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# Interaction between Yeast Sup45p (eRF1) and Sup35p (eRF3) Polypeptide Chain Release Factors: Implications for Prion-Dependent Regulation

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**The *SUP45* and *SUP35* genes of *Saccharomyces cerevisiae* encode polypeptide chain release factors eRF1 and eRF3, respectively. It has been suggested that the Sup35 protein (Sup35p) is subject to a heritable conformational switch, similar to mammalian prions, thus giving rise to the non-Mendelian [*PSI*$^+$] nonsense suppressor determinant. In a [*PSI*$^+$] state, Sup35p forms high-molecular-weight aggregates which may inhibit Sup35p activity, leading to the [*PSI*$^+$] phenotype. Sup35p is composed of the N-terminal domain (N) required for [*PSI*$^+$] maintenance, the presumably nonfunctional middle region (M), and the C-terminal domain (C) essential for translation termination. In this study, we observed that the N domain, alone or as a part of larger fragments, can form aggregates in [*PSI*$^+$] cells. Two sites for Sup45p binding were found within Sup35p: one is formed by the N and M domains, and the other is located within the C domain. Similarly to Sup35p, in [*PSI*$^+$] cells Sup45p was found in aggregates. The aggregation of Sup45p is caused by its binding to Sup35p and was not observed when the aggregated Sup35p fragments did not contain sites for Sup45p binding. The incorporation of Sup45p into the aggregates should inhibit its activity. The N domain of Sup35p, responsible for its aggregation in [*PSI*$^+$] cells, may thus act as a repressor of another polypeptide chain release factor, Sup45p. This phenomenon represents a novel mechanism of regulation of gene expression at the posttranslational level.**

The final step of protein biosynthesis consists of the termination codon-dependent release of nascent completed peptide chain from the ribosome. In bacteria, translation termination requires three release factors (RF) with different functions: RF1, catalyzing termination at stop codons UAA and UAG; RF2, recognizing UAA and UGA; and RF3, which stimulates in the presence of GTP the activities of RF1 and RF2 but lacks codon specificity (25, 37). Although it has long been known that higher eukaryotes possess a single RF (eRF), which is able to recognize all three nonsense codons in an in vitro termination assay in a GTP-dependent manner (14, 18), this factor was characterized only recently. A family of eukaryotic eRF1 was identified, and the structures of human and frog eRF1 were elucidated. This structural family includes the yeast Sup45 protein, and it was postulated that Sup45p is an eRF1 (12). Since the eRF1 protein family shows no homology with known GTP-binding sequence motifs, it was suggested that another eRF which confers GTP dependence to the termination process should exist (12). A second eRF (eRF3) which has an expected GTP-binding sequence motif and is highly homologous to yeast Sup35p was identified and shown to confer GTP dependence to the overall termination process (51). It was also demonstrated that higher eukaryotic eRF1 and eRF3 interact with each other (51). The same was true for their yeast homologs, Sup45p and Sup35p (41). Although the specific release factor activity of the Sup45 and Sup35 proteins has not been demonstrated in an appropriate biochemical assay, their role in translation termination in yeast is strongly supported by genetic data (41).

One of the factors controlling the efficiency of translation termination in yeast cells is the cytoplasmically inherited determinant [*PSI*$^+$] (reviewed in reference 7). The nature of this determinant was a puzzle for a long time. Recently it was suggested that [*PSI*$^+$] is related to a specific alternative conformational state of the Sup35 protein capable of self-propagation by an autocatalytic mechanism, analogous to that of mammalian prions (49). A similar mechanism was also suggested to explain the unusual genetic properties of another yeast non-Mendelian determinant, [*URE3*], which is presumably related to inheritable alteration of the Ure2 protein (49).

Prions are infectious agents responsible for some neurodegenerative diseases of humans and other animals, including human Creutzfeldt-Jakob disease, sheep scrapie, and bovine spongiform encephalopathy (reviewed in references 33 and 34). Unlike other infectious agents, prions are devoid of any detectable nucleic acid constituent (32). The only recognized component responsible for the prion infectivity represents an altered form of the host-encoded PrP protein. The infectious isoform ($PrP^{Sc}$) of this protein differs from its normal cellular form ($PrP^{C}$) physically by poor solubility in detergents, high resistance to proteolysis, and a marked propensity for aggregation (24, 26, 35). No covalent modifications were found to account for different properties of the prion protein and its normal isoforms (40). Instead, the conformation of $PrP^{Sc}$ was found to be dramatically altered, being primarily β-sheet, compared to that of $PrP^{C}$, which is largely α-helical (1, 28). The current explanation of the mechanism of prion propagation is based on the protein-only hypothesis (15) and suggests that $PrP^{Sc}$ can catalyze the conformational conversion of $PrP^{C}$ into $PrP^{Sc}$ form, thus ensuring prion multiplication (33).

Although the prion hypothesis for the yeast determinants

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[*PSI*$^+$] and [*URE3*] was primarily based on their unique genetic properties (reviewed in reference 50), biochemical evidence demonstrates that several traits characteristic of the mammalian prions are also exhibited by the yeast Sup35 protein: (i) the Sup35p molecules interact with each other in [*PSI*$^+$] but not [*psi*$^-$] cells, and this interaction is critical for [*PSI*$^+$] propagation, since its disruption leads to a loss of [*PSI*$^+$] (31); (ii) in [*PSI*$^+$] cells, Sup35p forms high-molecular-weight aggregates, accumulating most of this protein (30, 31); and (iii) Sup35p accumulated in [*PSI*$^+$] cells differs from Sup35p in [*psi*$^-$] cells by higher resistance to proteases (30, 31). Increased protease resistance was also demonstrated for the Ure2 protein from [*URE3*] cells (23). The aggregation of Sup35p in [*PSI*$^+$] cells should interfere with its function in translation termination, leading to the increased nonsense codon readthrough and the [*PSI*$^+$] nonsense suppressor phenotype (30, 31).

