Role for Hsp70 Chaperone in *Saccharomyces cerevisiae* Prion Seed Replication

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The *Saccharomyces cerevisiae* [PSI⁺] prion is a misfolded form of Sup35p that propagates as self-replicating cytoplasmic aggregates. Replication is believed to occur through breakage of transmissible [PSI⁺] prion particles, or seeds, into more numerous pieces. In [PSI⁺] cells, large Sup35p aggregates are formed by coalescence of smaller sodium dodecyl sulfate-insoluble polymers. It is uncertain if polymers or higher-order aggregates or both act as prion seeds. A mutant Hsp70 chaperone, Ssa1-21p, reduces the number of transmissible [PSI⁺] seeds per cell by 10-fold but the overall amount of aggregated Sup35p by only two- to threefold. This discrepancy could be explained if, in SSA1-21 cells, [PSI⁺] seeds are larger or more of the aggregated Sup35p does not function as a seed. To visualize differences in aggregate size, we constructed a Sup35-green fluorescent protein (GFP) fusion (NGMC) that has normal Sup35p function and can propagate like [PSI⁺]. Unlike GFP fusions lacking Sup35p’s essential C-terminal domain, NGMC did not form fluorescent foci in log-phase [PSI⁺] cells. However, using fluorescence recovery after photobleaching and size fractionation techniques, we find evidence that NGMC is aggregated in these cells. Furthermore, the aggregates were larger in SSA1-21 cells, but the size of NGMC polymers was unchanged. Possibly, NGMC aggregates are bigger in SSA1-21 cells because they contain more polymers. Our data suggest that Ssa1-21p interferes with disruption of large Sup35p aggregates, which lack or have limited capacity to function as seed, into polymers that function more efficiently as [PSI⁺] seeds.

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into more numerous pieces and by dismantling Sup35p aggregates into individual polymers.

Although SSA1-21 mutants have a large reduction in [PSI\(^+\)] seeds per cell, fractionation of Sup35p by centrifugation shows that there is only a slight reduction in the amount of aggregated Sup35p in SSA1-21 cells (23). This discrepancy could be explained if the [PSI\(^+\)] seeds in SSA1-21 cells are larger than normal or if more of the aggregated Sup35p in SSA1-21 cells does not function as [PSI\(^+\)] seeds.

To determine visually if Sup35p aggregated to a greater extent in live SSA1-21 cells, we replaced Sup35p with a Sup35-green fluorescent protein (GFP) fusion that functions normally in translation termination and in [PSI\(^+\)] propagation. In contrast to what is seen with widely used GFP fusions lacking the essential C-terminal domain Sup35p, visual differences in GFP fluorescence between [PSI\(^+\)] and [psi\(^-\)] were not obvious in log-phase cells. Using more sensitive techniques, however, we find evidence of Sup35p aggregates in the [PSI\(^+\)] cells and that these aggregates are larger in SSA1-21 cells. We further find that SSA1-2I has little effect on the size of Sup35p polymers in [PSI\(^+\)] cells, suggesting that Sup35p aggregates in SSA1-21 cells are larger because they contain more polymers. We present a new technique for analyzing aggregation of prion proteins in S. cerevisiae and provide insight into the role of Hsp70 in yeast prion propagation.

MATERIALS AND METHODS

Strains, media, and growth conditions. The yeast strains are listed in Table 1. Strain 779-6A is [PSI\(^+\)], which is the origin of [PSI\(^+\)] for all strains derived from 779-6A. Strain 74D-694 and its sup35::TRP1 derivative (designated 74-D694a) were gifts from Y. Nakamura (19). Where strains lacking [PSI\(^+\)] were used, [PSI\(^-\)] was eliminated by growth on medium containing 3 mM guanidine hydrochloride. The SSA1-2 allele of SSA1 encodes a tryptophan in place of leucine at codon 483 (23). Gene replacements at the chromosomal SSA1 and SSA2 loci have been described (21).

