

Evidence for Separable Functions of Srp1p, the Yeast Homolog of Importin α (Karyopherin α): Role for Srp1p and Sts1p in Protein Degradation

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Srp1p (importin α) functions as the nuclear localization signal (NLS) receptor in *Saccharomyces cerevisiae*. The *srp1-31* mutant is defective in this nuclear localization function, whereas an *srp1-49* mutant exhibits defects that are unrelated to this localization function, as was confirmed by intragenic complementation between the two mutants. *RPN11* and *STS1* (*DBF8*) were identified as high-dosage suppressors of the *srp1-49* mutation but not of the *srp1-31* mutation. We found that Sts1p interacts directly with Srp1p in vitro and also in vivo, as judged by coimmunoprecipitation and two-hybrid analyses. Mutants of Sts1p that cannot interact with Srp1p are incapable of suppressing *srp1-49* defects, strongly suggesting that Sts1p functions in a complex with Srp1p. *STS1* also interacted with the second suppressor, *RPN11*, a subunit of the 26S proteasome, in the two-hybrid system. Further, degradation of Ub-Pro- β -galactosidase, a test substrate for the ubiquitin-proteasome system, was defective in *srp1-49* but not in *srp1-31*. This defect in protein degradation was alleviated by overexpression of either *RPN11* or *STS1* in *srp1-49*. These results suggest a role for Srp1p in regulation of protein degradation separate from its well-established role as the NLS receptor.

Nucleocytoplasmic transport is a complex process mediated by interaction of transport cargoes with components of the nuclear pore complex along with other soluble factors (reviewed in references 9, 28, and 30). Import of most karyophilic proteins into the nucleus is energy dependent and mediated by short stretches of amino acids known as nuclear localization signals (NLSs). Proteins with classical NLSs, as exemplified by the simian virus 40 (SV40) large T antigen NLS and the nucleoplasmin NLS, are transported to the nucleus by a heterodimer composed of Srp1p (the *Saccharomyces cerevisiae* homolog of importin α [Kap60p]) and Kap95p (an *S. cerevisiae* homolog of importin β) (6, 10). Srp1p is the NLS receptor component that binds to the NLS itself, while Kap95p interacts with some components of the nuclear pore complex to facilitate movement of the Srp1p-Kap95p complex carrying a cargo protein from the cytoplasm to the nucleus (35, 36, 37).

There are several other receptor proteins, which have amino acid sequence similarity to importin β (Kap95p) and are grouped in the importin β family. These proteins directly bind cargo proteins such as ribosomal proteins and carry out a protein import function independently of Srp1p (reviewed in references 9, 28, and 30).

SRP1 was originally identified as a suppressor of certain temperature-sensitive (ts) mutations in RNA polymerase I (Pol I) in *S. cerevisiae* (54). Specific mutations in *SRP1* were able to suppress ts mutations in the zinc-binding domains of *RPA190* and *RPA135*, genes encoding the largest and second largest subunits of Pol I, respectively, but not other *rpa190* and *rpa135* ts mutations outside of this domain. These *SRP1* suppressor mutations displayed no apparent phenotype on their

own. Additional work has shown that different ts mutations in *SRP1* cause different defects, which include protein import defects, nuclear segregation defects, altered microtubule morphology, and altered nucleolar morphology (24, 44, 55). Although the role of Srp1p in protein import is firmly established, it is not clear whether all of the diverse and allele-specific phenotypes displayed by these mutations are the consequences of defects in protein import. For example, while defects in NLS binding and NLS protein import were clearly demonstrated for the *srp1-31* mutation (24, 44), no or only slight defects in NLS binding and protein import were observed for the *srp1-49* mutation, which showed various other phenotypes (44, 55; unpublished data). Likewise, suppression of Pol I ts mutations by *SRP1* mutations is difficult to explain on the basis of the Srp1p function as the NLS receptor (54, 55).

For these reasons, we considered and tested the possibility that Srp1p might have an additional function(s) distinct from the NLS receptor function in protein import. We now report our discovery that two mutations, *srp1-49* and *srp1-31*, show intragenic complementation, indicating that the function affected by *srp1-49* is clearly different from the function affected by *srp1-31*, i.e., the NLS receptor function. To obtain clues to the functions affected by *srp1-49*, high-dosage suppressors of *srp1-49* were isolated. Two suppressors identified, *STS1* and *RPN11*, suppressed only *srp1-49* and not *srp1-31*. *STS1* was originally identified (22) as a dosage-dependent suppressor of a mutation in *SEC23*, a gene required for endoplasmic reticulum (ER)-Golgi protein transport (56). Independently, *DBF8*, which is identical to *STS1*, was also identified as a gene essential for nuclear segregation and division (12). *RPN11* was recently identified as a component of the regulatory particle of the 26S proteasome (8), suggesting a link between *SRP1* and the ubiquitin-proteasome system. Further experiments designed to examine such a link have led to the conclusion that Srp1p, together with Sts1p, carries out a function in the regulation of protein degradation by the ubiquitin-proteasome system and this function is separate from its established function

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in NLS-dependent protein import into the nucleus. We report these experiments and also discuss previous observations which support this conclusion.

