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Propagation of the yeast prion-like [*psi*$^+$] determinant is mediated by oligomerization of the *SUP35*-encoded polypeptide chain release factor

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The Sup35p protein of yeast *Saccharomyces cerevisiae* is a homologue of the polypeptide chain release factor 3 (eRF3) of higher eukaryotes. It has been suggested that this protein may adopt a specific self-propagating conformation, similar to mammalian prions, giving rise to the [*psi*$^+$] nonsense suppressor determinant, inherited in a non-Mendelian fashion. Here we present data confirming the prion-like nature of [*psi*$^+$]. We show that Sup35p molecules interact with each other through their N-terminal domains in [*psi*$^+$], but not [*psi*$^-$] cells. This interaction is critical for [*psi*$^+$] propagation, since its disruption leads to a loss of [*psi*$^+$]. Similarly to mammalian prions, in [*psi*$^+$] cells Sup35p forms high molecular weight aggregates, accumulating most of this protein. The aggregation inhibits Sup35p activity leading to a [*psi*$^+$] nonsense-suppressor phenotype. N-terminally altered Sup35p molecules are unable to interact with the [*psi*$^+$] Sup35p isoform, remain soluble and improve the translation termination in [*psi*$^+$] strains, thus causing an antisuppressor phenotype. The overexpression of Hsp104p chaperone protein partially solubilizes Sup35p aggregates in the [*psi*$^+$] strain, also causing an antisuppressor phenotype. We propose that Hsp104p plays a role in establishing stable [*psi*$^+$] inheritance by splitting up Sup35p aggregates and thus ensuring equidistribution of the prion-like Sup35p isoform to daughter cells at cell divisions.

Keywords: non-Mendelian inheritance/prion/ *Saccharomyces cerevisiae/SUP35/*translation termination

Introduction

Prions are infectious agents causing transmissible spongiform encephalopathies, such as human kuru, Creutzfeld–Jacob disease and sheep scrapie. These diseases can be familial or sporadic in origin (for review, see Prusiner 1991, 1994). All attempts to detect a nucleic acid genome associated with prion infectivity were unsuccesful (Prusiner, 1982). The only recognized component of the infectious agent is a protein referred to as PrP^{Sc}, which is a specific isoform of host-encoded protein PrP^C and differs from it physically by poor solubility in detergents, high resistance to proteolysis and a marked propensity for aggregation (Prusiner *et al.*, 1983; Oesch *et al.*, 1985; Meyer *et al.*, 1986). Extensive studies have failed to reveal any covalent modifications to account for the different properties of PrP^C and PrP^{Sc} isoforms (Stahl *et al.*, 1993). Instead, the conformation of PrP^{Sc} was found to be altered dramatically, being primarily β-sheet, compared with that of PrP^C which is largely α-helical (Pan *et al.*, 1993). The current hypothesis explaining the mechanism of propagation of prions suggests that the PrP^{Sc} isoform is able to self-propagate its abnormal conformational state. Pursuing this concept, once a prion molecule has been introduced into the cell or has arisen in the cell spontaneously, it will convert all cellular PrP^C molecules into the PrP^{Sc} state. Two models have been suggested for the conversion process. In accordance with the refolding model, PrP^{Sc} is an inherent, stable property of PrP monomers, and the conversion reaction occurs between monomeric PrP^C and PrP^{Sc} molecules (Cohen *et al.*, 1994), while the PrP^{Sc} aggregation is a secondary process. The nucleation model views the process as PrP polymerization: the properties of PrP^{Sc} are acquired within the framework of the polymer and the conformational rearrangement occurs during binding of PrP^C to PrP^{Sc} polymer (Brown *et al.*, 1991; Jarrett and Lansbury, 1993).

The prion phenomenon is probably not restricted to mammalian PrPs. Recently, two examples of extrachromosomally inherited determinants in the yeast *Saccharomyces cerevisiae*, [*URE3*] and [*psi*$^+$], have been ascribed to an underlying prion-like mode of inheritance (Wickner, 1994). [*URE3*] is phenotypically expressed by derepression of nitrogen catabolite enzymes that normally would be repressed by a good nitrogen source (Aigle and Lacroute, 1975; Magasanik, 1992). [*psi*$^+$] increases the efficiency of certain nonsense suppressor tRNAs (Cox *et al.*, 1988) and may itself also cause weak nonsense suppression (Liebman and Sherman, 1979). Both determinants demonstrate a cytoplasmic mode of inheritance, being transmissible to all the haploid products of meiosis in a sexual cycle, or to haploid mitotic products of 'cytoduction' which is a form of mating without fusion of parental nuclei. No extrachromosomal DNA or RNA have been found to be associated with [*URE3*] or [*psi*$^+$] phenotypes (Tuite *et al.*, 1982; Cox *et al.*, 1988; Wickner, 1994). Moreover, conventional nucleic acid-damaging agents eliminate these determinants much less efficiently than some non-mutagenic compounds such as the protein-denaturing agent guanidine hydrochloride (GuHCl) (Tuite *et al.*, 1981; Cox *et al.*, 1988, Wickner, 1994). [*psi*$^+$] may also be eliminated by exposure to stress-inducing agents (Singh *et al.*, 1979; Cox *et al.*, 1988). The latter property of these determinants correlates well with the demonstration of the critical role of the chaperone protein Hsp104p in propagation of [*psi*$^+$] (Chernoff *et al.*, 1995). The curing of both determinants is reversible since they can be obtained easily *de novo*, a trait which is entirely compatible with the prion hypothesis (Wickner, 1994; Chernoff *et al.*, 1995).