Strain J370 is a [PSI\(^+\)] diploid formed by a cross of strains 1001 and 1013, in which one SUP35 allele was replaced with KanMX by transformation with DNA amplified by PCR (2, 47). To obtain strains with wild-type and GFP-tagged SUP35 (designated NGMC), strain J370 was first transformed by p501 (see below), which is a HIS3-based plasmid carrying wild-type SUP35. J370/p501 meiotic segregants with p501 that are sup35::KanMX and either wild-type (780-1D) or SSA1-21 (780-1C) were selected. To replace SUP35 with NGMC, 780-1C and 780-1D were first transformed with p501 (see below), which is a LEU2-based plasmid with NGMC. Sup35p is essential for growth, so transformants must maintain either p501 or p510. Moreover, when cells have either p501 or p510, the plasmid is maintained without selection (i.e., on rich medium). After growing transformants having both plasmids in rich medium, clones were identified that had lost p501 and retained p510. All such clones retained a typical [PSI\(^+\)] phenotype, indicating that the resident [PSI\(^+\)] is formed of the untagged Sup35p (expressed from p501) induced conversion of NGMC to a stable [PSI\(^+\)] form (see below). A similar plasmid shuffle was used to replace Sup35p with NGMC in strain 74D694a.

1/2YPD medium contains 0.5% yeast extract, 2% peptone, and 2% dextrose. YPAD is similar but contains 1% yeast extract and 400 mg of adenine/liter. 1/2YPD/G3 is 1/2YPD containing 3 mM guanidine. Solid media contained 2% 2,2-dimethoxyphenol and 4% agar. Growth conditions were as described (23) or as indicated. Cytoplasmic transmission of prions between strains (cytoduction) was done as described (23).

ESTIMATION OF [PSI\(^+\)] SEED NUMBERS. Quantitative assays were a slight modification of a previously described method (7). Cells grown on selection plates lacking adenine at 25°C were streaked onto 1/2YPD plates and grown for 1, 2, and 4 days at 30°C. Cells were then restreaked onto 1/2YPD/G3 and grown for 40 h at 30°C. Entire single colonies were picked up and diluted in 200 μl of water, spread onto selection plates lacking adenine, and grown for 2 weeks at 25°C. The resulting colonies were replica plated onto 1/2YPD and 1/2YPD/G3 plates, which were then incubated for 2 days at 30°C followed by 3 or more days at 25°C.
RESULTS

Functional Sup35-GFP fusion protein. Sup35p is a translation termination factor that catalyzes release of nascent peptides when ribosomes encounter stop codons. In [PSI+] cells, much Sup35p is in an aggregated form, which reduces translation termination efficiency and causes nonsense suppression. We monitored the presence of [PSI+] by its ability to suppress the ade2-1 nonsense allele when the SUQ5 tRNA is present (8). Nonsuppressed ade2-1 mutants require adenine and are red due to accumulation of a substrate of Ade2p. [PSI+] restores adenine prototrophy and white colony color.

Sup35p can be subdivided into three regions: a dispensable amino-terminal domain (N) rich in polar amino acids that is necessary for Sup35p prion formation, a highly charged middle domain (M) of unknown function that is also dispensable, and a C-terminal domain (C) that is necessary and sufficient for Sup35p release factor function (45, 52). Fusion proteins with GFP appended to the N or N and M domains of Sup35p have been used widely to observe Sup35p aggregation in [PSI+] cells (3, 10, 36, 53). They are useful for diagnosing the presence or absence of [PSI+] because fluorescence of the fusion proteins is diffuse in [psi−] cells but coalesces into foci in [PSI+] cells, apparently because the fusions incorporate into existing Sup35p aggregates through N domain interactions. Such reporters provide only indirect information on the state of endogenous Sup35p, however, and the foci might also represent self-aggregation of the fusion protein alone that is “seeded” by Sup35p prion aggregates. Moreover, these fusion proteins can induce [PSI+] to appear when expressed in [psi−] cells. This induction likely reflects an efficient ability of the fusions to drive aggregation, complicating interpretations regarding properties of the intact Sup35p.

Our earlier observations that SSA1-21 mutants have many fewer transmissible [PSI+] particles or seeds per cell but only a slight decrease in the amount of aggregated Sup35p could be explained if more aggregated Sup35p does not function as seeds or if the seeds are larger than normal (23). In an attempt to visualize differences in Sup35p aggregation directly in vivo and avoid the complications of truncated Sup35p-GFP fusions, we created a functional full-length Sup35-GFP fusion protein. The fusion protein has GFP inserted between the N and M domains and is designated NGMC to distinguish it from Sup35p, which we refer to as NMC (Fig. 1A). Cells lacking chromosomal SUP35 and expressing NMC or NGMC from the SUP35 promoter on a single-copy plasmid grew at similar rates (106 and 108 min/cell division, respectively; also see Fig. 1B for rate of colony formation). Therefore, NGMC performs as well as NMC with respect to essential Sup35p function.