MATERIALS AND METHODS

Media, strains, and plasmids. YEPD medium consists of 2% yeast extract, 1% Bacto-peptone (Difco Laboratories, Detroit, Mich.), and 2% glucose. Synthetic glucose (SGlu) medium (2% glucose, 0.67% yeast nitrogen base [Difco], and 0.5% Casamino acids [Difco]) was supplemented with required bases or tryptophan as described by Sherman et al. (42). Synthetic galactose (SGal) and synthetic raffinose (SRaff) media are the same as SGlu but with 2% galactose or 3% raffinose substituted for glucose, respectively. To make solid medium, 2% agar was added.

The yeast strains and plasmids used in this study are described in Table 1. Yeast plasmid transformations were performed as described by Gietz et al. (7). DNA sequencing was performed using an ABI373A DNA sequencer (PE Applied Biosystems). The *SRP1* chromosomal locus was replaced with the *srp1-31* or *srp1-49* mutant allele (55) by plasmid integration and pop-out (24, 51).

The *STS1* gene was amplified by PCR using lambda genomic clone 70732 (American Type Culture Collection) as the template with the primers 5' GGT ACA TAC TAG CGG CAG 3' and 5' CCG ACG ATG ACC ACT CAC 3'. The PCR product was cloned into the *SmaI* site of pUC19, yielding pNOY3234. A *SalI* digest was performed to remove *STS1* and move it into the *SalI* site of pRS5314 and pRS5316 (45), creating pNOY470 and pNOY471, respectively. For *STS1* genomic disruption, pNOY3234 was digested with *PflMI* and *NsiI* to remove most of the *STS1* coding region, and this deletion was replaced with the *HIS3* gene to make pNOY3235. pNOY3235 was digested with *SalI*, and the 1,934-bp fragment was gel isolated and digested with *PvuII*. The *SalI-PvuII sts1::HIS3* fragment was then transformed into NOY397, and His⁺ transformants were selected (NOY726). NOY726 was sporulated, and tetrads were dissected and showed a 2:2 segregation of His⁻ to His⁺, confirming that *STS1* is an essential gene (12). NOY726 was transformed with pNOY470 or pNOY471, and Trp⁺ or Ura⁺ transformants were selected, respectively. Then the strains were sporulated and tetrads were dissected to create NOY943 and NOY944.

Two-hybrid system plasmids were prepared as derivatives of pAS1 and pACT2 (4). SFY526 (Clontech) was used as the reporter strain for all two-hybrid experiments. pNOY472 was constructed by inserting the *NcoI-SacI* and *SacI-SalI* *SRP1* fragments from pNOY3198 into the *NcoI* and *SalI* sites of pAS1. *STS1* was amplified using PCR with the template lambda clone 70732 and the primers 5' CAT GCC ATG GGA TTT GAA TGG GGT TTT AAA CCC 3' and 5' CGG AAT TCT TAG TTA AAG GGC GAA TCA GTA G 3'. The PCR fragment was digested with *NcoI* and *EcoRI* and cloned into the corresponding sites of pACT2 (pNOY473). The *NcoI-XhoI* fragment from pNOY473 containing the *STS1* gene was ligated into the *NcoI* and *SalI* sites of pAS1, creating pNOY474. An *RPN11*-containing fragment, obtained by digesting pNOY285 with *SalI*, was treated with T4 DNA polymerase (New England Biolabs) to fill in the overhang and then *SacI* digested. The blunt *SacI* fragment was ligated into the *SmaI* and *SacI* sites of pACT2, creating pNOY475.

pNOY476 was constructed by cloning a *NotI* fragment containing the open reading frame for green fluorescent protein (GFP) (a gift from Pamela Silver) into the *NotI* site of pNOY477. For constructing pNOY478, the *GFP-STS1* fusion gene was derived from pNOY476 as a *KpnI* blunted *EagI* fragment. This fragment was ligated into pNOY258 previously digested with *BamHI*, blunt ended with T4 DNA polymerase, and then digested with *EagI*. pNOY479 was constructed by cloning the *PflMI-BamHI* fragment from pNOY476 into pNOY471. pNOY480 was constructed using site-directed mutagenesis and the oligonucleotide 5' CTT GCT CCT CGT TGG CGT AGA CTC CAG CAG TTG GAA TC 3' to remove NLS1 (21). pNOY481 was constructed by digesting pNOY258 with *PflMI* and *SacII* and replacing the *STS1* fragment with the *sts1ΔNLS1 PflMI-SacII* fragment from pNOY480. pNOY482 is a derivative of pNOY478 made by exchanging the *STS1 SacII* fragment for the *sts1ΔNLS1 SacII* fragment from pNOY480. pNOY483 is a derivative of pNOY258 but with E43G and I162T. pNOY484 was constructed by moving the *PflMI-SacI* fragment from pNOY483 into pNOY478 digested with *PflMI* and *SacI* to remove *STS1*.

pNOY291 (glutathione-S-transferase [*GST-STS1*]) was constructed by PCR using 5' TTT GGA TCC CAT ATG ATG GGC TTT GAA TGG GGT TTT AAA CCG AGC AGC AAA AT 3' and 5' CGC AAT TCT TAG TTA AAG GGC GAA TC 3' and pNOY258 as the template. The fragment was digested with *BamHI* and *EcoRI* and cloned into the *BamHI* and *EcoRI* sites of pGEX2T (Pharmacia). pNOY3280 was produced similarly but with pNOY480 as the template. pNOY488 was constructed by ligating the 0.1-kb triple hemagglutinin epitope tag (HA-tag) *NotI* fragment derived from the GTEP plasmid (39) into the *NotI* site of pNOY478.

