In Vitro Propagation of the Prion-Like State of Yeast Sup35 Protein

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The yeast cytoplasmically inherited genetic determinant [*PSI*$^+$] is presumed to be a manifestation of the prion-like properties of the Sup35 protein (Sup35p). Here, cell-free conversion of Sup35p from [*psi*$^-$] cells (Sup35p^{psi-}) to the prion-like [*PSI*$^+$]-specific form (Sup35p^{PSI+}) was observed. The conversion reaction could be repeated for several consecutive cycles, thus modeling in vitro continuous [*PSI*$^+$] propagation. Size fractionation of lysates of [*PSI*$^+$] cells demonstrated that the converting activity was associated solely with Sup35p^{PSI+} aggregates, which agrees with the nucleation model for [*PSI*$^+$] propagation. Sup35p^{PSI+} was purified and showed high conversion activity, thus confirming the prion hypothesis for Sup35p.

Transmissible spongiform encephalopathies, such as human Kuru, Creutzfeldt-Jakob disease, and sheep scrapie, are thought to be caused by prions, the infectious agents that lack a nucleic acid genome (*1*). Many lines of evidence support the "protein only" hypothesis (*2*), which proposes that the prion represents a pathogenic conformer (PrP^{Sc}) of endogenous host protein (PrP^{C}), able to propagate its abnormal conformational state by an autocatalytic mechanism (*1*, *3*). Conversion of PrP^{C} to PrP^{Sc} was reproduced in a cell-free system and was used to show that PrP^{Sc} derives from the direct interaction of PrP^{C} and PrP^{Sc} (*3*).

Because of their genetic properties, two non-Mendelian genetic elements of the yeast *Saccharomyces cerevisiae*, [*URE3*] and [*PSI*$^+$] (*4*), have been proposed to arise, like prions, from the ability of the Ure2 and Sup35 proteins, respectively, to exist in alternative conformational and functional states (*5*–*7*). Sup35p belongs to a family of proteins related to eukaryotic polypeptide release factor 3 (*8*). It is a multidomain protein, in which the COOH-terminal (C) domain shows similarity in amino acid sequence to translation elongation factor EF-1α and is essential for translation termination and cell viability (*9*, *10*). The Sup35p NH_2-terminal region of 253 amino acids is inessential for viability and can be subdivided into the NH_2-terminal (N) domain of 123 amino acids, required for [*PSI*$^+$] maintenance (*11*), and the middle (M) domain, for which no function has been ascribed (Fig. 1). Some properties typical of mammalian prions, such as aggregation and resistance to proteases, have also been demonstrated for the Sup35 protein in the [*PSI*$^+$] state (*6*, *7*). The propagation of the [*PSI*$^+$] determinant is mediated in vivo by the oligomerization of Sup35p, which requires its NH_2-terminal domain (*6*). These results provide a simple explanation for the nonsense-suppressor [*PSI*$^+$] phenotype: The aggregation of Sup35p inhibits its function in translation termination. Although these findings support the prion model for propagation and phenotypic manifestation of yeast [*PSI*$^+$], proving this model would require the demonstration that Sup35p^{PSI+} alone, rather than any other cellular component, causes the prion-like conversion of Sup35p^{psi-}. We found that Sup35p^{psi-} is convertible to an aggregated protease-resistant form if incubated with preexisting Sup35p^{PSI+} and that Sup35p^{PSI+} is the only protein required for causing this conversion.