To obtain the NGMC strains, we replaced NMC with NGMC by a plasmid shuffle technique (see Materials and Methods). The resulting wild-type and SSA1-21 strains, which express NGMC as the only source of Sup35p, retained typical [PSI+] phenotypes, indicating that NGMC could propagate as a prion. The letter G is added to the prion designation of NGMC cells ([GPSI+]) to distinguish it from [PSI+]. [GPSI+] therefore arose from conversion of NGMC by [PSI+] prions composed of NMC while the two proteins were both present in the same cell. Thus, the [PSI+] and [GPSI+] prions are compatible and are of the same “strain” origin. The [PSI+] and [psi−] states are normally very stable: spontaneous appearance or mitotic loss of [PSI+] occurs very rarely. We did not quantify such events but observed no difference in spontaneous appearance or loss of [PSI+] or [GPSI+] during routine handling and storage of cultures. Thus, NGMC was as stable as NMC in both the prion and nonprion states.

Compared to [PSI+] cells, [GPSI+] cells had a very faint pink color when grown at 30°C (Fig. 1B), which is indicative of a slight reduction in nonsense suppression. [PSI+] and [GPSI+] phenotypes were otherwise indistinguishable. [GPSI+] was similarly inhibited by SSA1-21, displaying comparable reductions in nonsense suppression and mitotic stability (Fig. 1B). [PSI+] and [GPSI+] also were the same regarding curability by deletion and overexpression of Hsp104 or by including millimolar amounts of guanidine hydrochloride in the growth media, which inactivates Hsp104 (data not shown) (5, 15, 24, 46). Finally, [PSI+] and [GPSI+] could both be transmitted cytoplasmically (by cytoduction, see above) with equal efficiency to both NGMC and NMC strains (data not shown). These results demonstrate that the GFP insertion did not significantly affect prion interactions of the N domains of NGMC.
or of those between NGMC and NMC. Strains discussed here-after, unless indicated otherwise, express NGMC in place of NMC.

**NGMC forms fluorescent foci only in [GPSI+]**

 cells of aging cultures. GFP fluorescence in [Gpsi–] wild-type and SSA1-21 cells was diffuse throughout the cytoplasm in both log-phase and stationary-phase cultures (Fig. 2). Unexpectedly, for both wild-type and SSA1-21 strains, GFP fluorescence in [GPSI+] cells from 1-day colonies of both strains is indistinguishable from that in [Gpsi–] cells. Foci of NGMC fluorescence are apparent only in [GPSI+] cells from older colonies of the same cultures.

NM-GFP fusion in our strains. When NMC was replaced with NGMC in another strain (74-D694) commonly used to monitor [PSI+], we again saw the appearance of [GPSI+] foci only in stationary-phase cells (Fig. 3A). Additionally, in our wild-type and SSA1-21 log-phase [PSI+] cells, fluorescent foci were clearly present after expressing NM-GFP for only 2 h (Fig. 3B). NM-GFP did not form foci in [psi–] variants of the same strains or of those between NGMC and NMC. Strains discussed here-after, unless indicated otherwise, express NGMC in place of NMC.

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 cells of aging cultures. GFP fluorescence in [Gpsi–] wild-type and SSA1-21 cells was diffuse throughout the cytoplasm in both log-phase and stationary-phase cultures (Fig. 2). Unexpectedly, for both wild-type and SSA1-21 strains, GFP fluorescence in [GPSI+] cells was uniformly diffuse throughout the cytoplasm, similar to that of [Gpsi–] cells. Thus, aggregation of NGMC into particles of a size detectable as fluorescent foci was not required for propagation of [GPSI+], suggesting that [PSI+] similarly does not require such aggregation of NMC.

Numerous fluorescent foci appeared throughout the cytoplasm of both wild-type and SSA1-21 cells from aging [GPSI+] cultures (Fig. 2). In focusing through the cells, we estimated the number of foci, which streamed throughout the cytoplasm, to be several hundred to a thousand. There was no consistent difference in the size or number of aggregates between age-matched wild-type and SSA1-21 cells. No such foci appeared in [Gpsi–] cells in cultures of any age. Thus, foci were restricted to [GPSI+] cells and appeared only in stationary-phase cells.