Sup35p is a multidomain protein composed of the amino-terminal region and carboxy-terminal domain (domain C) of 253 and 432 amino acids, respectively. The evolutionarily conserved domain C is structurally similar to translation elongation factor EF-1α and essential for viability and translation termination, while the N-terminal region is not conserved and is not essential for viability. This region may be further subdivided into the N-terminal domain (N domain) of 123 amino acids required for [*PSI*$^+$] maintenance and the middle region (M domain), to which no function has been ascribed (11, 42, 43). The N-terminal region of Sup35p is responsible for its aggregation in the [*PSI*$^+$] state, and thus it may be regarded as a *cis*-acting repressor of the polypeptide chain release function of the C-terminal domain (31).

In this study, we define the regions within the Sup35p molecule that are responsible for the interaction with Sup45p and demonstrate that, together with Sup35p, a substantial amount of Sup45p in [*PSI*$^+$] cells is incorporated in high-molecular-weight aggregates, which should inhibit its release activity. Thus, the [*PSI*$^+$]-specific phenotype is a complex phenomenon, depending on the simultaneous inhibition of activity of both polypeptide chain release factors.

## MATERIALS AND METHODS

**Strains, media, and genetic methods.** *Escherichia coli* TG1 {*supE hsd*Δ*5 thi*Δ(*lac-proAB*) *F′* [*traD36 proAB*$^+$ *lacI*$^q$ *lacZ M15*]} was used for the expression of the glutathione S-transferase (GST)–Sup45, GST-Sup35C, and GST-Sup35NM fusion proteins (36). The *Saccharomyces cerevisiae* strains used in this study were 5V-H19 (*MAT***a** *ade2-1 SUQ5 can1-100 leu2-3,112 ura3-52* [*PSI*$^+$]) and its [*psi*$^-$] derivative. Strains 1-5V-H19, 2-5V-H19, and 3-5V-H19 were obtained by replacing the wild-type *SUP35* gene of 5V-H19 with the *sup35-C*, *sup35-MC*, and *sup35-*Δ*B* deletion alleles, respectively (42). These alleles causes dominant antisuppression and recessive inability to propagate [*PSI*$^+$] (42, 43). These and other *SUP35* deletion alleles are shown in Fig. 1b.

We used standard organic (YEPD) and synthetic (SC) media for yeast (38) and LB medium for bacteria (36). Appropriate amounts of amino acids, bases, and antibiotics were added when necessary. All solid media contained 2.5% (wt/vol) agar. Yeast cells were grown at 30°C, and bacteria were grown at 37°C.

Yeast strains were cured, when necessary, of the [*PSI*$^+$] determinant by growth on YEPD medium supplemented with 3 mM guanidine hydrochloride (48). The [*psi*$^-$] colonies of *ade2-1 SUQ5* carrying strains were chosen by pink color and adenine requirement because the weak serine-inserting tRNA suppressor *SUQ5* cannot suppress the *ade2-1* ochre mutation in the absence of the [*PSI*$^+$] determinant (6, 22, 27).

Earlier it was reported that multicopy plasmids encoding either complete Sup35p or its N-terminal region are able to induce the appearance of the [*PSI*$^+$] determinant (3, 9). This trait seems to be strain specific, since extra copies of *SUP35* gene cannot efficiently induce [*PSI*$^+$] in strain 5V-H19. Nevertheless, [*PSI*$^+$] could be readily induced by Sup35p overexpression in 5V-H19 derivatives carrying the chromosomal deletion corresponding to Sup35p N domain (17). To obtain the [*PSI*$^+$] strains expressing Sup35p N-terminal fragments, the [*psi*$^-$] strain 3-5V-H19 (*sup35-*Δ*B*) was transformed with a multicopy plasmid encoding Sup35N1p, Sup35N2p, or Sup35NMp. Overexpression of these proteins in the [*psi*$^-$] 3-5V-H19 strain carrying the *sup35-*Δ*B* allele causes the highly efficient generation of the [*PSI*$^+$] determinant (17). The [*PSI*$^+$] status of such transformants was confirmed genetically by transfer of the [*PSI*$^+$] determinant to a [*psi*$^-$] tester strain in "cytoduction" experiments as described earlier (31, 42) and biochemically by studying aggregation of Sup35N1p, Sup35N2p, and Sup35NMp (see Results). It should be noted that [*psi*$^-$] derivatives of these transformants could not be obtained, because the [*PSI*$^+$] state was continuously induced de novo (17). Conversely, only the [*psi*$^-$] variant of the original 5V-H19 (*SUP35*) strain could be used for the expression of Sup35p N-terminal fragments, because the corresponding [*PSI*$^+$] transformants were distinguished by very poor growth (43). Therefore, we used the 3-5V-H19 (*sup35-*Δ*B*) strain to express Sup35p N-terminal fragments in the [*PSI*$^+$] state and the original 5V-H19 (*SUP35*) strain to express these fragments in the [*psi*$^-$] state.

**Plasmids, DNA manipulations, and transformation.** All DNA manipulations and plasmid construction were carried out by standard protocols (36). The hybrid genes encoding N-terminal fusions of GST to Sup45p and Sup35Cp were constructed as follows. The *Xmn*I-*Sna*BI fragment containing the entire *SUP45* coding sequence was inserted into the *Sma*I site of plasmid pGEX2T (39). The sequence between the *Bam*HI site of GST and the first ATG codon of *SUP45* is GGA TCC ccc tcg gta ccc act tca ATG. The *Sna*BI site is located 185 bp downstream of the *SUP45* termination codon. The *Hpa*I-*Eco*RI fragment encoding amino acids 252 to 685 of Sup35p (Sup35Cp) was cloned into *Sma*I-*Eco*RI sites of pGEX3X (39). The plasmid encoding fusion of GST to Sup35NMp (amino acids 1 to 251 of Sup35p) was described earlier (31). Multicopy yeast plasmids carrying various *SUP35* deletion alleles (43) were based on the episomal plasmid pEMBLyex4 (2). The *SUP35* deletion alleles and the encoded truncated Sup35 proteins are depicted in Fig. 1b. The multicopy plasmid pFL44L-*HSP104* carrying *URA3* as a selectable marker was kindly provided by M. Boguta (Warsaw, Poland).