NH_2-terminal fragments of Sup35p (Fig. 1) expressed in [*PSI*$^+$] cells have the same properties as full-length Sup35p: high protease resistance and propensity to aggregate (*6*, *7*, *12*). This allowed us to use COOH-terminally deleted Sup35p variants to distinguish Sup35p originating from the [*psi*$^-$] and [*PSI*$^+$] lysates. We monitored conversion by detecting a shift of cytosolic Sup35p^{psi-} to the aggregated fractions. To test whether Sup35p^{psi-} could be converted in vitro to a prion-like state, we mixed lysates of [*psi*$^-$] transformants of the strain 5V-H19 [MATa *ade2-1 SUQ5 leu2-3,112 ura3-52* (*11*)] with multicopy plasmids encoding Sup35N2p or Sup35NMp with a lysate of 5V-H19 [*PSI*$^+$] cells expressing wild-type Sup35p (*13*). After incubation for 2 hours, Sup35N2p (Fig. 2) and Sup35NMp (*14*) as well as full-length Sup35p encoded by the chromosomal *SUP35* gene manifested high protease resistance (*15*) and sedimented as large molecular size aggregates upon centrifugation through a layer of sucrose (*16*). As controls, lysates of corresponding [*psi*$^-$] strains were incubated for 2 or 3 hours without the addition of [*PSI*$^+$] lysates. No Sup35p aggregation or protease resistance was observed (Fig. 2). The extent of the conversion reaction depended on the incubation time: After a 20-min incubation (minimal time from mixing of lysates until the start of fractionation) much of the Sup35N2 and Sup35NM proteins was converted, whereas after 2 hours the cytosolic form of Sup35p completely disappeared. In a reverse experiment, addition of lysates con-

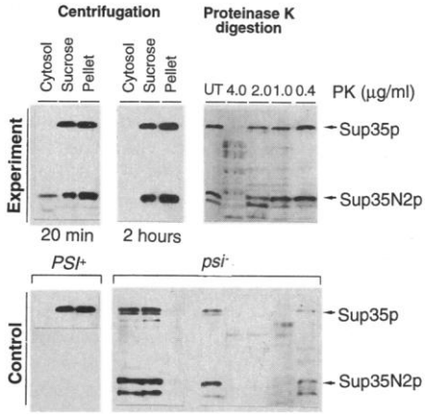

Fig. 2. Conversion of Sup35N2p^{psi-} to proteinase K (PK)–resistant and aggregated form in the presence of Sup35p^{PSI+}. Lysates of [*PSI*$^+$] and [*psi*$^-$] cells were mixed, incubated, and analyzed as described (*15*, *16*). Experiment: Lysates were mixed and incubated for 20 min or 2 hours and then treated with the indicated concentrations of PK for 30 min at 37°C (shown only for 20-min reaction). UT, untreated lysates. Control: Analysis of Sup35p in [*PSI*$^+$] and [*psi*$^-$] lysates before mixing and PK resistance of Sup35N2p in [*psi*$^-$] lysate after 2 hours of incubation.

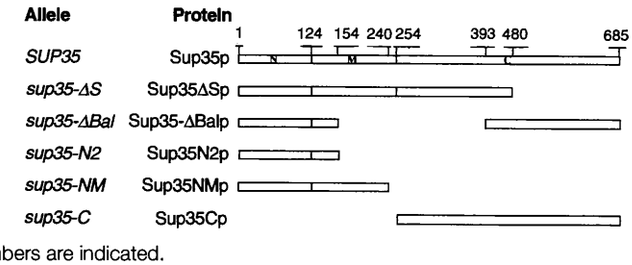

Fig. 1. Schematic representation of the Sup35 protein and its fragments. Designations of the *SUP35* deletion alleles and corresponding protein fragments are presented at left. Multicopy plasmids carrying the *SUP35* gene and its deletion alleles are described elsewhere (*10*). Amino acids numbers are indicated.

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taining Sup35N2p^{PSI+} or Sup35NMp^{PSI+} (Fig. 3) caused the conversion of Sup35p^{psi-}, although the yield was less than in the first case (~90% of Sup35p molecules were converted during a 2-hour incubation). This result shows that the NH_2-terminal region of Sup35p was sufficient to allow the in vitro conversion to occur. In these experiments a twofold excess of the Sup35 protein or its NH_2-terminal fragments from [*PSI*$^+$] lysates was added. Decrease of the ratio of Sup35p^{PSI+} to Sup35p^{psi-} to ~1:10 resulted in conversion of ~50% of Sup35p^{psi-} (Fig. 3).