Several observations indicate that [*URE3*] and [*psi*$^+$] are the prion-like isoforms of *URE2*- and *SUP35*-encoded proteins. The propagation of [*URE3*] and [*psi*$^+$] depends on the chromosomal *URE2* and *SUP35* genes, respectively (Ter-Avanesyan *et al.*, 1993a, 1994; Doel *et al.*, 1994; Wickner, 1994), while the phenotypes of these determinants are equivalent to repression or recessive mutations of the corresponding genes. Similarly, the *PrP* gene is required for the maintenance of the mouse prion (Bueler *et al.*, 1993; Prusiner *et al.*, 1993). Overexpression of the *URE2* and *SUP35* genes greatly increases the frequency of generation of corresponding determinants (Chernoff *et al.*, 1993; Wickner, 1994), which may be expected if their protein products are involved in prion-like mechanisms. Moreover, it was shown recently that, similarly to mammalian PrP^{Sc}, the prion-like form of yeast Ure2p demonstrates increased protease resistance over its wild-type form (Masison and Wickner, 1995).

The Sup35p protein belongs to the structural family, designated recently as an eukaryotic polypeptide chain release factor eRF3 (Zhouravleva *et al.*, 1995). Although its specific release factor activity has not been demonstrated biochemically, genetic studies strongly support its role in translation termination in yeast (Stansfield *et al.*, 1995). Sup35p interacts with the Sup45p protein, which is homologous to the vertebrate polypeptide chain release factor eRF1 (Frolova *et al.*, 1994), to form a functional termination complex *in vivo* (Stansfield *et al.*, 1995). Sup35p is composed of two parts: the amino-terminal region and carboxy-terminal domain of 253 and 432 amino acids, respectively (Kushnirov *et al.*, 1988; Ter-Avanesyan *et al.*, 1993b). The evolutionarily conserved C-terminal domain of Sup35p is structurally similar to translation elongation factor EF-1α and essential for cell viability, while its N-terminal region is not conserved and is not essential for viability. This region may be subdivided further into the N-terminal domain of 123 amino acids, required for [*psi*$^+$] maintenance, and the middle region, to which no function has been ascribed. The N-terminal domain represents a self-contained functional unit, able to act separately from the rest of Sup35p to support [*psi*$^+$] maintenance. The expression of N-terminally deleted Sup35p causes an antisuppressor phenotype (Kushnirov *et al.*, 1988; Ter-Avanesyan *et al.*, 1993a, 1994; Doel *et al.*, 1994).

Here we present results demonstrating the ability of Sup35p molecules to interact with each other and the role of such interaction in [*psi*$^+$] propagation and establishment of the [*psi*$^+$] phenotype.

Results

Sup35p molecules interact in vitro with each other in a [psi]-dependent manner

It was shown earlier that the N-terminal domain of the Sup35p protein is critical for the maintenance of [*psi*$^+$] (Ter-Avanesyan *et al.*, 1994). This suggests that the prion features of Sup35p may be dependent on the state of its N-terminal domain. Since the prion concept presumes that conversion of wild-type protein into prion form takes place during their interaction, we tested the ability of Sup35p molecules to interact with each other and the role of N-terminal part of Sup35p in this process.

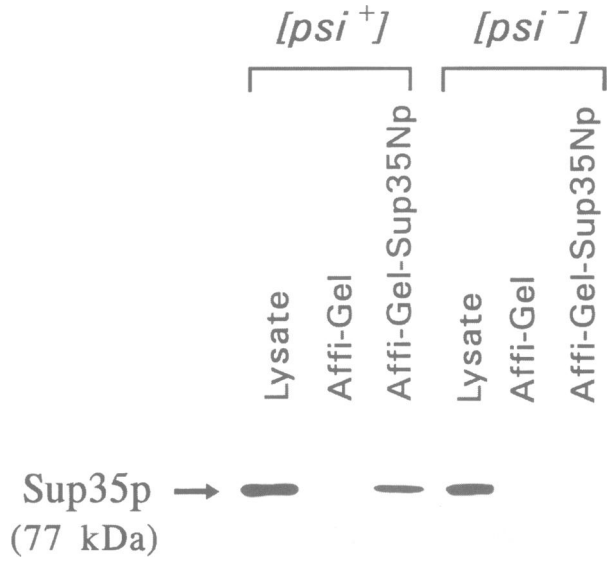

Fig. 1. Interaction of yeast Sup35p with bacterially expressed Sup35p N-terminal domain. The lysates of [*psi*$^+$] and [*psi*$^-$] variants of strain 5V-H19 were incubated with Affi-Gel 10 resin either loaded with Sup35Np or non-loaded control. Following washing, bound proteins were eluted and assayed for Sup35p by immunoblotting (Materials and methods). Lanes: Lysate, total lysates of [*psi*$^+$] or [*psi*$^-$] cells; Affi-Gel, Affi-Gel 10 after incubation with [*psi*$^+$] or [*psi*$^-$] lysates; Affi-Gel-Sup35Np, Affi-Gel 10 with immobilized Sup35Np after incubation with [*psi*$^+$] or [*psi*$^-$] lysates. The binding of Sup35p is observed in the case of [*psi*$^+$] but not for [*psi*$^-$], as is evident from the Affi-Gel-Sup35Np lanes.