DNA transformation of lithium acetate-treated yeast cells was performed as described previously (13). *E. coli* cells were transformed by standard methods (16).

**Preparation and fractionation of yeast cell lysates.** Yeast cultures were grown to an optical density at 600 nm 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) containing 1 mM phenylmethylsulfonyl fluoride to limit proteolytic degradation. Cell debris was removed by centrifugation at 15,000 × *g* for 10 min.

To analyze the size distribution of Sup45p and Sup35p, the lysates were underlaid with 1-ml 30% sucrose pads made in buffer A and centrifuged in a Beckman SW50 rotor at 45,000 rpm (200,000 × *g*) for 30 min at 4°C. Under these conditions, ribosomes were found mostly in the intermediate sucrose fraction and to a lesser extent in the pellet (31). The resulting supernatants, pellets, and intermediate fractions were analyzed by Western blotting.

**Purification of Sup35p fragments and assay for interaction with immobilized GST-Sup45p.** To isolate Sup35NMp from yeast, strain 1-5V-H19 was transformed with the Sup35NMp-encoding multicopy plasmid. The overexpression of Sup35NMp induces [*PSI*$^+$] clones in this strain (17). Lysate was prepared from such [*PSI*$^+$] clones, treated with RNase A (0.5 mg/ml), high salt (1M LiCl), and nonionic detergent (1% Triton X-100), and fractionated by centrifugation through a sucrose pad as described previously (31). The pellet was treated with 3 M guanidinium hydrochloride and precipitated again by centrifugation through a sucrose pad. The Sup35NMp aggregates were resistant to the treatments, while other proteins were solubilized (data not shown). Analysis of the obtained Sup35NMp preparation by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with subsequent Coomassie blue staining showed that Sup35NMp was approximately 70% pure. Western blot analysis with anti-Hsp104p and anti-Sup45p antibodies did not reveal any signals in purified Sup35NMp preparation, whereas high levels of these proteins were found in the original pellet (data not shown).

The GST-Sup45p, GST-Sup35NMp, and GST-Sup35Cp fusion proteins were expressed in *E. coli* and isolated by affinity chromatography on glutathione-Sepharose 4B (Pharmacia). The GST extensions of GST-Sup35NMp and GST-Sup35Cp were removed with thrombin (Sigma) and factor $X_a$ (Promega), respectively, as described elsewhere (39). The resin with immobilized GST-Sup45p was incubated with yeast lysates or with the purified Sup35NM and Sup35Cp proteins for 2 h at 4°C and then washed with a 40-fold resin volume of buffer A. Bound proteins were eluted with 2% SDS and analyzed by Western blotting.

**Study of the protease resistance of Sup45p, Sup35p, and Sup35p truncated variants.** Lysates were prepared as described above but without addition of phenylmethylsulfonyl fluoride. Each reaction was performed with 150 μg of total protein in a volume of 50 μl and 0.2 to 0.8 μg of proteinase K (Boehringer Mannheim) per ml. After 30 min of incubation at 37°C, 4-μl aliquots were removed and analyzed by Western blotting.

**Protein gel electrophoresis and Western blot analysis.** Protein samples were separated on an SDS–10 to 15% polyacrylamide gel as described previously (21) and electrophoretically transferred to nitrocellulose sheets (44). Western blots were probed with polyclonal rabbit anti-Sup35p (10) or anti-Sup45p (K. M. Jones, University of Kent) antibody. Bound antibodies were detected with the Amersham ECL system as instructed by the manufacturer. Prestained proteins (fructose-6-phosphate kinase [84 kDa], pyruvate kinase [63 kDa], fumarase [52 kDa], lactic dehydrogenase [35 kDa], and triosephosphate isomerase [32 kDa]; all from Sigma) were used as molecular mass standards.