To confirm the prion-like properties of the Sup35p aggregates resulting from the conversion reaction, we recovered these aggregates by centrifugation and used them to initiate new rounds of conversion. Such a cycle was repeated consecutively four times without a decrease in the efficiency of reaction. The first reaction was started by the addition of Sup35NMp^{PSI+} to the [*psi*$^-$] lysate containing full-length Sup35p; the two next cycles represented the conversion of Sup35p from the [*psi*$^-$] lysate caused by the seeds obtained in previous steps; and in the last cycle Sup35ΔSp from the [*psi*$^-$] lysate was converted (Fig. 4). In each cycle the amount of aggregated Sup35p increased four- to sixfold, and thus the overall amplification of the prion-like Sup35p aggregates was ~400-fold. The Sup35NMp^{PSI+}, which was used to start the first conversion cycle, was only barely detectable in the sedimented fraction obtained in the second reaction and could not be detected among the products of third reaction. This experiment provides functional evidence for the prion-like nature of the aggregates.

Two models have been suggested for the mechanism of PrP conversion to the prion isoform. In the refolding model, the PrP^{Sc} form is stable as a monomer, and the conversion occurs during interaction of the monomeric PrP^C and PrP^{Sc} molecules. PrP^{Sc} aggregation is considered as a secondary process (*17*). The nucleation model views the process as oligomerization of PrP: The properties of PrP^{Sc} are acquired within the framework of oligomer, and the conformational rearrangement occurs during binding of PrP^C to PrP^{Sc} oligomer (*18*, *19*). In vitro, the entity exhibiting the PrP-converting activity was many times larger than a soluble PrP monomer (*20*). This result supports the nucleation model for the PrP conversion. The in vitro system exploited here allowed us to test the applicability of the nucleation model for explaining the mechanism of a yeast

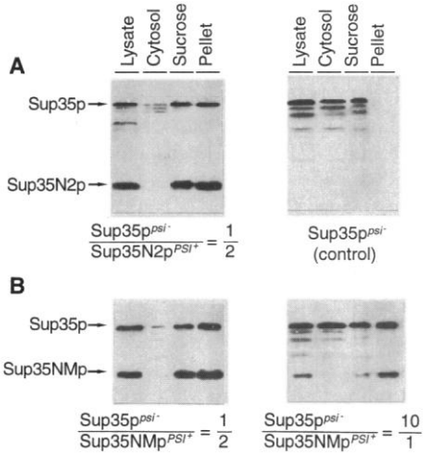

Fig. 3. Conversion of Sup35p^{psi-} to PK-resistant and aggregated form in the presence of Sup35N2p^{PSI+} and Sup35NMp^{PSI+}. The [*PSI*$^+$] lysates were obtained from the cells of 1-5V-H19 [*PSI*$^+$] strain, differing from 5V-H19 by the chromosomal 5′-deletion mutation *sup35-C* (*11*), which was transformed with multicopy plasmids carrying the *SUP35-N2* or *SUP35-NM* alleles. These [*PSI*$^+$] lysates were mixed with 5V-H19 [*psi*$^-$] lysate, incubated for 2 hours, and analyzed as described (*15*, *16*). The ratio of Sup35p^{PSI+} to Sup35p^{psi-} in lysate mixtures is indicated. (**A**) Excess (twofold) of Sup35N2p^{PSI+} over Sup35p^{psi-} in lysate mixture (left) caused prion-like conversion of Sup35p as revealed by centrifugation analysis and treatment with PK (as above). In a control experiment (right), Sup35p^{psi-} was not converted after 2 hours of incubation of the corresponding [*psi*$^-$] lysate. (**B**) Dependence of the efficiency of conversion reaction on the amount of Sup35NMp^{PSI+} in lysate mixture. The ratio of Sup35NMp^{PSI+} to Sup35p^{psi-} was 2:1 (left) or 1:10 (right).