A fusion of glutathione-*S*-transferase (GST) with the N-terminal region (amino acids 1–251) of Sup35p (GST–Sup35Np) was expressed in *Escherichia coli* and purified by binding to glutathione–agarose. The resin with immobilized fusion protein was used to precipitate Sup35p from lysates of [*psi*$^+$] and [*psi*$^-$] cells. Although no precipitation was observed (data not shown), we suggested that the N-terminally located GST sequence inhibits binding. When the GST extension was cleaved off by thrombin, the resultant Sup35Np fragment bound Sup35p from [*psi*$^+$] but not from [*psi*$^-$] lysates (Figure 1). This indicates that: (i) Sup35p may exist in two different forms, corresponding to a [*psi*$^+$] (Sup35p^{psi+}) and [*psi*$^-$] (Sup35p^{psi-}) state, differing in their ability to form oligomers; (ii) the interaction between Sup35p molecules proceeds via their N-terminal parts; and (iii) the N-terminal GST extension blocks this interaction. In the above experiment, the [*psi*] status of the bacterially expressed Sup35p could not be assigned correctly and this protein was only used to reveal the differential properties of Sup35p from lysates of [*psi*$^+$] and [*psi*$^-$] cells.

GST–SUP35 hybrid allele does not support [psi$^+$] propagation

The ability of Sup35p molecules to interact with each other is not just a distinctive feature of the Sup35p^{psi+} form, but it is critical for the maintenance of the [*psi*$^+$] determinant. Since this interaction depends on the N-terminal domain, it may be eliminated by either deleting the N-terminal region from complete Sup35p or, as shown above, by fusing a GST sequence to its N-terminus. It was demonstrated earlier that the *SUP35* deletion alleles, coding for proteins lacking amino acids 1–253, 1–123 or

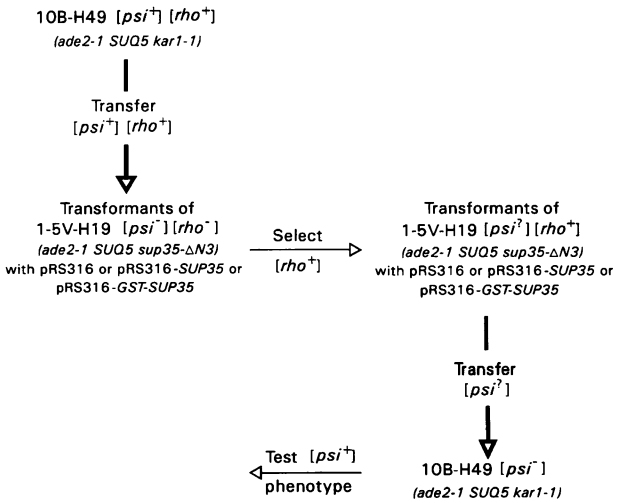

Fig. 2. Scheme of 'cytoduction' experiments demonstrating the inability of the *GST–SUP35* hybrid construct to support propagation of [*psi*$^+$]. Thick vertical arrows denote transfer of cytoplasmic factors by cytoduction, thin horizontal arrows represent selection procedures. [*psi*$^?$], the [*psi*] status is to be determined. The details of cytoduction procedures are given in Materials and methods and in the text.

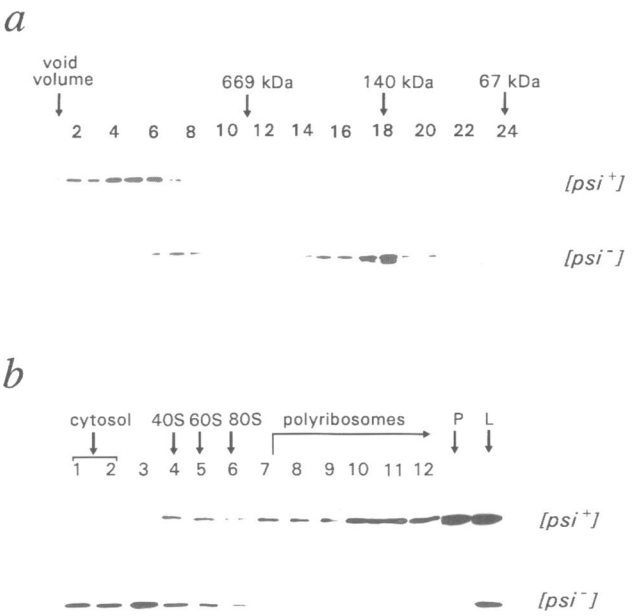

Fig. 3. Size fractionation of [*psi*$^+$] and [*psi*$^-$] lysates. Immunoblot analysis of Sup35p protein. (**a**) Gel filtration on Sepacryl S-300. Fraction numbers and the eluted positions of some protein standards are shown above the panel. (**b**) Sucrose density gradient centrifugation. Fractions are described above the panel. P, pellets obtained after centrifugation; L, original lysates.