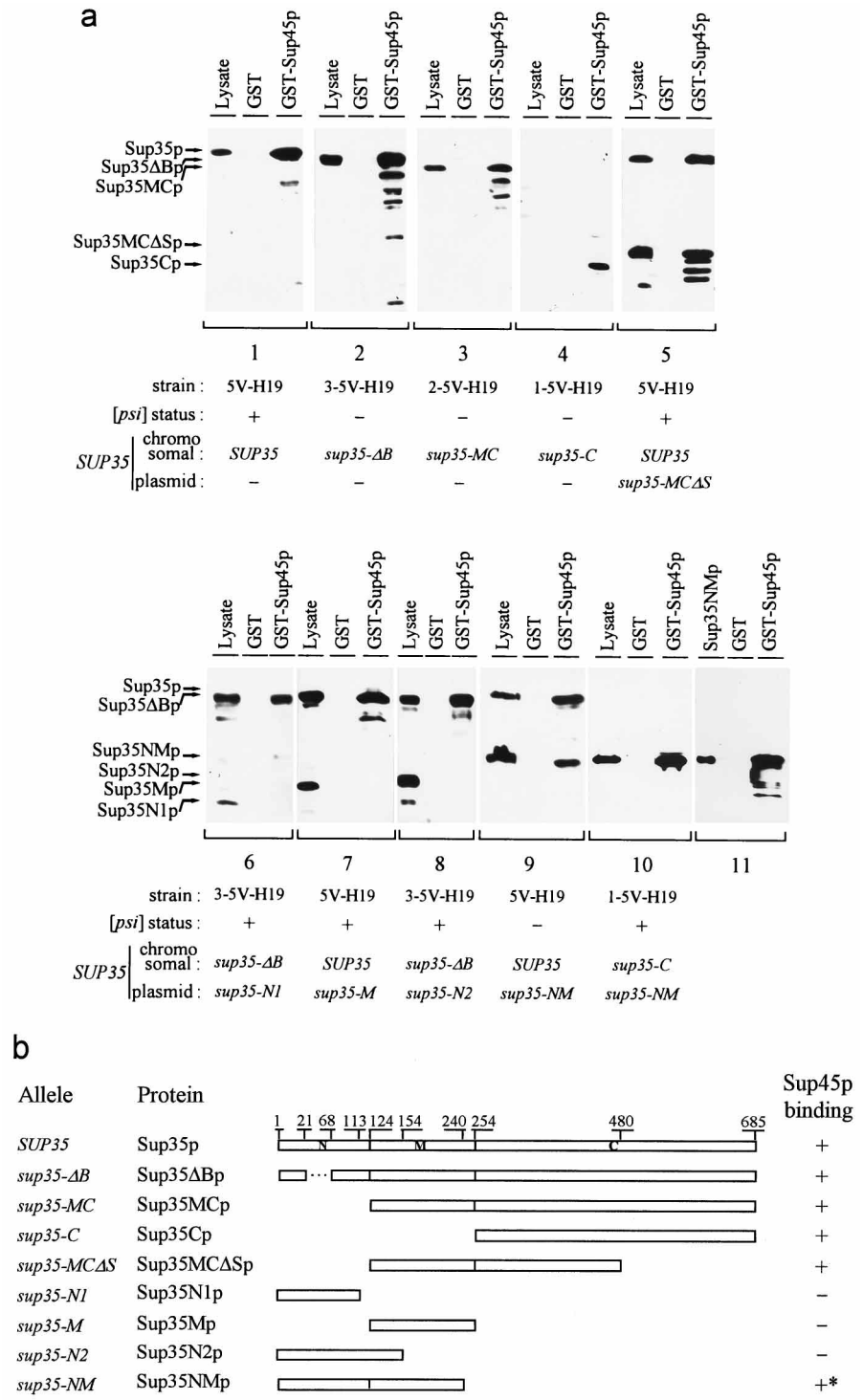


FIG. 1. (a) Interaction of Sup45p with Sup35p fragments, determined by immunoblot analysis of Sup35p. Yeast strains expressing different Sup35p fragments are described below the blots. The strain lysates were incubated with glutathione-Sepharose 4B with immobilized GST or GST-Sup45 protein. Following washing, bound proteins were eluted and analyzed. Each strain is represented by a panel of three lanes: lysate (total lysate of the strain); GST (proteins bound by immobilized GST); and GST-Sup45p (proteins bound by immobilized GST-Sup45p). Sup35Cp was not evident in lysates in panels 4 and 10 due to low specificity of antibody to this part of the protein (43), but it is evident due to enrichment in corresponding GST-Sup45p precipitates (the Sup35Cp band is located just above the Sup35NMp band in panel 10). In panel 11, a partially purified Sup35NMp preparation (Materials and Methods) was used instead of a total lysate. Minor bands not indicated by the arrows represent degradation products characteristic of Sup35p in the [*psi*$^-$] state (31). The [*PSI*] status of each of the strains used for panels 1, 5, 7, and 9 was determined by examining the efficiency of suppression of *ade2-1* ochre mutation in strain 5V-H19. This analysis was not complicated by the presence of a multicopy plasmid with the *SUP35-NM* allele (panel 9), since although multicopy plasmids carrying the *SUP35* gene or its 3′-deletion alleles cause suppression of nonsense mutations in yeast (4), they do not cause suppression of the *ade2-1* mutation. Strains used for panels 2 to 4 were [*psi*$^-$] since their chromosomal 5′-deletion mutations cause elimination of [*PSI*$^+$] (42). The [*PSI*$^+$] status of each of the strains used for panels 6, 8, 10, and 11 was confirmed by cytoduction experiments as described in Materials and Methods. (b) Schematic representation of the Sup35 protein and its fragments used in the binding experiments. The ability or inability to bind to Sup45p is indicated by + or −, respectively. ∗, Sup35NMp, expressed in yeast but not in *E. coli*, was able to bind to Sup45p.

## RESULTS

**The C-terminal domain of the Sup35 protein is involved in interaction with Sup45p.** It was shown earlier that the *S. cerevisiae* Sup35 and Sup45 proteins interact with each other in vivo and in vitro to form a heterodimeric release factor complex (41). To localize the regions of the Sup35 protein involved in interaction with Sup45p, we tested the ability of immobilized Sup45p to precipitate different truncated Sup35p molecules from the lysates of yeast strains expressing such molecules. To obtain the immobilized Sup45p, this protein was expressed in *E. coli* as a fusion to the C terminus of GST (GST-Sup45p) and purified by binding to glutathione-Sepharose 4B. The resin with bound GST-Sup45p was incubated with yeast lysates and then washed by buffer to remove nonspecific proteins. Bound proteins were eluted and analyzed by Western blotting. The resin with bound GST alone was used as a negative control. GST-Sup45p was able to precipitate Sup35p from [*PSI*$^+$] lysates (Fig. 1a, panel 1) as well as from [*psi*$^-$] lysates (data not shown). This result demonstrates that Sup45p-Sup35p interaction does not depend on the [*PSI*] state of Sup35p molecules. To check the ability of the essential C domain of Sup35p to bind to Sup45p, we tested three strains, carrying different chromosomal 5′-deletion alleles of *SUP35*, which encode Sup35p variants with partial or complete deletion of domains N and M. All of these truncated variants of Sup35p were able to bind to GST-Sup45p (Fig. 1a, panels 2 to 4), showing that the C domain of Sup35p, which is similar to EF-1α, contains a site for Sup45p binding. The Sup35C protein, purified from bacteria, also interacted with Sup45p (data not shown). This result indicates that the interaction was direct, i.e., not mediated by other molecules of yeast lysate.

For further mapping of this site, the [*PSI*$^+$] variant of strain 5V-H19 was transformed with a multicopy plasmid encoding the Sup35MCΔS protein, which includes the M domain and the first half (230 amino acids) of the C domain. The Sup35MCΔS protein was bound efficiently to GST-Sup45p (Fig. 1a, panel 5). The same result was obtained for the 5V-H19 [*psi*$^-$] strain transformed with the same plasmid (data not shown). This binding was not mediated by the chromosomally encoded Sup35 protein, because the N-terminally deleted Sup35MCΔS protein does not interact with complete Sup35p (31). Since the M domain of Sup35p is not able to bind to Sup45p (see below), one can conclude that the binding site for Sup45p is located within the first half of Sup35Cp.