To construct the *sts1(E43G) srp1-49* strain, NOY514 was mated with NOY945. The diploids were cured of the wild-type *STS1 URA3* plasmid by streaking onto SGlu with 5-fluoroorotic acid and the resulting strain was transformed with pNOY343, which contains the *sts1(E43G)* mutation. The resulting diploid was sporulated, and tetrads were dissected. Spores that were Trp⁺, Leu⁺, His⁺, and Ura⁺ were selected (NOY946).

For the experiments to study the stability of the ubiquitin-Pro-β-galactosidase

(Ub-P-βgal), strains were constructed by introducing plasmid pUb-P-e^k-βgal (3) (obtained from A. Goldberg), carrying the gene for Ub-P-βgal fused to a *GAL* promoter, into the strains to be tested.

NLS peptide binding assay. The NLS peptide binding assay was performed as described by Shulga et al. (44).

Protein pull-downs and immunoprecipitations. GST fusion proteins were produced and purified as described previously (2). Srp1p was prepared from GST-Srp1p by thrombin cleavage, and free GST was removed using glutathione agarose. For GST pull-down reactions, 1 μg of input proteins was mixed together in 100 μl of buffer M (25 mM ammonium sulfate, 50 mM Tris-HCl [pH 8.0], 5% glycerol, 1 mM dithiothreitol, and 1 mM EDTA) and allowed to incubate on ice for 30 min. Then, 200 μl of buffer M plus 0.2% bovine serum albumin and 0.5% Tween 20 was added along with a 15-μl bed volume of glutathione-agarose beads washed in buffer M, and the reactions were incubated further for 45 min at 4°C with rotation. Beads were washed four times with 400 μl of buffer M. Sodium dodecyl sulfate (SDS) sample buffer was added to the beads, which were then boiled and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were blotted onto Immobilon (Millipore, Bedford, Mass.), and the blot was probed with sheep anti-Srp1 antibodies (54). SV40 large T antigen NLS peptide and reverse large T antigen peptide were added to reactions before incubations on ice and were described previously (2).

For coimmunoprecipitation of HA-Sts1p and Srp1p, NOY944 and NOY947 cells were grown to an A_{600} of ~0.9 in 50 ml of synthetic glucose medium. Cells were harvested by centrifugation and washed with water. Extracts were prepared in buffer D (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 10% glycerol) by glass bead lysis. The protein concentrations were determined using Bradford reagent (Bio-Rad) and adjusted to be equal. Protein A-Sepharose and Srp1 antiserum were then added to extracts (containing approximately 1 mg of protein) and mixed for 4 h at 4°C. The beads were then washed with buffer A (50 mM HEPES [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100), and samples were resolved on an SDS-10% polyacrylamide gel and analyzed by Western blotting using anti-HA antibody. The use of equivalent amounts of extracts in these experiments was always confirmed by Coomassie staining of gels after electrophoresis of samples.

Microscopy. A Zeiss Axioplan 2 was used to visualize direct and indirect fluorescence. For direct fluorescence of strains containing galactose-inducible GFP constructs, strains were grown overnight in SRaff medium. Production of GFP fusion protein was induced by the addition of 3% galactose directly to the culture, and the cells were observed 3 to 4 h after induction.

Degradation of β-galactosidase test substrates. Actively growing cells (A_{600} <1.0) were harvested by centrifugation and labeled in 200 μl of labeling buffer (50 mM sodium phosphate [pH 7.0], 2% galactose) with 0.25 mCi of EXPRES³⁵S protein labeling mix (NEN) for 5 min at 30°C. Cells were then pelleted and resuspended in 400 μl of chase mix (2% yeast extract, 1% peptone, 2% glucose, 10 mM Met, 5 mM Cys, and 0.6 mg of cycloheximide per ml), and incubation was continued at 30°C. Aliquots were removed at specific time points and added to tubes containing 400 μl of buffer A with protease inhibitors (aprotinin, bestatin, pepstatin, leupeptin, and PMSF) and glass beads, and the tubes were rapidly frozen. The cells were lysed by vortexing, and radioactivity in trichloroacetic acid-precipitable material of the extracts was determined. Equal counts were used for immunoprecipitation reactions using anti-β-galactosidase antibodies (Promega) and protein A-Sepharose (Pharmacia). The protein A-Sepharose beads were washed with buffer A plus 0.1% SDS. Samples were analyzed by SDS-PAGE followed by autoradiography.

RESULTS

Intragenic complementation of *srp1-31* and *srp1-49*. In previous work, ts defects in both in vitro NLS peptide binding and in vivo NLS protein import were observed for the *srp1-31* mutation, whereas no or only very weak defects were observed for the *srp1-49* mutation (24, 44). In order to test the possibility that these two mutations affected separate functions of Srp1p, we carried out a complementation test. Strains containing each of these two mutations on the chromosome were constructed and transformed with *CEN* plasmids containing *SRP1*, *srp1-31*, or *srp1-49*. The strains were then streaked on selective medium at 38°C to check for complementation of the ts growth phenotype by a different allele of *srp1*.