The diffuse fluorescence in [GPSI+] log-phase cells was unexpected because other widely used GFP fusions, which lack the Sup35p C terminus, show clear focus formation after expressing the fusions for a relatively short time. Since our GFP fusion and strain background both differed from those used previously to visualize Sup35p in vivo, we tested both our GFP fusion in another strain background, and the commonly used NM-GFP fusion in our strains. When NMC was replaced with NGMC in another strain (74-D694) commonly used to monitor [PSI+], we again saw the appearance of [GPSI+] foci only in stationary-phase cells (Fig. 3A). Additionally, in our wild-type and SSA1-21 log-phase [PSI+] cells, fluorescent foci were clearly present after expressing NM-GFP for only 2 h (Fig. 3B). NM-GFP did not form foci in [psi–] variants of the same strains or of those between NGMC and NMC. Strains discussed here-after, unless indicated otherwise, express NGMC in place of NMC.
TABLE 2. Prion seed numbers in wild-type and SS1-21 cells

| Time of incubation on 3 mM guanidine (days) | Avg no. of seeds per cell ± SD | Wild type | SS1-21 mutant |
|-------------------------------------------|-------------------------------|-----------|--------------|
| 1                                         | 76 ± 9                        | 8 ± 2     |              |
| 2                                         | 51 ± 18                       | 2 ± 1     |              |
| 4                                         | 52 ± 21                       | 3 ± 1     |              |

* Data are averages of two or three independent experiments with five to nine colonies for each time point.

under the same conditions. Therefore, focus formation or lack thereof was a characteristic of the GFP fusion rather than the strain background.

Fluorescent foci do not directly represent [GPS1⁺] seeds. In stationary-phase cells, the number and appearance of NGMC aggregates that have attained a size visible by GFP fluorescence were very similar in wild-type and SS1-21 cells. Since SS1-21 mutants have fewer seeds per cell, we inferred that these foci do not represent prion seeds. To test this hypothesis, and to determine if stationary-phase cells had different numbers of seeds than log-phase cells, we used a recently described technique (7) based on guanidine inactivation of Hsp104 (15, 24) to estimate seed number per cell. Log-phase and stationary-phase cells, grown like those used for the fluorescence imaging, were spread onto rich medium containing 3 mM guanidine and allowed to form colonies. Inhibition of Hsp104 by guanidine blocks replication of [PSI⁺] seeds (33). As a cell grows into a colony, its limited number of seeds become distributed to only a portion of its progeny in the colony. The number of [PSI⁺] cells in the resulting colony, determined by plating the cells of the colony onto medium selecting for [PSI⁺] seeds, provides an estimate of the number of [PSI⁺] seeds that were present in the cell that began the colony.

In agreement with our earlier estimates of a 10-fold difference (23), we found about 80 seeds/cell for wild-type cells and about 10/cell for SS1-21 cells (Table 2). These seed numbers are much lower than the number of fluorescent foci. Moreover, this difference does not correlate with a difference in the number of foci between wild-type and SS1-21 cells. Together with the observation that [GPS1⁺] seeds propagate in the absence of foci in log-phase cells, these data suggest that the foci do not directly represent [GPS1⁺] seeds. Although these results do not rule out the possibility that aggregates seen as foci have some ability to act as [GPS1⁺] seeds, they imply that such ability is limited. On average, the seed numbers of stationary-phase cells were lower than those of log-phase cells for both wild-type and SS1-21 strains. Apparently, in stationary-phase [GPS1⁺] cells, aggregation of Sup35p continues but seed replication does not.

NGMC aggregates in [GPS1⁺] cells are larger in the SS1-21 mutant. We measured fluorescence recovery after photobleaching as a more sensitive technique to detect differences in the size of NGMC aggregates in wild-type and SS1-21 log-phase cells. Because the rate of diffusing particles is related to particle mass, the time required for recovery of fluorescence by diffusion of unbleached protein after photobleaching will be longer for particles having more mass. Therefore, increased time of fluorescence recovery can reflect increased size of NGMC aggregates. Compared with isogenic [Gpsi⁻] cells there was a significant increase in recovery time for wild-type [GPS1⁺] cells, suggesting that aggregates of NGMC were present (Fig. 4). Similar measurements showed that such aggregates were also present in SS1-21 [GPS1⁺] cells and that these aggregates were larger than those in wild-type [GPS1⁺] cells.