**The NM fragment of Sup35p also interacts with Sup45p.** The next step was to test whether the inessential N-terminal part of Sup35p, including N and M domains (NM fragment or Sup35NMp), is able to bind to GST-Sup45p. The resin with bound GST-Sup45p was incubated with the cell lysate of a 5V-H19 [*psi*$^-$] strain transformed with the multicopy plasmid encoding Sup35NMp. Both chromosome-encoded complete Sup35p and Sup35NMp were precipitated (Fig. 1a, panel 9). Since the Sup35NMp fragment does not interact with Sup35p in [*psi*$^-$] lysates (31), one can conclude that this fragment binds to Sup45p directly; i.e., this interaction is not mediated by the full-length Sup35p. The ability of Sup35NMp to interact with Sup45p did not depend on the [*PSI*$^+$] determinant. The immobilized GST-Sup45p precipitated both the plasmid-encoded Sup35NM and chromosome-encoded Sup35C proteins from the lysate of [*PSI*$^+$] transformants of the 1-5V-H19 (*sup35-C*) strain (Fig. 1a, panel 10). In this case, GST-Sup45p again bound both proteins independently because Sup35NMp does not interact with the N-terminally deleted Sup35Cp molecules (31).

To check that the interaction between Sup35NMp and Sup45p was direct, i.e., not mediated by other molecules, Sup35NMp was purified from a [*PSI*$^+$] yeast strain. The purified Sup35NMp was completely precipitated by GST-Sup45p, in contrast to its partial precipitation from the crude lysate (data not shown). This result indicates that these proteins interact directly. Unlike the native Sup35NM protein from yeast, Sup35NMp purified from *E. coli* did not interact with Sup45p (data not shown). The difference was not due to the requirement for some other protein mediating this interaction, since the result was the same in the presence and absence of supplementing yeast lysate. Rather, the difference might be attributed to a different modification or folding of Sup35NM proteins, expressed in yeast and bacteria.

**N and M domains of Sup35p expressed separately do not interact with Sup45p.** To assign the N-terminal Sup45p binding site to either the N or M domain of Sup35p, these domains were expressed separately in yeast. Two variants of the Sup35p protein were used to represent the N domain: Sup35N1p, slightly smaller than the N domain, and Sup35N2p, overlapping Sup35Mp by 30 amino acids (Fig. 1b). [*PSI*$^+$] transformants of strain 3-5V-H19 (*sup35-ΔB*) with a multicopy plasmid encoding either Sup35N1p or Sup35N2p were obtained as described in Materials and Methods. Only the Sup35ΔB protein was precipitated by the immobilized GST-Sup45p from the lysates of these transformants (Fig. 1a, panels 6 and 8), indicating that Sup35N1p and Sup35N2p cannot bind to Sup45p and Sup35ΔBp. Strain 5V-H19 [*PSI*$^+$] was transformed with a plasmid encoding the M domain (Sup35Mp). After incubation with immobilized Sup45p, only Sup35p, not Sup35Mp, was precipitated (Fig. 1a, panel 7). Similar results were obtained with lysates of 5V-H19 [*psi*$^-$] transformants carrying the plasmids encoding the Sup35N2 and Sup35M proteins (data not shown). This indicates that separately expressed N and M domains of Sup35p do not interact with Sup45p irrespective of the [*PSI*] status of the strain used for lysate preparation.

**Sup45p is aggregated in [*PSI*$^+$] cells.** It was shown earlier that Sup35p in [*PSI*$^+$] cells forms high-molecular-weight aggregates, cosedimenting with the heavy polyribosomal fraction and even larger structures (31). Since the Sup45 protein is able to bind to Sup35p, its distribution between the soluble and aggregated fractions could also be influenced by the [*PSI*] status of cells. To check this, the extracts of the [*PSI*$^+$] and [*psi*$^-$] variants of strain 5V-H19 were fractionated by centrifugation through a sucrose cushion, and the fractions were analyzed by immunoblotting using antibodies against Sup35p and Sup45p. Both proteins showed essentially similar, [*PSI*]-dependent distributions (Fig. 2a). In the lysate of [*psi*$^-$] cells, most of these proteins were found in the cytosolic and intermediate ribosomal fractions, whereas after centrifugation of the [*PSI*$^+$] lysate, Sup35p and Sup45p were found in the pellet and ribosomal fractions (Fig. 2a). This experiment indicates that the Sup45p molecules may be included into [*PSI*$^+$]-specific aggregates formed by the full-length Sup35p molecules, which possess two binding sites for Sup45p. The treatment of the complexes with high salt (1 M KCl), nonionic detergent (1% Triton X-100), and RNase A (500 μg/ml) failed to release Sup35p or Sup45p, although some other proteins were solubilized (data not shown). Surprisingly, the aggregation of Sup45p in [*PSI*$^+$] cells was not observed earlier (30). The reason for this discrepancy is unclear. Nevertheless, it is worth mentioning that [*PSI*$^+$]-dependent aggregation of Sup45p is not a specific trait of strain 5V-H19, because we observed the same phenomenon in a yeast strain with a different origin (data not shown).

