of occasional deformation of one end as the reason of the apparent unidirectional growth. These results indicate the polarity of Sup35 prion fibers and impose constraints on the models of fiber growth.

Prions are infectious proteins (1); an abnormally folded form of the prion protein causes an auto-catalytic conversion of a normal (soluble) form of prion protein, which has the same primary structure as the abnormal one, to the abnormal insoluble form and results in generation and accumulation of infectious insoluble prion particles (1, 2). In many cases, the particles are observed as amyloid fiber-like structures, which are called “prion fibers.” This concept originated from the studies of mammalian neurodegenerative diseases such as Creutzfeldt-Jacob disease of humans, scrapie of sheep, bovine spongiform encephalopathy of cattle, and so on but recently spread to include other proteinogenic genetic elements from yeast Saccharomyces cerevisiae (3), namely [PSI+](4–11) and [URE3](12–14). In particular, the prion-inducing fragment of yeast Sup35 (Sup35NM), a determinant of the yeast prion-like [PSI+](15), is comprised of a glutamine/asparagine-rich N-terminal and medium domains of Sup35 and is a valuable model protein for studying the mechanism of prion-fiber formation (6, 8–11, 16, 17). These and other studies have established that the prion fibers are made through two distinct processes, the generation of seeds of fibers and the elongating growth of fibers. Here, we report the observation of fiber growth of Sup35NM labeled by fluorescent dyes. Different from most methods for analysis of fiber growth, e.g., Congo red binding, sedimentation analysis, and circular dichroism, and electron microscopy, this method enabled us to watch growing fibers individually in aqueous conditions. By using Sup35NM labeled with either green or red fluorescent dye, directionality of fiber growth was clearly observed.

EXPERIMENTAL PROCEDURES

Expression System—For bacterial expression, DNA encoding Sup35NM was amplified by polymerase chain reaction using pYK807 (a gift of Akihiko Kikuchi), which contains whole sup35 gene (18), as a template. Primers used were 5'-GGG GGG GCA TAT GTC GGA TTC AAA CAA AGG C-3' and 5'-CCG GGA CGC TGT AAT TCT TAG CAG TGG TGA TGG TGA TGG TGA TGG TGA TGG TGA TGG TGA TGG TGA TGA ACA ACT TGG TCA TC-3', which introduces a histidine tag and a cysteine (His6-Cys) at the C terminus (Sup35NM contains no endogenous Cys). The polymerase chain reaction product was subcloned as an NdeI-EcoRI fragment into pET21c, and the fidelity of the construct (pET-Sup35NMHC, where HC means His6-Cys) was confirmed by DNA sequencing.

Purification of Sup35NM—Escherichia coli BL21 (DE3) cells were transformed with pET-Sup35NMHC, cultured at 37 °C to A600 of 0.7, and induced with 1.0 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cells were harvested, suspended, and broken by sonication in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl2, 0.33 mg/ml lysozyme, 0.1 mg/ml DNase I, and a tablet of proteinase inhibitor mixture (Roche Molecular Biochemicals). The suspension was centrifuged at 17,000 × g for 50 min at 4 °C. The pellets were suspended with 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 4% (v/v) Triton X-100, washed by centrifugation at 17,000 × g for 50 min at 4 °C, suspended in water, and washed again by centrifugation. The resultant pellets were solubilized in Buffer A (8 mM urea, 20 mM sodium phosphate (pH 7.4), 500 mM NaCl, 10 mM β-mercaptoethanol, 10 mM imidazole) and were applied to a nickel-nitric acid superflow (Qiagen) column. The column was washed with 5 column volumes of Buffer A containing 10 mM imidazole and eluted with a 10–500 mM imidazole gradient in Buffer A. The eluate containing Sup35NM was dialyzed with 0.05% (v/v) trifluoroacetic acid and was applied to reversed-phase high pressure liquid chromatography (Poros R2/H). The column was washed with 5 column volumes of Buffer A containing 10 mM imidazole and eluted with a 10–500 mM imidazole gradient in Buffer A. The eluate containing Sup35NM was dialyzed with 0.05% (v/v) trifluoroacetic acid and was applied to reversed-phase high pressure liquid chromatography (Poros R2/H). The column was washed with 5 column volumes of Buffer A containing 0.1% (v/v) trifluoroacetic acid and eluted with 10–95% (v/v) acetonitrile gradient in 0.1% trifluoroacetic acid, and fractions containing Sup35NM were collected. Protein concentration was determined by UV absorption at 276 nm. Extinction coefficient for Sup35NM (25720 M–1 cm–1) was calculated based on the amino acid composition using DNASTAR software. Purified Sup35NM was verified as an expected polypeptide by both N-terminal sequencing and mass spectrophotometric analysis.