21–68 were unable to propagate [*psi*$^+$] (Ter-Avanesyan *et al.*, 1994). Using a similar strategy, we demonstrated that the *GST–SUP35* fusion allele does not support the [*psi*$^+$] propagation (Figure 2). These experiments were based on the ability of the *kar1* mutation to block karyogamy, thus preventing mixing of nuclear, but not cytoplasmic, genetic material of mating cells (cytoduction). This allows one to transfer between cells only cytoplasmically located determinants (Conde and Fink, 1976). The strain 1-5V-H19 (*sup35-ΔN3*) was transformed with pRS316-*GST–SUP35* or with pRS316-*SUP35* (positive control) or the original pRS316 (negative control) centromeric plasmids and then the [*psi*$^+$] was transferred to these transformants by cytoduction from the 10B-H49 (*kar1*) [*psi*$^+$] donor strain. The *sup35-ΔN3* allele of the 1-5V-H19 strain does not support [*psi*$^+$] (Ter-Avanesyan *et al.*, 1994) and the maintenance of [*psi*$^+$] in this strain must depend on the plasmid *SUP35* constructs. However, the determination of [*psi*$^+$] status is not possible in this strain because the *sup35-ΔN3* allele causes a dominant antisuppressor effect (Ter-Avanesyan *et al.*, 1993b). Therefore, a second round of cytoduction was performed to transfer the determinants from the 1-5V-H19 transformants to a tester strain 10B-H19 (*kar1*) [*psi*$^-$]. The transformants of 1-5V-H19 with the pRS316-*GST–SUP35* and pRS316 plasmids did not transfer [*psi*$^+$] to the tester strain in contrast to transformants with the control pRS316-*SUP35* plasmid. Therefore, it was concluded that the *GST–SUP35* hybrid allele is unable to support [*psi*$^+$] propagation. This result was confirmed by an alternative strategy. It was demonstrated that the replacement of the chromosomal *SUP35* gene with the *GST–SUP35* hybrid allele causes the elimination of the [*psi*$^+$] determinant (data not shown).

Sup35p forms aggregates in [psi$^+$] cells

The observed ability of Sup35p to form complexes suggests that Sup35p in [*psi*$^+$] cells may aggregate similarly to mammalian PrP^{Sc} protein (Prusiner *et al.*, 1982, 1983). The analysis of lysates by Sephacryl S-300 chromatography (Figure 3A) showed that in a [*psi*$^+$] lysate Sup35p was associated with a high molecular weight fraction and was almost not detectable in fractions below 1000 kDa. In contrast, in a [*psi*$^-$] lysate, ~70% of the Sup35p was found in the 140 kDa fraction which probably contains a complex of Sup35p with Sup45p which is functional in translation termination (Stansfield *et al.*, 1995). The centrifugation of the [*psi*$^+$] lysate in a sucrose density gradient showed that Sup35p co-sedimented with polyribosomes, with more than half being found in the pellet, i.e. sedimenting faster than 8–10 ribosomes (Figure 3B). The polyribosome levels may be increased by cycloheximide addition, preventing ribosome run-off (Stanners, 1966) or reduced by either RNase A treatment of cell lysates or by blocking translation initiation with NaN_3 (Van der Zeist *et al.*, 1972). Neither treatment altered the Sup35p distribution, indicating that polyribosomes were not an essential component of these complexes. The treatment of the complexes with 1 M KCl, 1 M LiCl or 1% Triton X-100 also failed to release Sup35p, although some other proteins were solubilized (data not shown).

Antisuppression is caused by the soluble form of Sup35p

Since the N-terminal Sup35p domain is critical for the [*psi*$^+$]-dependent formation of Sup35p oligomers, one can suggest that the N-terminally altered Sup35p molecules do not have a propensity to become incorporated into Sup35p^{psi+} aggregates. To check this, the 5V-H19 [*psi*$^+$] strain was transformed with a multicopy plasmid carrying the *sup35-ΔN2* allele that encodes a Sup35p protein lacking amino acids 1–123, and the cell extracts fractionated by centrifugation through a sucrose cushion. Most of truncated Sup35p was found in the soluble fraction, whereas the

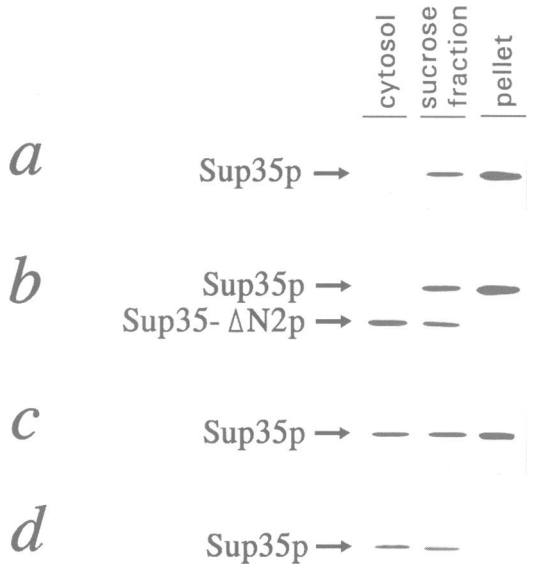

Fig. 4. Distribution of Sup35p between the soluble and aggregated forms in 5V-H19 strains. Immunoblot analysis of Sup35p protein. The lysates of the following strains were fractionated by centrifugation through a sucrose cushion: (**a**) 5V-H19 [*psi*$^+$]; (**b**) 5V-H19 [*psi*$^+$] transformed with pEMBL-*sup35-ΔN2* plasmid; (**c**) 5V-H19 [*psi*$^+$] transformed with pFL44L-*HSP104* plasmid; (**d**) 5V-H19 [*psi*$^-$].