**Sup35p N-terminal domains form aggregates in [*PSI*$^+$] cells.** Genetic data showed that the sequence of the 113 N-

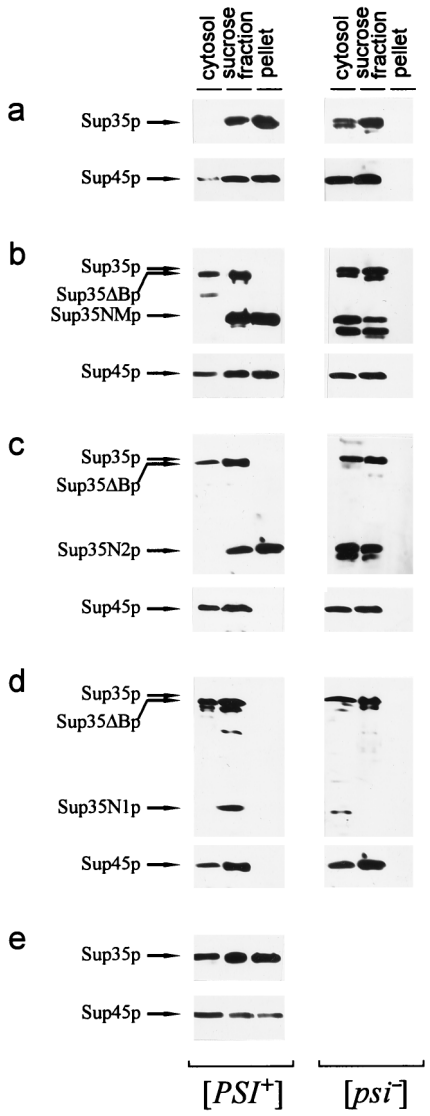


FIG. 2. Distribution of Sup35p and Sup45p between the soluble and aggregated forms, determined by immunoblot analysis of the Sup35 and Sup45 proteins. Lysates of the following strains were fractionated by centrifugation through sucrose cushion: (a) 5V-H19 [*PSI*$^+$] and [*psi*$^-$]; (b) 3-5V-H19 [*PSI*$^+$] and 5V-H19 [*psi*$^-$] transformed with a multicopy plasmid encoding Sup35NMp; (c) 3-5V-H19 [*PSI*$^+$] and 5V-H19 [*psi*$^-$] transformed with a multicopy plasmid encoding Sup35N2p; (d) 3-5V-H19 [*PSI*$^+$] and 5V-H19 [*psi*$^-$] transformed with a multicopy plasmid encoding Sup35N1p; and (e) 5V-H19 [*PSI*$^+$] transformed with a multicopy plasmid encoding Hsp104p.

terminal amino acids of Sup35p plays a key role in [*PSI*$^+$] propagation (42). However, it was not demonstrated that this part of Sup35p is sufficient for its oligomerization. To show this, pairs of [*PSI*$^+$] and [*psi*$^-$] strains expressing the Sup35p N-terminal fragments were compared by centrifuging their lysates. To obtain the [*PSI*$^+$] variants, the [*psi*$^-$] strain 3-5V-H19 (*sup35-ΔB*) was transformed with a multicopy plasmid encoding either Sup35N1p, Sup35N2p, or Sup35NMp, and the [*PSI*$^+$] clones were selected as described in Materials and Methods. The [*psi*$^-$] strains were obtained by transformation of 5V-H19 [*psi*$^-$] with the same plasmids. The lysates of transformants were fractionated by centrifugation through sucrose cushion, and the fractions were analyzed by immunoblotting. In agreement with previous studies (31), the Sup35NMp fragment was found in the soluble and sucrose fractions of [*psi*$^-$] lysates, while in [*PSI*$^+$] lysates it was shifted to the intermediate and pellet fractions (Fig. 2b). The difference in Sup35NMp distribution is similar to that of full-length Sup35p in the [*psi*$^-$] and [*PSI*$^+$] lysates (Fig. 2a). Sup35N2p also showed a similar difference in distribution (Fig. 2c). Sup35N1p was shifted from the cytosolic fraction in the case of [*psi*$^-$] lysates to the ribosomal fraction in the case of [*PSI*$^+$] lysates (Fig. 2d). Thus, in [*PSI*$^+$] cells, all three Sup35p N-terminal fragments can form aggregates. A minimal tested region sufficient for the oligomerization corresponds to Sup35N1p or the first 113 amino acids of Sup35p. The rest of Sup35p has no potential for oligomerization (31), which is confirmed by the solubility of Sup35ΔBp (Fig. 2b to d) and Sup35Mp (data not shown) in [*PSI*$^+$] lysates. Interestingly, the aggregates of Sup35N1p sedimented more slowly and therefore were of significantly lower molecular weight than the aggregates of full-length Sup35p, Sup35NMp, or Sup35N2p. This may be due to the following reasons: (i) Sup35N1p is expressed at levels approximately 10-fold lower than those of Sup35NMp; (ii) Sup35N1p is of lower molecular weight; and (iii) some additional proteins, such as Sup45p, may be included in the aggregates of Sup35NMp but not Sup35N1p.

To find out whether Sup45p is included in the aggregates of the Sup35p N-terminal fragments, the preparations shown in Fig. 2 were probed with anti-Sup45p antibody. In the cells expressing Sup35NMp, the [*PSI*$^+$] determinant caused a shift of Sup45p to the pellet simultaneously with Sup35NMp (Fig. 2b). This result indicates that Sup45p is included in Sup35NMp aggregates. The inclusion of Sup45p in the aggregates may be related to the fact that Sup35NMp is able to bind Sup45p. A small portion of Sup45p in this [*PSI*$^+$] strain was present in the soluble fraction, probably bound to the soluble Sup35pΔB molecules. Sup45p distribution did not depend on [*PSI*] in the strains expressing Sup35N2p or Sup35N1p (Fig. 2c and d). This result could be expected, since Sup45p cannot bind to Sup35N2p and Sup35N1p and thus should not be incorporated in the aggregates of these molecules. This finding also does not allow us to explain the difference in the molecular weights of aggregates formed by Sup35N1p and Sup35N2p in [*PSI*$^+$] strains by incorporation of Sup45p.