Labeling with Fluorescent Dyes—Sup35NM that had additional His6-Cys at the C terminus was used for the labeling, and because several proteins can be fused to the C terminus of the Sup35NM fragment without significant loss of the prion infectivity, we introduced Cys (and the fluorescent dye) at the C terminus of the Sup35NM fragment. Sup35NM was incubated either with an excess of tetramethylrhodamine-5-maleimide (TMR) or Oregon GreenTM 488-maleimide (OG; Molecular Probes) in 8 mM guanidine HCl and 25 mM HEPES (pH 7.5) for 24 h at 4 °C. Unreacted fluorescent dyes were removed by microcon-5 (Millipore) filtration and washed with 8 mM guanidine HCl repeatedly until filtrate did not show fluorescence. Labeled Sup35NM was concentrated with microcon-5. On average, a polypeptide was labeled by 0.3 dyes. For biotinylation, biotin–fluorescent dyes (OG, Oregon GreenTM 488-maleimide).

1 The abbreviations used are: TMR, tetramethylrhodamine-5-maleimide; OG, Oregon GreenTM 488-maleimide.
guanidine HCl was passed through a 100-kDa molecular-cut filter (micron-100; Millipore). The fiber formation was initiated by dilution of Sup35NM into Buffer B (5 mM potassium phosphate (pH 7.4), 150 mM NaCl) 100-fold or more, and the solution was incubated at 25 °C with gentle agitation. At the indicated times, the protein solution was mixed with Congo red solution, and the absorption spectrum was measured. Congo red bound to the amyloid fibers was estimated by the equation established by Klunk et al. (19). Time-lapse binding of Congo red to fibers of either TMR (red)- or OG (green)-labeled Sup35NM showed similar propagation as that of unlabeled Sup35NM with correction of a small contribution of labeling dyes to the absorbance (data not shown).

**Observation of Fluorescently Labeled Sup35NM Fibers—**We confirmed that fibers were formed similarly from guanidine HCl-denatured Sup35 and from urea-denatured Sup35NM by electron microscopic analysis (data not shown). However, because amorphous aggregates tended to appear in the urea solution of red-Sup35NM, we routinely started fiber formation from the guanidine HCl-denatured Sup35NM. Typically, red-Sup35NM (200 μM) in 8 M guanidine HCl were diluted 1:200 into Buffer B containing 1 mM dithiothreitol. Buffer B was filtered with an 0.22-μm filter and was previously degassed. After a 3-h incubation at 20 °C with gentle stirring by 10-rpm rotation, red-Sup35NM (200 μM) in 8 M guanidine HCl were diluted 1:200 into the mixture, and incubation was continued. At appropriate times, an aliquot of the incubated mixture was flowed into an observation chamber assembled from slide glasses (20), and fibers were allowed to adhere to the glass surface for 5 min. The free protein was removed by washing with excess Buffer B. To minimize bleaching, the solution in the chamber was exchanged with Buffer B containing 2,500 units/ml catalase (Fluka), 0.2 mg/ml glucose oxidase (Sigma), 6 mg/ml glucose, and 0.5% (v/v) 2-mercaptoethanol (21). Red- and green-labeled Sup35NM fibers were observed on an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan) with fluorescence cubes, WIG, and WIB (Olympus) for TMR and OG, respectively. The images were recorded with a SIT camera (G2741-08; Hamamatsu Photonics, Shizuoka, Japan) on a videotape and analyzed with NIH image and Adobe Photoshop. When indicated, a mixture of OG-Sup35NM fibers (1 μM) was subjected to sonication for 1 s at 4 °C with a Branson Sonifier 250.

**RESULTS**

Polarity of Fiber Growth Tested with Red and Green-Sup35NM—Because fusion of several proteins to the C terminus of Sup35NM does not significantly impair its ability to form fibers (6, 8, 22, 23), we introduced fluorescent dyes at the C-terminal cysteine of Sup35NM. Fiber formation was started by diluting the labeled Sup35NM in 8 M guanidine HCl into buffer. Fibers formed from fluorescent Sup35NM with kinetics similar to unlabeled Sup35NM, as tested by Congo red binding, light scattering, and electron microscopy (data not shown). When the fibers were immobilized on the glass surface, fibers made of the labeled Sup35NM were clearly visible with fluorescence microscopy. This procedure produced fibers segmented with two colors. If the fibers elongate unidirectionally, green segment should be found at only one end of the individual red fibers, producing “red-green” fibers. On the other hand, bidirectional growth of the fibers predicts the generation of “green-red-green” fibers.