Ts growth of the *srp1-31* strain could be complemented by a plasmid containing *srp1-49*, and ts growth of the *srp1-49* strain could be complemented by a plasmid carrying *srp1-31* (Fig. 1, sectors 956 and 959). Both mutant strains were complemented by the wild-type *SRP1*, confirming the recessive nature of the mutations, but not by plasmids containing the identical mutation or by the vector plasmid (Fig. 1). These results indicate

TABLE 1. Yeast strains and plasmids used

Strain or plasmid	Description ^a	Reference or source
Strains		
SFY526	<i>MATa ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 can⁺ gal4-542 gal80-538 URA3::GAL1-lacZ</i>	Clontech
NOY388	(W303-1a) <i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</i>	
NOY397	<i>MATa/α ade2-1/ade2-1 ura3-1/ura3-1 his3-11/his3-11 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/ can1-100</i>	
NOY514	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 Δsrp1::LEU2</i> pNOY166 (<i>srp1-49 TRP1</i>)	
NOY520	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 Δsrp1::LEU2</i> pNOY162 (<i>SRP1 TRP1</i>)	
JLY543 (=NOY612)	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 srp1-31</i>	24
JLY555 (=NOY613)	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 srp1-49</i>	24
NOY726	<i>MATα/α ade2-1/ade2-1 ura3-1/ura3-1 his3-11/his3-11 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/ can1-100 Δsts1::HIS3/STS1</i>	
NOY934	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100</i> pNOY485 pUb-P-e ^k -βgal	
NOY937	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 srp1-49</i> pNOY485 pUb-P-e ^k -βgal	
NOY938	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 srp1-49</i> pNOY486 pUb-P-e ^k -βgal	
NOY939	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 srp1-49</i> pNOY487 pUb-P-e ^k -βgal	
NOY940	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 srp1-31</i> pNOY485 pUb-P-e ^k -βgal	
NOY943	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 Δsts1::HIS3</i> pNOY471	
NOY944	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 Δsts1::HIS3</i> pNOY470 (<i>STS1 TRP1</i>)	
NOY945	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 Δsts1::HIS3</i> pNOY479	
NOY946	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 Δsts1::HIS3 Δsrp1::LEU2</i> pNOY343 [<i>sts1-11(E43G) URA3</i>], pNOY166 (<i>srp1-49 TRP1</i>)	
NOY947	<i>MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 Δsts1::HIS3</i> pNOY488 (<i>HA-STS1</i>)	
NOY948	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-1,112 can1-100</i> pNOY478 (<i>GAL-GFP STS1</i>)	
NOY949	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-1,112 can1-100</i> pNOY482 (<i>GAL-GFP-sts1ΔNLS1</i>)	
NOY950	<i>MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-1,112 can1-100</i> pNOY484 [<i>GAL-GFP sts1-11,12 (E43G, I162T)</i>]	
NOY992	Strain JLY543 containing plasmid pRS316-GAL	
NOY990	Strain JLY543 containing plasmid pNOY258	
NOY991	Strain JLY543 containing plasmid pNPY285	
Plasmids		
pACT2	<i>GAL4</i> AD vector (<i>LEU2</i> 2 μ m Amp ^r) and carries the <i>GAL4</i> transcriptional AD [<i>GAL4</i> (768–881)], encoding Gal4p (768–881), which is under control of the <i>ADH</i> promoter and followed by the region encoding an HA1-flu epitope tag and multicloning sites	4
pAS1	<i>GAL4</i> DBD vector (<i>TRP1 CHY2</i> 2 μ m Amp ^r) and carries the <i>GAL4</i> DBD [<i>GAL4</i> (1–147)], encoding Gal4p(1–147), which is under control of the <i>ADH</i> promoter and followed by the region encoding an HA1-flu epitope tag and multicloning sites	4
pUC19	2,686-bp plasmid cloning vector	53
pRS314	<i>E. coli</i> -yeast shuttle vector carrying <i>TRP1</i> , <i>CEN6</i> , and <i>ARSH4</i>	53
pRS316	<i>E. coli</i> -yeast shuttle vector carrying <i>URA3</i> , <i>CEN6</i> , and <i>ARSH4</i>	45
pUb-P-e ^k -βgal	p <i>GALI</i> ::Ub-Pro-βgal 2 μ m Amp ^r	45
pRS316-GAL	<i>E. coli</i> -yeast shuttle vector carrying <i>URA3</i> , <i>CEN6</i> , and <i>ARSH4</i> with the <i>GALI</i> promoter followed by multicloning sites	23
pNOY162	Derivative of pRS314 carrying <i>SRP1</i>	
pNOY163	Derivative of pRS314 carrying <i>srp1-31</i>	
pNOY166	Derivative of pRS314 carrying <i>srp1-49</i>	
pNOY258	Derivative of pRS316-GAL with <i>STS1</i> fused to the <i>GALI</i> promoter	
pNOY285	Derivative of pRS316-GAL with <i>RPN11</i> fused to the <i>GALI</i> promoter	
pNOY291	Derivative of pGEX2T carrying <i>GST-STS1</i>	
pNOY343	Derivative of pNOY471 carrying <i>sts1(E43G)</i> instead of <i>STS1</i>	
pNOY470	Derivative of pRS314 carrying <i>STS1</i>	
pNOY471	Derivative of pRS316 carrying <i>STS1</i>	
pNOY472	Derivative of pAS1 carrying <i>SRP1</i>	
pNOY473	Derivative of pACT2 carrying <i>STS1</i>	
pNOY474	Derivative of pAS1 carrying <i>STS1</i>	
pNOY475	Derivative of pACT2 carrying <i>RPN11</i>	
pNOY476	Derivative of pRS314 carrying <i>GFP-STS1</i>	
pNOY477	Derivative of pNOY470 with a <i>NotI</i> site added after the ATG of <i>STS1</i>	
pNOY478	Derivative of pNOY258 carrying <i>GFP-STS1</i> instead of <i>STS1</i>	
pNOY479	Derivative of pRS316 carrying <i>GFP-STS1</i>	
pNOY480	Derivative of pRS314 carrying <i>sts1ΔNLS1</i>	
pNOY481	Derivative of pNOY258 carrying <i>sts1ΔNLS1</i> instead of <i>STS1</i>	
pNOY482	Derivative of pNOY478 carrying <i>GFP-sts1ΔNLS1</i> instead of <i>GFP-STS1</i>	
pNOY483	Derivative of pNOY248 carrying <i>sts1-11,12(E43G, I162T)</i> instead of <i>STS1</i>	
pNOY484	Derivative of pNOY478 carrying <i>GFP-sts1-11,12(E43G, I162T)</i> instead of <i>STS1</i>	
pNOY485	Derivative of pRS314 containing <i>GALI</i> promoter	
pNOY486	pNOY485 vector containing <i>RPN11</i> gene fused to <i>GALI</i> promoter	
pNOY487	pNOY485 vector containing <i>STS1</i> gene fused to <i>GALI</i> promoter	
pNOY488	Derivative of pRS314 carrying <i>HA-STS1</i>	
pNOY3198	Derivative of pGEX2T carrying <i>GST-SRP1</i>	Pharmacia (2)
pNOY3234	Derivative of pUC19 carrying <i>STS1</i>	
pNOY3235	Derivative of pNOY3234 with <i>HIS3</i> replacing the <i>PfIMI-NsiI</i> <i>STS1</i> fragment	
pNOY3280	Derivative of pGEX2T carrying <i>GST-sts1ΔNLS1</i>	