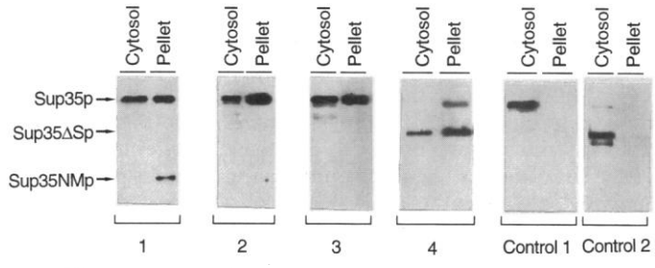

Fig. 4. Consecutive cycles of prion-like Sup35p conversion. Immunoblot analysis of Sup35p. (Panel 1) First cycle. The [*PSI*$^+$] lysate was obtained from the strain 1-5V-H19 [*PSI*$^+$] transformed with the Sup35NMp-encoding multicopy plasmid and mixed with 5V-H19 [*psi*$^-$] lysate with the ratio of 1 Sup35NMp^{PSI+} to 10 Sup35p^{psi-}. (Panels 2 and 3) In the second and third cycles the sedimented materials obtained in previous cycles were mixed with 5V-H19 [*psi*$^-$] lysate (with the ratio of 1 molecule of Sup35p from the pellet to approximately seven molecules of Sup35p^{psi-}). (Panel 4) Fourth cycle. The sedimented material obtained in the third cycle was mixed with the lysate of 5V-H19 [*psi*$^-$] transformed with Sup35ΔSp-encoding plasmid (at Sup35p ratio of 1 to 4). Sup35p^{psi-} from 5V-H19 lysate used in cycles 1 to 3 (Control 1) and Sup35ΔSp^{psi-} used in the cycle 4 (Control 2) were not aggregated after 3 hours of incubation.

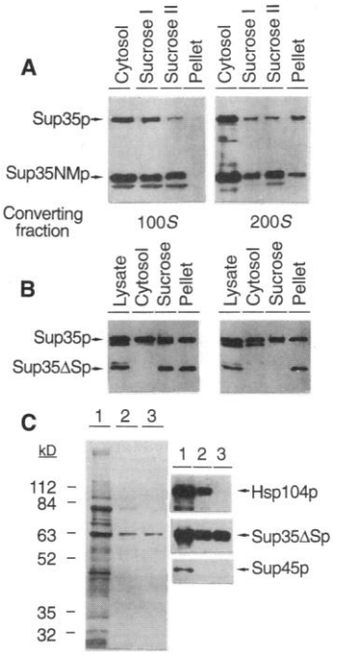

Fig. 5. Size fractionation and purification of Sup35p^{PSI+}-converting activity. (**A**) Prion-like converting activity was found only in fractions containing complexes of large molecular size. The lysate of 5V-H19 [*PSI*$^+$] was fractionated by centrifugation (*21*). The fractions obtained were normalized with respect to their Sup35p content and mixed with the lysate of 5V-H19 [*psi*$^-$] strain transformed with multicopy plasmid encoding Sup35NMp. The mixtures were incubated for 2 hours and analyzed as described (*16*). The sucrose I and sucrose II denote upper and lower halves of the sucrose fraction. The conversion extent did not exceed 20%, because the ratio of Sup35p^{PSI+} to Sup35NMp^{psi-} in these experiments was less than 1:20. (**B**) Purified Sup35ΔSp^{PSI+} manifests the prion-like converting activity. Sup35ΔSp^{PSI+} obtained after treatment with 2.5 M GuHCl (*22*) or lysate of 1-5V-H19 [*PSI*$^+$] transformed with Sup35ΔSp-encoding plasmid (control) was mixed with 5V-H19 [*psi*$^-$] lysate (right and left, respectively), incubated for 3 hours at 4°C, and analyzed as described (*16*). The ratio of Sup35ΔSp^{PSI+} to Sup35p^{psi-} was ~1:8 in both cases. Sup35p from 5V-H19 [*psi*$^-$] lysate did not aggregate after 3 hours of incubation (as in Fig. 3). (**C**) Purification of Sup35ΔSp^{PSI+} (*22*). Sup35ΔSp^{PSI+} preparation was analyzed by SDS–polyacrylamide gel electrophoresis with subsequent Coomassie blue staining (left) and protein immunoblotting with antibodies to Hsp104p (Bioquote, York, United Kingdom), Sup35p, and Sup45p (right). Lanes 1, 2, and 3 represent sedimented fraction before GuHCl treatment, and after treatment with 1.25 M and 2.5 M GuHCl, respectively. No contaminants were detected after 2.5 M GuHCl treatment, but small amounts of contaminating proteins including Hsp104p were present after treatment with 1.25 M GuHCl. Positions of molecular size markers (Sigma) are indicated on the left.