The slower recovery times could also reflect a difference in other factors that influence diffusion, such as association of NGMC with subcellular structures. To confirm that the difference in fluorescence recovery after photobleaching was due to increased size, we fractionated lysates of log-phase cells on a sizing column (Fig. 5). Consistent with the conclusion that SS1-21 [GPS1⁺] cells have larger NGMC aggregates, NGMC from SS1-21 lysates eluted from the column earlier than NGMC from wild-type lysates (Fig. 5B, fraction 31) and was more abundant than that from wild-type lysates in the highest-molecular-weight fractions (Fig. 5B and 5C). Very similar results were obtained with a different column (see Materials and Methods; data not shown). Thus, two independent lines of evidence suggest that NGMC aggregates in [GPS1⁺] cells are larger in SS1-21 mutants. We also observed more soluble NGMC in the SS1-21 lysate than in the wild-type lysate (Fig. 5A, fractions 64 and higher), which agrees with previous centrifugation fractionation experiments (23).

Kryndushkin et al. recently proposed that large aggregates of Sup35p in [PSI⁺] cells are formed by the coalescence of a number of smaller SDS-insoluble Sup35p “polymers” that range from about 800 to 3,000 kDa, or about 8 to 50 Sup35p monomers in typical [PSI⁺] cells (27). The SDS-insoluble polymers migrate as large aggregates under nondenaturing conditions, such as those used in the sizing column. When treated with SDS, however, the larger aggregates fall apart into individual polymers that remain intact. The polymers can then be resolved by electrophoresis in semidenaturing agarose gels.

Data are averages of two or three independent experiments with five to nine colonies for each time point.
Semidenaturing agarose gel electrophoresis does not provide information about the size of the larger aggregates but is useful for comparing the sizes of the SDS-insoluble polymers from different strains. The increased size of NGMC aggregates could be due to an increase in the size of such polymers or of the larger aggregates composed of them or both.

We used semidenaturing agarose gel electrophoresis to determine if the increased size of NGMC aggregates in \textit{[GPSI$^+$]} SSA1-21 cells was due to increased size of NGMC polymers. When lysates of log-phase \textit{[GPSI$^+$]} cells were fractionated on semidenaturing agarose gels, there was no significant difference in mobility of SDS-insoluble NGMC polymers between SSA1-21 and wild-type cells (Fig. 6). These results indicate that the increased size of aggregates in SSA1-21 cells is not due to increased size of individual SDS-insoluble polymers. Rather, it is likely due to an increase in the average number of these polymers that are present in the larger aggregates, which would imply that there are fewer free polymers in SSA1-21 cells. In agreement with the column fractionation (Fig. 5), semidenaturing agarose gel electrophoresis also showed that \textit{[GPSI$^+$]} SSA1-21 cells had more soluble Sup35p than wild-type \textit{[GPSI$^+$]} cells.

\textbf{SSA1-21 does not affect resolubilization of denatured luciferase by Hsp104.} Reactivation of denatured protein by Hsp104 requires the assistance of Hsp70 and Hsp40 (18). Although we earlier found that \textit{SSA1-21} did not impair Hsp104’s ability to confer tolerance to exposure to lethal heat (23), we wished to quantify more precisely the ability of Hsp104 to reactivate heat-denatured protein in vivo. To do this, we used a thermolabile luciferase reporter protein whose solubilization from aggregates depends upon Hsp104 (13, 35). The contribution of Hsp70 to this process in vivo cannot be tested directly since Ssa protein is essential for growth. In wild-type and \textit{SSA1-21} strains lacking \textit{SSA2}, however, Ssa1p and Ssa1-21p, respectively, are the only detectable Ssa proteins. The rate of reactivation and the yield of active luciferase in cells expressing Ssa1-21p were similar to that of wild-type cells (Fig. 7). These results demonstrate that Ssa1-21p was not significantly affecting Hsp104 activity in luciferase reactivation in vivo, nor was it significantly altered in Hsp70 activity important for this process.