^a AD, activation domain; DBD, DNA binding domain.

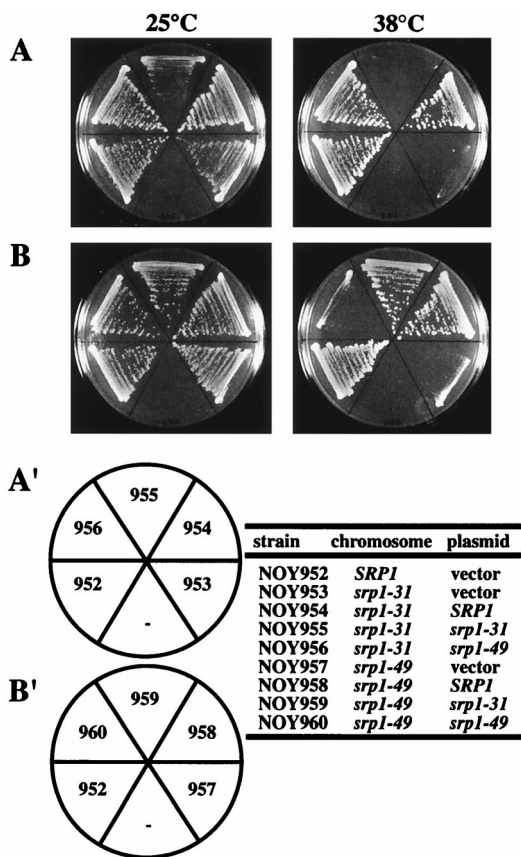


FIG. 1. Complementation of *srp1-31* and *srp1-49* mutations. Strains carrying *SRP1*, *srp1-31*, or *srp1-49* on the chromosome were transformed with plasmids containing *SRP1*, *srp1-31*, or *srp1-49*. The resulting strains, as listed in the figure, were streaked on synthetic glucose minus tryptophan plates and incubated at the indicated temperatures for 3 days. Vector represents pRS314.

that the functions affected by *srp1-31* and *srp1-49* are clearly different.

Isolation of high-dosage suppressors of *srp1-49*. In order to understand the nature of the defect in the *srp1-49* mutant, high-dosage suppressors of *srp1-49* were isolated. The *srp1-49* strains NOY514 and NOY613 were transformed with a galac-

tose-inducible yeast cDNA library (23) and plated on glucose selective medium at 25°C for 2 days. Transformants were then replica plated onto galactose selective medium to induce expression of the cDNAs and allowed to grow at 38°C for an additional 3 to 5 days. Plasmid DNA was isolated from colonies that grew on galactose but not on glucose at 38°C, and DNA sequencing was performed to identify the dosage-dependent suppressors. Two suppressors of *srp1-49* were identified in this way, *STSI* and *RPN11* (Fig. 2A). Plasmids carrying the *STSI* or *RPN11* suppressor were then transformed into strains carrying the *srp1-31* mutation. No suppression was observed (Fig. 2B). Therefore, *STSI* and *RPN11* are allele-specific high-dosage suppressors of *srp1-49*.