full-length Sup35p was in the precipitate (Figure 4). In contrast to the original [*psi*$^+$] strain, the transformants obtained showed an antisuppressor phenotype (Ter-Avanesyan *et al.*, 1993b). We suggest that the aggregation of Sup35p in [*psi*$^+$] cells inhibits its function in translation, increasing the rate of nonsense codon readthrough and giving rise to the [*psi*$^+$] phenotype. However, when Sup35p with an altered N-terminal region is expressed in such cells, it remains soluble, performing the Sup35p function and preventing the expression of the [*psi*$^+$] phenotype. Analogous properties were demonstrated by two other Sup35p variants. Sup35-ΔNBp, lacking amino acids 21–68, and heterologous Sup35Pp from yeast *Pichia pinus*, which shows significant divergence in its N-terminal domain, remained soluble in [*psi*$^+$] cells and their expression there caused an antisuppressor phenotype (data not shown).

Another case in which [*psi*$^+$] cells show an antisuppressor phenotype was described by Chernoff *et al.* (1995), who demonstrated that the 2- to 4-fold overexpression of Hsp104p protein in a [*psi*$^+$] strain gave an antisuppressor effect, but did not eliminate the [*psi*$^+$] determinant. Higher overexpression of Hsp104p caused [*psi*$^+$] loss. Since Hsp104p acts to dissolve protein aggregates (Parsell *et al.*, 1994), we have suggested that moderately increased levels of Hsp104p may partially dissolve the Sup35p^{psi+} aggregates and cause the appearance of Sup35p in the soluble fraction, giving rise to an antisuppressor effect. To check this suggestion, the strain 5V-H19 [*psi*$^+$] was transformed with a multicopy plasmid carrying the *HSP104* gene. Northern blot analysis showed that the *HSP104* mRNA was 20-fold overexpressed compared with the parental strain (data not shown). The transformants manifested an antisuppressor phenotype and gradual accumulation of [*psi*$^-$] cells. The cells of one transformant were collected on the 5th day after the transformation and lysed. Fractionation of the lysate revealed significant levels of soluble Sup35p (Figure 4). The appearance of Sup35p in the soluble fraction cannot be explained by the accumulation of [*psi*$^-$] cells, since the latter constituted only 2% of the analysed yeast culture. This observation indicates that Hsp104p acts to dissolve Sup35p aggregates. Initially this results in the appearance of Sup35p in the soluble fraction and antisuppression and later leads to the loss of [*psi*$^+$], probably due to complete dissolution of the aggregates.

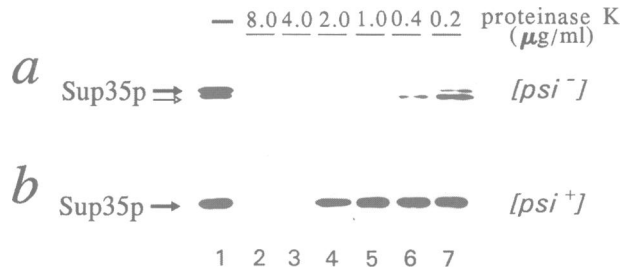

Fig. 5. Comparison of Sup35p degradation in [*psi*$^-$] (**a**) and [*psi*$^+$] (**b**) lysates. Immunoblot analysis of Sup35p protein. Lane 1, untreated lysates; lanes 2–7, lysates treated with 8.0, 4.0, 2.0, 1.0, 0.4 and 0.2 μg/ml of proteinase K, respectively. The thin arrow indicates the most characteristic of Sup35p^{psi-} degradation products, which is not observed in the case of [*psi*$^+$]. Lane 1 demonstrates higher Sup35p levels in the [*psi*$^+$] lysate, since the Sup35p content was equalized by loading 8-fold less [*psi*$^+$] lysate on the gel. The treated [*psi*$^+$] and [*psi*$^-$] lysates were loaded on the gel in the same proportion.

Sup35p^{psi+} shows increased resistance to proteases

Comparison of [*psi*$^+$] and [*psi*$^-$] cell lysates by Western blotting revealed that the Sup35p content was up to 10-fold higher in [*psi*$^+$] cells. In addition, Sup35p was reproducibly more degraded in [*psi*$^-$] lysates than in [*psi*$^+$] (Figure 5). These differences were less evident at early exponential phase, but more pronounced at stationary phase of growth. Since the *SUP35* mRNA levels did not differ in [*psi*$^+$] and [*psi*$^-$] cells (Ter-Avanesyan *et al.*, 1994), greater protein stability is the most probable reason for the accumulation of Sup35p in [*psi*$^+$] cells. Proteinase K digestion experiments also revealed the increased resistance of Sup35p^{psi+} (Figure 5). However, the [*psi*]-dependent difference in resistance was not so dramatic as between PrP^{Sc} and PrP^{C} proteins (Meyer *et al.*, 1986). Moreover, in contrast to mammalian PrP^{Sc} protein, no submolecular protease-resistant core was observed for Sup35p^{psi+}. The difference in the resistance of Sup35p between [*psi*$^+$] and [*psi*$^-$] lysates could be explained by poor accessibility of Sup35p^{psi+} due to its aggregation.