**Aggregated forms of Sup35p N-terminal fragments but not of Sup45p show increased resistance to proteases.** It was shown earlier that Sup35p in the [*PSI*$^+$] state shows an increased resistance to cellular proteases and proteinase K (31). The increased resistance of Sup35p to cellular proteases was inferred from the observation that the content of Sup35p was up to eightfold higher in [*PSI*$^+$] cells than in [*psi*$^-$] cells. Similarly, in this study we observed in [*PSI*$^+$] cells an eightfold excess of Sup35NMp, a fourfold excess of Sup35N2p, and a threefold excess of Sup35N1p in comparison with the levels of these proteins in [*psi*$^-$] cells (Fig. 3a, b, and c, respectively). This result suggests that the [*PSI*$^+$] state increases the resistance of Sup35p N-terminal fragments to degradation by cellular proteases. Proteinase K digestion experiments also revealed an increased resistance of the Sup35N1p, Sup35N2p, and Sup35NMp fragments expressed in [*PSI*$^+$] cells (Fig. 3). Notably, the Sup35ΔBp molecules present in the same cells in soluble form did not manifest increased resistance to proteinase K. Thus, only those variants of Sup35p which form aggregates in [*PSI*$^+$] cells demonstrate increased proteinase K resistance. Furthermore, in the case of Sup35NMp, a protease-resistant fragment was clearly evident. This result indicates that, similarly to the mammalian $PrP^{Sc}$ protein, the Sup35NM protein in the [*PSI*$^+$] state possesses a submolecular protease-resistant region (Fig. 3a). It is noteworthy that such a region

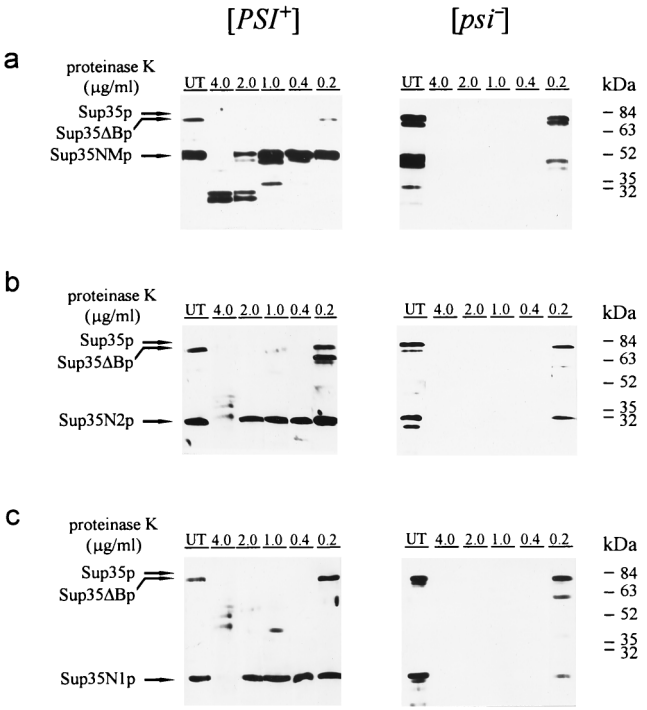


FIG. 3. Comparison of degradation by proteinase K of the Sup35NM, Sup35N2, and Sup35N1 proteins in [*PSI*$^+$] and [*psi*$^-$] lysates. Shown is immunoblot analysis of the Sup35 protein. Lysates were treated with the indicated concentrations of proteinase K for 30 min at 37°C. UT, untreated lysates. Lysates of the 3-5V-H19 [*PSI*$^+$] and 5V-H19 [*psi*$^-$] strains transformed with multicopy plasmids encoding Sup35NMp (a), Sup35N2p (b), Sup35N1p (c) were analyzed. The lanes with untreated lysates demonstrate higher levels of the Sup35p N-terminal fragments in [*PSI*$^+$] lysates, since the content of these proteins was equalized by loading eightfold (for Sup35NMp), fourfold (for Sup35N2p), and threefold (for Sup35N1p) less [*PSI*$^+$] lysates on the gel. The treated [*PSI*$^+$] and [*psi*$^-$] lysates were loaded on the gel in the same proportion. Positions corresponding to molecular mass markers are indicated on the right.

was not observed for the Sup35N1, Sup35N2 (Fig. 3c and b), and full-length Sup35 (31) proteins from the [*PSI*$^+$] lysates. In contrast to Sup35p, the Sup45 protein, which is also found in high-molecular-weight aggregates in [*PSI*$^+$] cells, did not show an increased resistance to proteinase K digestion (data not shown). Also, the content of Sup45p in [*PSI*$^+$] cells was not higher than in [*psi*$^-$] cells. Thus, the aggregation alone does not cause the protease protection of proteins.

**Overexpression of Hsp104p chaperone in [*PSI*$^+$] cells causes partial release of Sup45p from aggregates.** It was observed that a two- to fourfold overexpression of the Hsp104 chaperone protein, which acts to dissolve denatured protein aggregates in yeast (29), causes an antisuppressor phenotype in [*PSI*$^+$] cells (5). It was also shown that the overexpression of Hsp104p dissolves Sup35p aggregates in [*PSI*$^+$] cells, leading to the appearance of soluble Sup35p and thus causing an antisuppressor phenotype (30, 31). We tested whether the increased expression of Hsp104p in [*PSI*$^+$] cells would also solubilize Sup45p. The preparations used to show Sup35p solubilization (31) were analyzed by immunoblotting with anti-Sup45p antibody. The fractions tested were obtained by centrifugation through a sucrose cushion of the lysate of strain 5V-H19 [*PSI*$^+$] transformed with a multicopy plasmid carrying the *HSP104* gene. The *HSP104* mRNA was 20-fold overexpressed in this transformant compared to its level in the untransformed parental strain (31). The analysis revealed the increased levels of soluble Sup45p in this lysate in comparison with a lysate of a nontransformed [*PSI*$^+$] strain (Fig. 2d). The appearance of Sup45p in the soluble fraction cannot be explained by the accumulation of [*psi*$^-$] cells, since the latter constituted only 2% of the analyzed yeast culture (31). Thus, the Sup35 and Sup45 proteins are simultaneously liberated from the [*PSI*$^+$]-specific aggregates by the action of excess Hsp104p (Fig. 2e). The liberation of Sup45p could represent an additional reason for the antisuppressor effect of Hsp104p overexpression.