Interaction of *SRP1* with *STSI*. We first examined the possibility of whether the dosage-dependent suppression of *srp1-49* by *STSI* reflected a physical interaction of the two proteins encoded by these genes. An interaction of the two proteins was first indicated by the yeast two-hybrid system (Table 2). Second, we detected in crude cell extracts a complex containing Srp1p and Sts1p by coimmunoprecipitation. Extracts were prepared from a strain containing HA-tagged *STSI* (NOY947) and a control strain without the HA-tag (NOY944). Srp1p was precipitated with Srp1p antiserum, and the precipitates were subjected to SDS-PAGE followed by Western blot using an anti-HA monoclonal antibody. As shown in Fig. 3A, HA-Sts1p was coimmunoprecipitated with Srp1p (lane 5; lane 3 is a negative control using extracts without Srp1p antiserum, which gave a very weak background signal). These two experiments strongly indicate that Srp1p and Sts1p interact in vivo.

In order to examine whether these two proteins interact directly in vitro, a *GST-STSI* fusion gene was constructed. Purified Srp1p and GST-Sts1p, which were both produced in *Escherichia coli* as recombinant proteins, were mixed, and GST-Sts1p was pulled down using glutathione-agarose. Pulled-down proteins were separated by SDS-PAGE followed by Western blot analysis of Srp1p using anti-Srp1p antibodies. As shown in Fig. 3B (lane 4), a direct interaction between Srp1p and GST-Sts1p was indeed observed.

As described below, Sts1p is mostly localized to the nucleus. To determine if the interaction between Srp1p and Sts1p could be through an NLS in Sts1p, SV40 NLS peptide was added to the reaction mixture as a competitor in the GST pull-down experiments. NLS peptide was able to decrease the binding of GST-Sts1p to Srp1p (Fig. 3B, compare lane 6 to lane 4), while

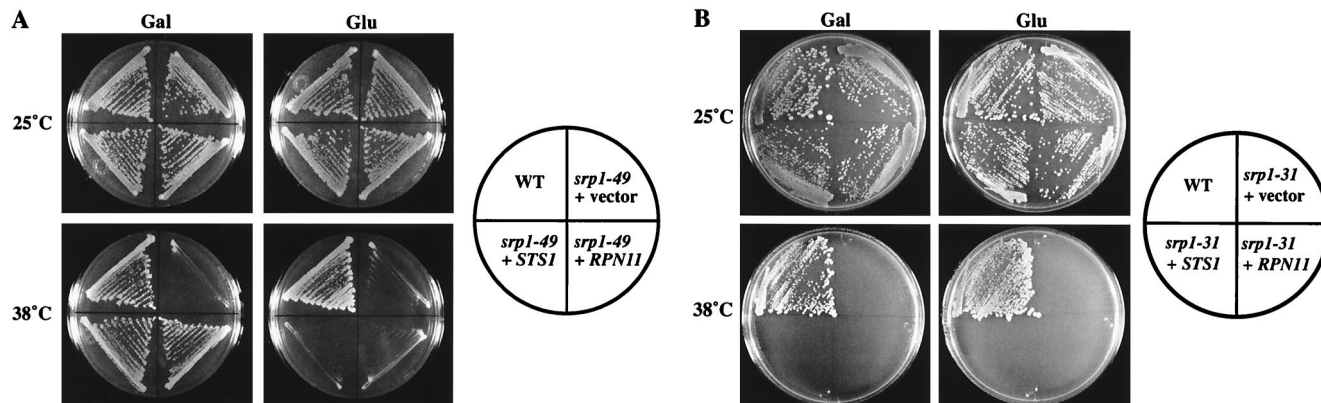


FIG. 2. Dosage-dependent suppression of *srp1-49* but not *srp1-31* by *STSI* and *RPN11*. (A) The *srp1-49* strains containing vector only (pRS316-GAL), a *GAL*-driven *STSI* plasmid (pNOY258), and a *GAL*-driven *RPN11* plasmid (pNOY285) are shown after growth on synthetic complete galactose (Gal) or glucose (Glu) plates lacking uracil as indicated for 3 days at 25 or 38°C. (B) The *srp1-31* strains containing vector only, a *GAL*-driven *STSI* plasmid, and a *GAL*-driven *RPN11* plasmid were examined in the same way. WT, wild type.

TABLE 2. Summary of two-hybrid assays^a

Gene or mutation		β-Galactosidase activity
AD	DBD	
<i>STS1</i>	<i>SRP1</i>	+++
<i>sts1(E43G I162T)</i>	<i>SRP1</i>	—
<i>RPN11</i>	<i>SRP1</i>	—
<i>RPN11</i>	<i>STS1</i>	++
<i>RPN11</i>	None (pAS1)	—
<i>STS1</i>	None (pAS1)	—
None (pACT2)	<i>STS1</i>	—
None (pACT2)	None (pAS1)	—
p53	T antigen	+++

^a Strain SFY526 was transformed with plasmids pACT2 (AD) and pAS1 (DBD) carrying the genes indicated. After growth on synthetic complete glucose medium lacking leucine and tryptophan for 4 to 5 days, β-galactosidase filter assays were performed as described by Steffan et al. (48). Color development was rated on a scale of + (blue color development after 1 h at 37°C) to +++ (blue color development in less than 20 min at 37°C). AD, activation domain; DBD, DNA binding domain.

a peptide containing the same amino acids in reverse sequence was not (lane 7). Thus, the direct interaction of Srp1p with GST-Sts1p in vitro appears to take place through an interaction similar to that of Srp1p with classical NLS peptides.