Discussion

The infectious form of the prion protein, PrP^{Sc}, differs from its wild-type isoform, PrP^{C}, by an increased tendency to aggregate, high protease resistance and accumulation at higher levels (Prusiner, 1991, 1994). Here we observed that Sup35p^{psi+} shows similar differences to Sup35p^{psi-}: marked propensity for aggregation, increased resistance to cellular proteases and proteinase K and accumulation in cells. Thus, the basic properties of prions are reproduced by Sup35p^{psi+}. These data support the prion model for Sup35p and confirm the applicability of the [*psi*$^+$] phenomenon as a model for prion studies.

The prion propagation model is based on the presumption that direct protein–protein interaction, and possibly complex formation between PrP^C and PrP^{Sc}, is required for the prion conversion reaction to occur. The [*psi*$^+$] system gives us the possibility to test the significance of Sup35p complex formation for the process of yeast prion propagation. Since previous studies have shown that the only region of the Sup35p molecule responsible for the [*psi*$^+$] maintenance is its N-terminal domain (Ter-Avanesyan *et al.*, 1993a, 1994; Doel *et al.*, 1994), this suggests that the N-terminal region is critically involved in the complex formation. Here we show that Sup35p can interact with a bacterially expressed Sup35p N-terminal domain. Importantly, such complex formation is a [*psi*]-dependent process, since the N-terminal Sup35p fragment interacted with Sup35p in [*psi*$^+$] lysates but not [*psi*$^-$] lysates. The ability of the Sup35p molecules to interact with each other is not just a characteristic feature of the [*psi*$^+$] state, but it is also critical for the propagation of the [*psi*$^+$] determinant. The fusion of GST to the amino-terminus of Sup35p blocked its interaction with $Sup35p^{psi+}$. At the same time, the *GST–SUP35* hybrid allele was not able to support [*psi*$^+$] maintenance. This demonstrates the importance of Sup35p–Sup35p interaction in the propagation of the [*psi*$^+$] state.

The ability of $Sup35p^{psi+}$ molecules to interact with each other is a possible reason for the formation of Sup35p aggregates, observed in [*psi*$^+$] cells. These aggregates accumulate most of the cellular Sup35p protein. This allows us to suggest a simple model, explaining the phenotypic expression of the [*psi*$^+$] determinant by inhibition of the polypeptide chain release activity of the Sup35p protein. It has been reported that there is a >20-fold molar excess of ribosomes over the Sup35p molecules (Didichenko *et al.*, 1991) and therefore the latter should circulate between the terminating ribosomes to perform the release factor function. The aggregation of $Sup35p^{psi+}$ should decrease the amount of circulating Sup35p, thus inhibiting the translation termination reaction, increasing the level of nonsense readthrough and giving rise to a [*psi*$^+$] nonsense-suppressor phenotype. The N-terminal domain of Sup35p, responsible for the aggregation, may thus be regarded as a *cis*-acting repressor of the polypeptide chain release function of the C-terminal domain. Such repression does not occur with the Sup35p variants having a deleted or altered N-terminal domain and unable to undergo complex formation with $Sup35p^{psi+}$. The introduction of corresponding alleles into [*psi*$^+$] cells, possessing wild-type Sup35p, causes a dominant antisuppressor phenotype, although cells retain [*psi*$^+$] (Ter-Avanesyan *et al.*, 1993b). Such cells contain aggregated Sup35p in the [*psi*$^+$] form and the N-terminally altered Sup35p in a soluble state. The latter should perform the release function of Sup35p efficiently, which in turn should decrease the nonsense readthrough thus causing an antisuppressor phenotype.

Another candidate for the yeast prion-like protein is Ure2p, a regulator of nitrogen catabolism. Its prion state produces the [*URE3*] phenotype, equivalent to the lack of Ure2p function. Similarly to Sup35p, Ure2p is composed of two domains: the N-terminal region is not essential for Ure2p function, but its covalent attachment to the C-terminal domain is required for inactivation of the latter by the [*URE3*] prion state (Masison and Wickner, 1995). Thus, in their prion state, the N-terminal domains of both Ure2p and Sup35p act as *cis* repressors of the adjoining functional domains. Such repression is mediated by aggregation in the case of Sup35p protein, but this has yet to be demonstrated for Ure2p. Aggregation is not the only possible mechanism of such repression, but it seems to us to be the most probable in the case of Ure2p. Similarly to $Sup35p^{psi+}$ and PrP^{Sc}, the prion form of Ure2p shows increased resistance to proteinase K (Masison and Wickner, 1995), an indication in favour of an aggregated state for Ure2p in [*URE3*] strains.

It was noted that the mammalian PrP protein may also show the two-domain organization of Sup35p, since the N-terminal regions of both proteins possess tandem amino acid repeats of rather similar sequence and length (Cox, 1994; Tuite, 1994). However, unlike for Sup35p, the C-terminal part of PrP plays an important role in prion conversion and undergoes a conformational alteration, since it forms a protease-resistant core, known as PrP^{27-30} (McKinley *et al.*, 1983). Thus, the functional relationship between the PrP domains is not consistent with the above scheme of one domain being a repressor of the other, functional domain.