Fluorescent images of individual fibers were categorized into two main variations, “green-only” fibers and red-green ones (Fig. 1A). Statistical analysis showed that, among 1343 fibers, 252 (19%) were red-green fibers, whereas only 7 (0.5%) were green-red fibers (Fig. 1B). The green-only fibers amounted to 79% of the total. We compared the average length of the green-only fibers to that of the green segment of the red-green fibers and found that the former (mean, ~1.0 μm) was shorter than the latter (mean, ~1.6 μm) (Fig. 1C). This indicates that the green-only fibers are the products formed de novo after the addition of the green-Sup35NM, because the green-Sup35NM monomers themselves can assemble into the seeds and generate green-only fibers after lag period, in addition to binding to and elongating the preformed red fibers.

Fusion of two fibers did not account for the red-green fiber formation, because simple mixing of preformed red fibers and green fibers did not produce the red-green fibers. In addition, the apparent polarity of the fiber growth is not because of any bias introduced by the attached fluorescent dyes. This was verified by the reciprocal experiment with the reverse order of addition; addition of red-Sup35NM monomers to the preformed green fibers resulted in production of red-only and red-green fibers.
fibers in a ratio similar to the above (data not shown).

**Observation of Growth of Immobilized Sup35NM Fibers**—To follow the growth of the same fibers, we developed another observation system (Fig. 2A). First, the preformed green-fibers containing biotinylated Sup35NM were immobilized on the glass surface through biotin-streptavidin linkers, and then red-Sup35NM monomers were introduced, and fibers were allowed to grow further. After a 25 min-incubation, unincorporated red-Sup35NM monomers were washed, and the fluorescent images were observed as shown in Fig. 1A. This cycle was repeated at 25-min intervals for 5 cycles. **B**, time-lapse imaging of fiber growth. After the immobilization of preformed green fibers containing biotinylated Sup35NM, red-Sup35NM monomers were introduced into the observation chamber and imaged at the indicated intervals. **Bar** indicates 10 μm.

**DISCUSSION**

We have developed a method to observe growth of the prion fibers under a microscope by labeling proteins with fluorescent dyes, and we have indicated polarity of fiber growth; the majority of fibers grew unidirectionally, and only a small fraction of fibers grew to both directions. Our result is in contrast with a recent report by Scheibel et al. (11) in which bidirectional growth of Sup35NM was proposed to be dominant based on electron microscopic observation.2 However, they also found fibers growing unidirectionally but did not present the results of a quantitative analysis. The real reason for the presence of bidirectional growing fibers in our experiments is not known, but two comments are worth mentioning. The bidirectional growth could be explained by some combinations of unidirectional growth, such as side-by-side association of two fibers.

2 Because Scheibel et al. (11) used the Sup35NM fragment without a histidine tag, we also investigated the directionality of the yeast prion fibers grown from Sup35NM without any histidine tag. Again, the growth of the prion fibers revealed the strong polarity (A. Kishimoto, H. Taguchi, and M. Yoshida, unpublished observation).
The unidirectional fiber growth of yeast prion reminds us of bacterial flagella fibers, which also grow unidirectionally both in vivo and in vitro (24). As discussed previously (2), the bacterial flagella are in some ways analogous to Sup35NM. Indeed, this is suggested by the fact that the N and C termini of flagellin, a building block of flagella, are disordered as monomers but become ordered upon polymerization into flagella fibers (25).

Several models have been proposed for the prion conversion process, and they belong to two general classes (Fig. 4), differing in the critical step, that is, whether conversion takes place after (Model 1) or before (Model 2) association with the fiber. Model 1 is better able to explain unidirectional growth; a monomer has a single fiber-binding site, and another binding site, which is necessary to accept the next monomer, is generated only after the monomer associates to the fiber. If a prion monomer has two fiber-binding sites, a fiber should grow bidirectionally as in Model 2.

As time-lapse AFM analysis has revealed a bidirectional growth of fiber-forming amylin and β-amyloid peptides (26, 27), it is not clear that fiber growth with strong polarity, unidirectional growth as an extreme case, is a general feature of prion fibers. Nevertheless unidirectional growth polarity of the yeast Sup35NM prion fibers seems clear.

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