\textbf{DISCUSSION}

We describe a new Sup35-GFP fusion (NGMC) that supports cell growth and prion phenotypes in ways nearly indistinguishable from those of native Sup35p. NGMC allows direct visual monitoring of Sup35p in live cells, which can be used with fluorescence recovery after photobleaching analysis to analyze the aggregation status of Sup35p in vivo. This ability to monitor Sup35p in real time under a variety of conditions will be useful for studying the dynamics of Sup35p aggregation and transmission in \textit{[PSI$^+$]} cells. With this fusion, we show by directly measuring in vivo diffusion rates that NGMC aggre-
gates in\textit{PSI}/H11001\textsuperscript{−} cells are larger than normal in SSA1-21 mutants. We also find that the size of NGMC polymers that make up the aggregates is unchanged in SSA1-21 cells, suggesting that the aggregates are larger because they contain more polymers per aggregate. Furthermore, we find that aggregation of NGMC to the extent that it forms visible fluorescent foci is not required for prion propagation and that aggregates of NGMC seen as fluorescent foci lack the ability or have limited capacity to function as [\textit{PSI}‡] seeds.

Our data are consistent with earlier studies showing that prions can be present in cells lacking fluorescent foci. After overexpressing a Sup35NM-GFP fusion to induce [\textit{PSI}‡] in [\textit{psi–}] cells, the number of cells with foci was smaller than the number of cells induced to become [\textit{PSI}‡] (53). This difference indicates that visible aggregates containing the NM-GFP fusion were not present in many cells that later would be scored as [\textit{PSI}‡]. The \textit{S. cerevisiae} [\textit{URE3}] prion, which is formed of Ure2 protein, is also capable of propagating in cells lacking foci when maintained by a Ure2-GFP fusion protein (14).

Propagation of [\textit{PSI}‡] requires both growth and replication of Sup35p prion aggregates. [\textit{PSI}‡] prion seeds can be defined as inheritable self-propagating units of Sup35p, one of which is sufficient to establish the prion phenotype. This concept alludes to the particulate character of [\textit{PSI}‡] inheritance. Ample in vitro and in vivo evidence suggests that \textit{S. cerevisiae} prions propagate by an amyloid-like mechanism, in which highly structured fibrous aggregates recruit protein into the fibers (6, 9, 17, 25, 26, 28, 41, 43, 44, 51). Also, Sup35p amyloid produced in vitro can seed [\textit{PSI}‡] propagation in vivo (25, 43). Although the precise structure of the physical entities that represent infectious [\textit{PSI}‡] seeds remains uncertain, seed replication is generally and most simply envisioned as occurring by the breakage of such self-propagating fibers into more numerous pieces. For naturally occurring yeast prions, efficient seed replication...
requires the disaggregating activity of Hsp104 (5, 11, 31, 37–39).

Kryndushkin et al. showed that aggregated Sup35p in [PSI+] cells is found as individual SDS-insoluble polymers and as large aggregates formed by the coalescence of a number of such polymers (27). They propose that Hsp104 generates new [PSI+] seeds by severing the polymers and by dismantling the large Sup35p aggregates into individual polymers, both of which increase the number of free polymers. Additionally, it was suggested that Sup35p polymers within the aggregates could be severed without generating new seeds if the resulting polymer fragments remained associated with the aggregate.

The implications of this work are that the Sup35p polymers act efficiently as [PSI+] seeds and that higher-order aggregates composed of them, while less efficient, can act as [PSI+] seeds by being a source of Sup35p polymers that are liberated by the action of cellular chaperones. Although this study provides very useful insight into the physical basis of [PSI+] seeds, it should be emphasized that the precise properties of seeds is uncertain, as they are quantified genetically as the transmissible entities that perpetuate the [PSI+] state.

The interpretation that individual Sup35p polymers act most efficiently as [PSI+] seeds also provides a simple explanation for our observations. Our conclusion that NGMC aggregates are larger in SSA1-21 cells because there are more NGMC polymers per aggregate would explain the concomitant decrease in [GPSI+] seeds. If a larger proportion of NGMC polymers were associated with large aggregates, then there would be fewer free NGMC polymers. Moreover, since the efficiency with which Sup35p is recruited into prion aggregates depends upon the number of seeds (33), the increase in Sup35p solubility in SSA1-21 cells can be explained by the reduced number of free polymers capable of recruiting and therefore depleting Sup35p. A similar conclusion relating free polymers to Sup35p solubility has been reported by others (1, 27).