prion propagation. The lysate of 5V-H19 [*PSI*$^+$] strain was fractionated by centrifugation through a sucrose density gradient (*21*), and Sup35p from various fractions was tested for conversion activity by addition to [*psi*$^-$] lysate containing Sup35NMp and Sup35p. The cytosolic and 100*S* fractions did not cause aggregation of Sup35NMp, but all fractions of greater density—that is, 200*S*, 270*S*, and pellet—contained the conversion activity (Fig. 5). Thus, the converting agent is not the Sup35p^{PSI+} monomer; rather, it appears to be Sup35p^{PSI+} aggregates. These results are compatible with the nucleation model for the Sup35p prion-like conversion. The absence of converting activity in the soluble fraction also suggests that the converting agent is not a cytosolic enzyme that modifies Sup35p. Purification of Sup35Sp^{PSI+} to apparent homogeneity (*22*) showed that the converting activity copurified with Sup35p aggregates (Fig. 5). This confirms the basic assumption of the prion model for [*PSI*$^+$], that the converting agent is an altered form of Sup35p.

The above data allow us to explain the results of in vitro studies of the [*PSI*$^+$] phenomenon. Lysates of [*PSI*$^+$] strains produce nonsense codon readthrough in a cell-free translational reaction, whereas lysates of [*psi*$^-$] strains do not. Mixing of these lysates shows that the [*psi*$^-$] lysate is dominant, and prevents readthrough (*23*). This result contrasts with the dominance of [*PSI*$^+$] over [*psi*$^-$] in vivo. However, our results suggest that the soluble fraction of [*PSI*$^+$] lysates obtained by centrifugation at 100,000*g* that was used for cell-free translation reactions does not have the converting activity.

Our results demonstrate the general similarity of yeast and mammalian prions and provide a support for the "protein only" hypothesis for the inheritance of yeast [*PSI*$^+$] phenotype. However, conversion of yeast Sup35p proceeds much more efficiently than that of mammalian PrP. The in vitro conversion of PrP^C to PrP^{Sc} requires more than 50-fold excess of PrP^{Sc} over PrP^C and incubation of mixed PrP^C and PrP^{Sc} for at least 2 days (*3*). These differences may be quantitative rather than qualitative and may be related to the in vivo properties of these prions. Yeast are rapidly dividing unicellular organisms, and the stable inheritance of [*PSI*$^+$] requires that the time of its replication be less than that of a cell generation, that is, less than 2 hours during an exponential phase of growth. There is no such restriction for nondividing cells of brain tissues, in which the PrP^{Sc} accumulation requires months (*1*).