An important finding, related to the prion-like phenomena in yeast, is the dependence of [*psi*$^+$] maintenance on the expression of the chaperone Hsp104p. A 2- to 4-fold excess of Hsp104p causes an antisuppressor effect but does not cure [*psi*$^+$]. High levels of overexpression of Hsp104p eliminate this determinant and, surprisingly, the disruption of the *HSP104* gene causes the same effect (Chernoff *et al.*, 1995). These data were considered to imply a direct role for Hsp104p in the process of conformational conversion of the $Sup35p^{psi-}$ protein into the $Sup35p^{psi+}$ isoform. However, these results may have a simpler explanation in view of the observation of Sup35p aggregation in [*psi*$^+$] cells. It is known that Hsp104p acts to dissolve protein aggregates in yeast cells (Parsell *et al.*, 1994). In accordance with this, we have shown that Hsp104p overexpression partially dissolves $Sup35p^{psi+}$ aggregates, thus increasing the levels of soluble Sup35p and causing antisuppression in [*psi*$^+$] cells. However, as long as the $Sup35p^{psi+}$ aggregates are not dissolved completely, the cells are not cured of [*psi*$^+$]. In agreement with the nucleation model of prion replication (Brown *et al.*, 1991; Jarrett and Lansbury, 1993), we suggest that the conversion of $Sup35p^{psi-}$ into the $Sup35p^{psi+}$ isoform takes place during the formation of oligomers which represent seeds for further aggregation. The wild-type levels of Hsp104p are required to split up the $Sup35p^{psi+}$ aggregates, thus increasing the number of $Sup35p^{psi+}$ oligomeric seeds. The lack of Hsp104p would decrease strongly the number of such seeds and this, in turn, would reduce the probability of $Sup35p^{psi+}$ transmission to the daughter cells following cell divisions. This suggests that Hsp104p is required to ensure stable [*psi*$^+$] inheritance and it is not necessarily involved in the conversion of $Sup35p^{psi-}$ molecules to the $Sup35p^{psi+}$ isoform.

Materials and methods

Strains and media

The *S.cerevisiae* strains used in this study were: 5V-H19 (MATa *ade2-1 SUQ5 can1-100 leu2-3,112 ura3-52* [*psi*$^+$]); 10B-H49 (MATα *ade2-1*

SUQ5 leu2-3,112 lys1 his3-11,15 kar1-1 [*psi*$^+$]) and their [*psi*$^-$] derivatives. The strain 1-5V-H19 was obtained by replacing the wild-type *SUP35* gene of 5V-H19 with the *sup35-ΔN3* deletion allele encoding truncated Sup35p protein lacking amino acids 1–253 (Ter-Avanesyan *et al.*, 1994). This allele causes dominant antisuppression and recessive inability to propagate [*psi*$^+$] (Ter-Avanesyan *et al.*, 1993a,b, 1994). *E.coli* strain DH5α [*supE*44 Δ*lac* U169 (φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1] was used for cloning experiments and TG1 [*supE hsd*Δ5 *thi*Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15)] for the expression of GST–Sup35Np fusion protein (Sambrook *et al.*, 1989). We used standard organic (YEPD) and synthetic (SC) media for yeast (Sherman *et al.*, 1986) and LB medium for bacteria (Sambrook *et al.*, 1989). Appropriate amounts of amino acids, bases and antibiotics were added when necessary. The 5-fluoroorotic acid (5FOA) medium was prepared by the method of McCusker and Davis (1991). The final concentration of 5FOA was 400 μg/ml. Non-fermentable media contained glycerol (24 ml/l) as a sole carbon source. All solid media contained 2.5% (w/v) agar. Yeast cells were grown at 30°C and bacteria at 37°C.

Plasmids, DNA manipulation and transformation

All DNA manipulations and plasmid construction techniques were carried out by standard protocols (Sambrook *et al.*, 1989). The hybrid gene, encoding a fusion of GST with N-terminal amino acids 1–251 of Sup35p was constructed as follows. An artificial *Bam*HI site was introduced before the first ATG codon of *SUP35* to give a sequence GGA TCC CCC CTC GAT CTA GCA ACA ATG. A *SUP35* fragment from the *Bam*HI to *Hpa*I at codon 251 was inserted between *Bam*HI and *Sma*I sites of pGEX2T (Smith and Johnson, 1988). The *GST–SUP35* allele encoding a fusion of GST with the entire Sup35p was constructed as follows. The GST coding sequence was taken from plasmid pEG(KG) (Mitchell *et al.*, 1993) as the *Ecl*136II–*Sma*I fragment and inserted in the *SUP35* gene at position −10 before the first ATG in-frame with the downstream *SUP35* coding sequence. The whole *GST–SUP35* construct was excised by *Xho*I and *Xba*I and either inserted in plasmid pRS316 (Sikorski and Hieter, 1989) or used for replacement of the chromosomal *SUP35* gene. The multicopy plasmid pFL44L-*HSP104* carrying *URA3* as a selectable marker was kindly provided by M.Boguta (Warsaw, Poland). Multicopy pEMBLyex4-based plasmid (Cesareni and Murray, 1987) carries the *sup35-ΔN2* deletion allele that encodes truncated Sup35p protein lacking amino acids 1–123 (Ter-Avanesyan *et al.*, 1993a,b). DNA transformation of lithium acetate-treated yeast cells was performed as described (Gietz *et al.*, 1995). *E.coli* cells were transformed by standard method (Hanahan, 1985).