An explanation for the effects of SSA1-21 is that Ssa1p (Hsp70) normally acts in [PSI+] seed propagation by dismantling polymers from Sup35p aggregates. Our earlier work showed that Ssa1p has enhanced substrate binding compared with Ssa1p (20, 21). By binding more avidly to Sup35p aggregates, Ssa1p might be impaired in an Hsp70 activity that promotes the disassembly of aggregates into polymers, either directly or through interaction with Hsp104. Although an inhibitory effect on Hsp104 would be expected to cause an increase in size of Sup35p aggregates, those that inhibit Hsp104 alone, such as guanidine inactivation, increase the size of Sup35p polymers (22, 27). The effect of Ssa1p on [PSI+] could be due to altered interaction of Ssa1p with Hsp104 in the dismantling process or to steric hindrance of Hsp104 access to Sup35p aggregates by increased amounts of bound Ssa1p. Any of these direct or indirect effects would also explain the ability of Ssa1p to impair [PSI+] in the presence of the functionally redundant Ssa2p. Since cells expressing Ssa1p as the only Ssa protein have no apparent defect in thermostolerance or reactivation of denatured luciferase in vivo, the process of breaking highly ordered Sup35p aggregates to generate new prion seeds should be different than disaggregation of amorphous aggregates of thermally denatured protein. Alternatively, replication of prion aggregates might be much more sensitive to disruptions of Hsp70 or Hsp104 machinery function.

An alternative explanation also consistent with the dominant effects of Ssa1-21p is that it is hyperactive in an Hsp70 function that promotes aggregation of the Sup35p polymers into higher-order aggregates. Work showing that excess wild-type Ssa1 causes fluorescent Sup35NM-GFP aggregates in [PSI+] cells to increase in size led to the proposal that Ssa1p helps assemble Sup35p into larger aggregates that are less sensitive to the disaggregating activity of Hsp104 (3). In contrast to SSA1-21p, however, excess Ssa1p increases nonsense suppression and does not cause [PSI+] instability (34). Moreover, unlike SSA1-21, excess Ssa1p increases the size of Sup35p polymers in [PSI+] cells (1). Although Ssa1p might help Sup35p polymerize in vivo, the effects of SSA1-21p on [PSI+] are not consistent with its being more efficient at a wild-type Hsp70 function.

Fluorescent foci in [PSI+] cells expressing Sup35NM-GFP fusions are interpreted as reflecting the extent of aggregation of endogenous Sup35p. Depletion of Hsp104 causes such foci to increase in size while decreasing in number (48). Similarly, foci in cells harboring variants of [PSI+] that appear partially resistant to Hsp104 are larger and fewer (3). These differences correlate with reduced [PSI+] stability, which is consistent with the notion that Hsp104 acts in [PSI+] propagation by breaking Sup35p aggregates. Furthermore, large visible aggregates of GFP fusion of both Sup35-NM and Ure2p (the determinant of the yeast [URE3] prion) have been suggested to be dead-end products of prion propagation that lack or have limited seeding capacity (3, 38, 48).

The appearance of fluorescent NGMC foci in cells of aging cultures shows that a functional Sup35-GFP fusion protein also can aggregate to the degree that it forms visible fluorescent foci, but our data also show that [GPSI+] propagation does not require aggregation of NGMC to such an extent. Therefore, fluorescent foci do not necessarily represent [GPSI+] seeds. In fact, wild-type and SSA1-21 cells had similar numbers of foci but different numbers of seeds. Moreover, the large number of foci in stationary-phase cells does not correlate with the small number of seeds estimated to be present in these cells. Thus, the number of the NGMC aggregates represented by foci lack seeding capacity.

Our data showing that there are many fewer [GPSI+] seeds than foci are consistent with the conclusions drawn by Kryndushkin et al., who proposed that foci seen in cells expressing Sup35-GFP fusions represent the largest aggregates and that the majority of seeds should be below detection by GFP fluorescence (27). Moreover, since detection of aggregates by fluorescence recovery after photobleaching in the diffuse fluorescence of log-phase cells implies that most of the fluorescent material is in an aggregated form, the number of aggregates in these cells must be much higher than the number of foci seen in stationary-phase cells. Thus, most Sup35p aggregates in [PSI+] cells do not function as prion seeds. Alternatively, current methods for determining [PSI+] seed numbers per cell significantly underestimate such numbers.

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