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13. For preparation of cell lysates, yeast cultures were grown in liquid complete medium (YPD) or in medium selective for plasmid markers in the case of transformants to an absorbance at 600 nm of 1.5, collected, washed in water, and lysed by mixing with glass beads in buffer A [25 mM tris-HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol] containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture as described [*Short Protocols in Molecular Biology*, F. M. Ausubel *et al.*, Eds. (Wiley, New York, 1992)]. Cell debris was removed by centrifugation at 15,000*g* for 20 min.
14. S. V. Paushkin, unpublished data .
15. Lysates were prepared as described (*13*), but without addition of protease inhibitors. Each reaction contained 150 μg of total protein and proteinase K (0.4 to 4.0 μg/ml) (Boehringer Mannheim) in a volume of 50 μl. After a 30-min incubation at 37°C, portions (4 μl) were removed and analyzed by protein immunoblotting with antibody to Sup35p as described (*6*).
16. The lysates were mixed with the indicated proportion of Sup35p^{PSI+} and Sup35p^{psi-} and after incubation with slow rotation at 4°C, placed on a layer of sucrose (1 ml, 30%) made in buffer A and centrifuged at 200,000*g* for 30 min at 4°C. Resulting fractions were electrophoretically separated, and Sup35p was analyzed by protein immunoblotting as described (*6*). Ribosomes were found mostly in the intermediate sucrose fraction and to a lesser extent in the sedimented material. Sup35p of sucrose fraction of lysate mixes could represent small Sup35p^{PSI+} aggregates or ribosome-bound Sup35p^{psi-} (*6*) or both. Therefore, the extent of conversion reaction was estimated as the Sup35p ratio between cytosolic fraction and sedimented material.
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21. The lysate (0.3 ml) of 5V-H19 [*PSI*$^+$] strain was loaded onto a 15 to 40% linear sucrose gradient made in buffer A and centrifuged at 180,000*g* for 3 hours at 4°C. The gradient was fractionated into 0.4-ml portions. The sedimented material was dissolved in a loading volume of buffer A.
22. The lysate of the transformant of 1-5V-H19 [*PSI*$^+$] with Sup35ΔSp-encoding multicopy plasmid (*13*) was treated with ribonuclease A (500 g/ml) for 15 min at 20°C, a high salt concentration (1 M LiCl), and nonionic detergent (1% Triton X-100) for 20 min at 4°C and sedimented by centrifugation through a sucrose layer as described (*16*). The sedimented material was treated again as above, resuspended in buffer A, made either 1.25 M or 2.5 M with GuHCl, incubated for 30 min at 4°C, precipitated by centrifugation through a sucrose layer, and resuspended in buffer A for use in the conversion reaction.
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Mating Type Switching in Yeast Controlled by Asymmetric Localization of *ASH1* mRNA

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Cell divisions that produce progeny differing in their patterns of gene expression are key to the development of multicellular organisms. In the budding yeast *Saccharomyces cerevisiae*, mother cells but not daughter cells can switch mating type because they selectively express the *HO* endonuclease gene. This asymmetry is due to the preferential accumulation of an unstable transcriptional repressor protein, Ash1p, in daughter cell nuclei. Here it is shown that *ASH1* messenger RNA (mRNA) preferentially accumulates in daughter cells by a process that is dependent on actin and myosin. A cis-acting element in the 3′-untranslated region of *ASH1* mRNA is sufficient to localize a chimeric RNA to daughter cells. These results suggest that localization of mRNA may have been an early property of the eukaryotic lineage.

During early development, cellular diversity is achieved by differences between cells in their patterns of gene expression. A good example of differential gene expression in lower eukaryotes occurs during the diploidization of homothallic strains of the budding yeast *Saccharomyces cerevisiae* (*1*). Upon germination, haploid spores grow to a critical size and then produce buds. Anaphase takes place at the bud neck, and a complete set of chromosomes is delivered to both the mother cell and the daughter cell (bud). The mother cell can switch its mating type, but the daughter cell cannot. This difference is due to mother cell–specific transcription of the *HO* gene, which encodes an endonuclease that initiates gene conversion at the mating type locus (*2*).

Transcription of *HO* in mother cells is due to the unequal accumulation within