Examination of the Sts1p sequence revealed two basic regions in the amino-terminal end which could potentially serve an NLS function, NLS1 (₃₅KQKRR₃₉) and NLS2 (₅₈KYGGVSKRR₆₆) (Fig. 4A). Both regions were individually deleted using oligonucleotide-directed mutagenesis, and each deletion was tested for complementation of the *sts1* genomic deletion. While the mutant carrying the deletion of NLS2 could still complement an *sts1* deletion strain, one carrying the deletion of NLS1 did not (data not shown). The production of Sts1ΔNLS1 protein in yeast cells was confirmed by Western blot analysis using anti-HA antibody directed against an N-terminal HA epitope engineered into the deletion protein construct. Therefore, the failure of complementation was not due to lack of protein production. To further characterize NLS1, a fusion protein containing the NLS1 deletion, GST-Sts1ΔNLS1p, was isolated from *E. coli* as a recombinant protein and tested for its interaction with Srp1p in vitro. The mutant fusion protein was unable to interact with Srp1p (Fig. 3B, lane 5). These experimental results strongly suggest that Sts1p carries an NLS that resembles SV40 NLS and that the in vitro interaction of GST-Sts1p with Srp1p is through this NLS-like sequence of Sts1p. Thus, Sts1p may be a nuclear protein and its nuclear import from the cytoplasm may involve its binding to Srp1p. However, based on the results obtained by fractionation of cell extracts and by indirect immunofluorescence microscopy, it was previously reported that Sts1p was localized to the cytoplasm (1, 22). Therefore, the question of localization of Sts1p was reexamined.

Analysis of nuclear localization of Sts1p in the wild-type and *srp1* mutant strains. We constructed a strain containing a gene encoding a tagged GFP-Sts1 fusion protein under control of the galactose promoter (NOY948) and analyzed localization of the fusion protein after induction by galactose. As shown in Fig. 4B, GFP-Sts1 showed a clear nuclear localization in vivo by its fluorescence. This nuclear localization was also confirmed by indirect immunofluorescence using anti-GFP antibodies with a strain containing *GFP-STs1* in an *sts1* deletion background (data not shown). Effects of the NLS1 deletion on protein localization were then tested in vivo using a galactose-inducible *GFP-sts1ΔNLS1* fusion gene. Compared to the nu-

clear localization of GFP-Sts1p, GFP-Sts1ΔNLS1p showed a more cytoplasmic distribution (Fig. 4B). The fact that GFP-Sts1ΔNLS1p was not excluded from the nucleus may suggest that there is another region in Sts1p with some NLS function. Taken together, these data strongly suggest that Sts1p is mainly localized to the nucleus and interacts with Srp1p via an NLS sequence resembling the SV40 NLS sequence to gain entry into the nucleus.

To prove that Sts1p indeed uses Srp1p for nuclear import, a plasmid containing galactose-inducible *GFP-STs1* was transformed into an *srp1-31* ts strain, which shows defects in NLS-dependent protein import at nonpermissive temperatures, as mentioned above. Localization of GFP-Sts1p was then followed after a temperature shift by direct fluorescence signal. GFP-Sts1p was found in both the nucleus and the cytoplasm after 4 h at 37°C (data not shown) (48a). Therefore, Sts1p uses Srp1p, at least in part, to gain entry into the nucleus. In contrast to the *srp1-31* mutation, no defect in the localization of GFP-Sts1p in an *srp1-49* strain was observed during up to 8 h of incubation at 37°C (data not shown) (48a), when many other drastic mutational defects such as an alteration in the nucleolar morphology had already taken place. Thus, suppression of *srp1-49* by a high dosage of *STS1* is not due to compensation of a (hypothetical) decrease in the nuclear transport of Sts1p in *srp1-49* mutants.

Interaction between Srp1p and Sts1p required for suppression of *srp1-49*. As described above, there is an interaction of Sts1p with Srp1p through the NLS1 sequence of Sts1p. We have found that this interaction may be prerequisite to suppression of *srp1-49* by *STS1* but is not sufficient for suppression. An additional interaction not involved in the protein import function must take place for suppression. The mutant gene *sts1ΔNLS1* was put under control of the galactose promoter and transformed into the *srp1-49* strain. Transformants were then tested for their ability to grow at the nonpermissive temperature in the presence of galactose. A high dosage of *sts1ΔNLS1* was unable to suppress the growth defect of *srp1-49* at 38°C on galactose (Fig. 5, strain 965 compared to 963). The results show that direct interaction with Srp1p through the