Genetic methods

Yeast strains were cured of the [*psi*$^+$] determinant by growth on YEPD medium supplemented with 3 mM GuHCl (Tuite *et al.*, 1981). The [*psi*$^-$] colonies of *ade2-1 SUQ5*-carrying strains were chosen by their pink colour and adenine requirement because the weak serine-inserting tRNA suppressor *SUQ5* (also called *SUP16*) cannot suppress the *ade2-1* ochre mutation in the absence of the [*psi*$^+$] determinant (Cox, 1965; Liebman *et al.*, 1975; Ono *et al.*, 1979).

Non-suppressive petites ([*rho*$^-$]) in transformants of strain 1-5V-H19 were obtained by ethidium bromide treatment (Goldring *et al.*, 1970). 'Cytoduction' experiments (Figure 2) were performed as described by Ter-Avanesyan *et al.* (1994). Strains of opposite mating type, one of which carries the *kar1-1* mutation that blocks karyogamy (Conde and Fink, 1976), were mated by mixing them together on the surface of a YEPD plate and incubated for 1 day at 30°C. In the first round of cytoduction, the [*rho*$^+$] and [*psi*$^+$] determinants were transferred from the 10B-H49 [*rho*$^+$] [*psi*$^+$] *kar1-1* strain to the strain 1-5V-H19 (*sup35-*ΔN3) [*rho*$^-$] [*psi*$^-$] that had been transformed with different plasmids of interest. [*rho*$^+$] and [*psi*$^+$] show high coincidence of transfer (Cox *et al.*, 1988). Therefore, cytoductants of transformants of 1-5V-H19 were selected from the mating mixtures of cells by transfer to histidine omission medium containing glycerol as a sole carbon source. Respiratory-competent colonies phenotypically indistinguishable in other respects from the transformants of strain 1-5V-H19 were scored as cytoductants. The [*psi*] status of the cytoductants was revealed in the second round of cytoduction. Only those [*rho*$^+$] cytoductants of transformants that could transfer [*psi*$^+$] by cytoduction to the [*psi*$^-$] tester strain, 10B-H49, were considered to possess the [*psi*$^+$] determinant. The use of strain 10B-H49 allowed the selective isolation of cytoductants, since only those cells of the recipient strain 10B-H49 that received the [*psi*$^+$] determinant could grow on the adenine omission medium.

To estimate the percentage of [*psi*$^-$] cells in the culture of the 5V-H19 [*psi*$^+$] transformant with the pFL44L-*HSP104* plasmid, an aliquot of this culture was spread on a YEPD plate, colonies were grown and replica plated to a media containing 5FOA to eliminate the *URA3*-carrying pFL44L-*HSP104* plasmid. Plasmid-less colonies were then scored for the presence of [*psi*$^+$] by examining their growth on adenine omission medium.

Preparation and fractionation of yeast cell lysates

Yeast cultures were grown to an OD_{600} of 1.5, harvested, washed in water and lysed by vortexing with glass beads in buffer A [25 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT)] containing 1 mM phenylmethylsulphonyl fluoride (PMSF) to limit proteolytic degradation. Cell debris was removed by centrifugation at 15 000 *g* for 10 min.

Lysates were fractionated on a Sephacryl S-300 (Pharmacia) column (80×2 cm) equilibrated with buffer A. The fractions of 2 ml were collected, concentrated by trichloracetic acid precipitation and analysed by Western blotting. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa) and ovalbumin (43 kDa) were used as molecular mass standards for gel filtration.

For the fractionation by centrifugation, lysates were loaded on a 15–40% (mass/volume) linear sucrose gradient, made in buffer A, and centrifuged at 38 000 r.p.m., 4°C for 3 h in a Beckman SW41 rotor. The pellet was dissolved in a loading volume of buffer A. The gradient was fractionated into 0.4 ml portions which were analysed similarly to the gel filtration fractions. To confirm the location of ribosomes and their subunits, the fractions were made up to 1% with SDS, and rRNAs analysed by electrophoresis in 0.8% agarose gels. To analyse the size distribution of Sup35p by differential centrifugation, the lysates were underlaid with 1 ml of 30% sucrose pads made in buffer A and centrifuged in a Beckman SW50 rotor at 45 000 r.p.m., 4°C for 30 min. The resulting supernatants, pellets and intermediate fractions were analysed by Western blotting.

Immobilization of Sup35p N-terminal domain

The GST–Sup35Np fusion protein was expressed in *E.coli*, isolated by affinity chromatography on glutatione–agarose, cleaved from GST by thrombin as described by Smith and Johnson (1988), eluted and fixed on Affi-Gel 10 (Bio-Rad) as recommended by the manufacturer. The resin was incubated with lysates for 2 h at 4°C and then washed by a 40-fold resin volume of buffer A. Bound proteins were eluted with 2% SDS and analysed by Western blotting.

Study of the protease resistance of Sup35p

Lysates were prepared as described above, but without addition of PMSF. Each reaction contained 150 μg of total protein and 0.2–8 μg/ml of proteinase K (Boehringer Mannheim) in a volume of 50 μl. After 30 min incubation at 37°C, 4 μl aliquots were removed and analysed by Western blotting.

Protein gel electrophoresis and Western blot analysis

Protein samples were separated on a 10–15% SDS–polyacrylamide gel according to Laemmli (1970) and electrophoretically transferred to nitrocellulose sheets (Towbin *et al.*, 1979). Western blots were probed with polyclonal rabbit anti-Sup35p antibody (Didichenko *et al.*, 1991). Bound antibody was detected using the Amersham ECL system according to the manufacturer's instructions.

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