## DISCUSSION

It is known that the molecules of Sup35p and Sup45p form a heterodimeric complex that is functional in translation termination (41). Sup35p is a three-domain protein, the essential termination function of which is limited to its C domain (42, 43, 51), while the only function ascribed so far to the rest of the protein is [*PSI*$^+$] maintenance (11, 42). In this study, we demonstrated that Sup35p possesses two sites for Sup45p binding, one of which is located within the C domain. The interaction of Sup45p with the C domain of Sup35p is probably crucial for the termination, since this domain is essential for the termination function of Sup35p. Further analysis indicated that the first half of the C domain retains the ability to bind Sup45p. It should be noted that this region corresponds to the region of prokaryotic elongation factor EF-Tu exhibiting GTP and aminoacyl-tRNA binding motifs (19, 20).

The other site for Sup45p binding was found within the nonessential Sup35NMp fragment. However, neither the N nor M domain expressed separately could bind to Sup45p. This finding may indicate that both the N and M domains contribute to this binding site. It is also possible that the NM binding site is located within either one of the N and M domains, but their proper folding or modification requires the integrity of the NM fragment. The interaction of Sup45p with Sup35NMp is not critical for translation termination, because the NM fragment itself is not essential for the cell viability. Nevertheless, it is possible that this fragment also participates in termination and influences the efficiency or specificity of this process.

In [*PSI*$^+$] cells, Sup35p exists mostly in the form of oligomers (30, 31). The Sup35p N domain plays a key role in the propagation of [*PSI*$^+$], since it may act as a separate functional unit, required to support the propagation of [*PSI*$^+$] determinant (42), and it is essential for Sup35p oligomerization (31). However, it was difficult to show that the N domain alone is sufficient for [*PSI*$^+$] maintenance, because the C domain is essential for cell growth and must be expressed in the same cell (42). Here we demonstrated the existence of [*PSI*$^+$]-specific Sup35p aggregates composed solely of the N or NM fragment and not containing the C domain. This observation shows that the N domain is the only region of Sup35p required for its oligomerization and, therefore, for [*PSI*$^+$] maintenance. Thus, the Sup35p molecules are kept together in aggregates by interactions of their N-terminal domains, which represent a core region of the aggregate. The region of Sup35p, composed of the M and C domains, is attached to the core but is not required for aggregate integrity and thus may be designated the side chain of the aggregate. Sup35p aggregates may also include other molecules interacting with Sup35p, such as Sup45p. The data presented here indicate that Sup45p is included in aggregates of the full-length Sup35p or Sup35NMp fragment which possess sites for Sup45p binding. At the same time, Sup45p is not included in the aggregates of the N domain lacking a site for its binding. Unlike Sup35p, aggregate-bound Sup45p does not show increased resistance to proteinase K and

cellular proteases. This may indicate its location at the side chains of Sup35p aggregates.

The results obtained in this study provide new details for understanding the molecular background of the nonsense suppressor phenotype generated by the [*PSI*$^+$] determinant. Earlier it was suggested that the aggregation of Sup35p in [*PSI*$^+$] cells inhibits Sup35p activity and leads to a [*PSI*$^+$] nonsense suppressor phenotype (31). In this study, we demonstrated that the Sup45 protein is also incorporated in these aggregates. This should lower the levels of the free form of Sup45p, providing an additional reason for a decrease in the efficiency of translation termination in [*PSI*$^+$] strains. It is difficult to evaluate the extent to which the [*PSI*$^+$] phenotype depends on Sup45p aggregation, since it may also depend on the aggregation of Sup35p. However, some phenotypes, observed earlier in two [*PSI*$^+$] strains having a decreased ratio of Sup45p to Sup35p, may be explained only by the insufficient Sup45p function. It was found that overexpression of Sup35p in [*PSI*$^+$] strains is lethal, while simultaneous overexpression of Sup35p and Sup45p is not (8, 41). It is reasonable to suggest that the increased amount of aggregated Sup35p absorbs Sup45p more efficiently, thus bringing the levels of soluble Sup45p below a certain threshold and causing lethality. This case also demonstrates that the interaction of Sup35p and Sup45p is rather strong, since it may result in very low levels of noncomplexed Sup45p. The other strain was a [*PSI*$^+$] diploid heterozygous for *SUP45* but not a *SUP35* null allele, where some dominant phenotypes, such as slow growth and inability to sporulate, were evident (8). Since the complete inactivation of one of the two *SUP45* wild-type alleles of a diploid strain altered the physiology of [*PSI*$^+$] but not [*psi*$^-$] cells, it was suggested that the [*PSI*$^+$] determinant can inactivate a significant proportion of the *SUP45*-encoded protein. This interpretation agrees well with the current results: the most of Sup45p in [*PSI*$^+$] cells is presumably inactivated due to absorption by Sup35p aggregates. Based on all these findings, the [*PSI*$^+$] suppressor phenotype may result from the partial inactivation of both yeast translation termination factors, which represent subunits of a single functional complex (41). The N-terminal domain of Sup35p responsible for the aggregation was regarded as a *cis*-acting repressor of the polypeptide chain release function of the Sup35p C-terminal domain (31). In a similar way, the N domain may be considered a *trans*-acting repressor of the function of the Sup45p polypeptide chain release factor. It is also possible that Sup45p is not the only example of proteins subjected to a prion-dependent inactivation. The [*PSI*$^+$]-dependent aggregates of Sup35p could absorb other interacting proteins, thus decreasing their activity in a [*PSI*]-dependent manner.

At present it is not clear whether the [*PSI*$^+$] state is biologically meaningful for yeast. However, since the [*PSI*$^+$] determinant increases the readthrough of nonsense codons (45, 46), it may allow translation of natural stop codons on certain mRNAs to produce proteins with C-terminal extensions with modified or new functions (47). Although for yeast no such examples are known yet, it is possible that they exist and that under certain conditions, the [*PSI*$^+$] cells with an increased level of nonsense codon readthrough have selective advantages.

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