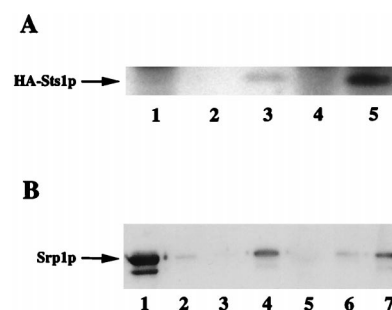


FIG. 3. Interaction of Srp1p and Sts1p as demonstrated by coimmunoprecipitation from cell extracts (A) and by the use of GST-Sts1p fusion protein (B). (A) Extracts were prepared from the strain carrying the HA-tagged *STS1* gene (lanes 3 and 5) or the control strain without the HA-tag (lanes 2 and 4). The extracts were treated with anti-Srp1p antibodies (lanes 4 and 5) and protein A-Sepharose, and precipitated proteins were subjected to SDS-PAGE followed by Western analysis using a monoclonal anti-HA antibody to detect HA-Sts1p. Lane 1 is an antibody control without extracts. Lanes 2 and 3 are negative controls without antibody. (B) Srp1p was incubated with GST (lane 3), GST-Sts1p (lane 4), GST-Sts1ΔNLS1p (lane 5), GST-Sts1p plus 200 μM SV40 NLS peptide (lane 6), or GST-Sts1p plus 200 μM reverse NLS peptide (lane 7). Lane 2 did not receive any GST fusion protein. GST or GST fusion proteins were pulled down using glutathione-agarose beads and then subjected to SDS-PAGE followed by Western blot using anti-Srp1p antibodies to detect Srp1p. Lane 1 is 10% of the input Srp1p preparation.

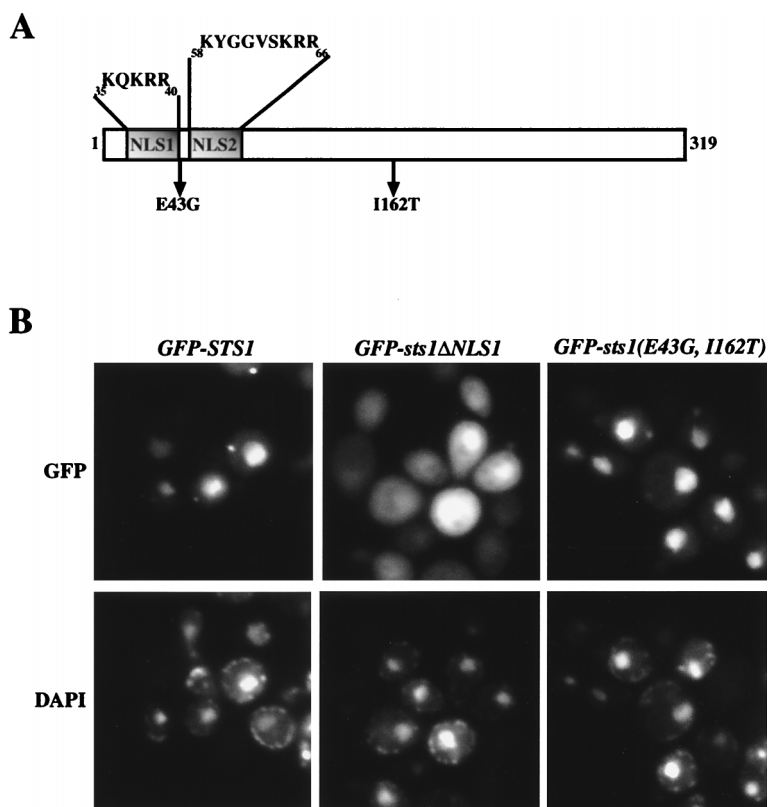


FIG. 4. Localization of Sts1p assayed by the use of GFP-Sts1 fusion protein. (A) Schematic representation of Sts1 protein, showing locations of NLS1 and NLS2 as well as two point mutations, E43G and I162T. (B) Strains carrying *GFP-STSI*, *GFP-sts1ΔNLS1*, and *GFP-sts1-11,12(E43G, I162T)* were grown in SRaff at 30°C, and synthesis of the fusion proteins was induced by galactose. Localization of the protein was analyzed by direct fluorescence microscopy. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI).

NLS1 sequence may be required for a high dosage of *STSI* to suppress *srp1-49*. Since Sts1ΔNLS1 protein is clearly present in the nucleus in addition to the cytoplasm, as mentioned above (see Fig. 4B), it appears that the direct interaction required for the suppression is probably not simply to achieve nuclear transport of Sts1p. This inference was further supported by the following experiments.

We isolated *sts1* mutants that did not show an interaction with *SRP1* in the two-hybrid assay. These mutants were then screened for loss of the ability to suppress *srp1-49* under high-dosage expression. In this way, we found that an *sts1* mutation containing a two-amino-acid substitution, *sts1-11,12(E43G I162T)*, abolished both the ability to interact with *SRP1* in the two-hybrid system (Table 2) and high-dosage suppression of *srp1-49* (Fig. 5, strain 961; for the locations of the mutations, see Fig. 4A). We then examined whether this double mutation inhibited the nuclear localization of Sts1p. Like the wild-type GFP-Sts1p, the mutant GFP-Sts1(E43G, I162T)p was found to be localized to the nucleus (Fig. 4B). Thus, Sts1p localization to the nucleus is not sufficient for suppression of *srp1-49*. An additional interaction between Sts1p and Srp1p, which is detected by the two-hybrid system and is abolished by the E43G I162T double mutation, appears to be required for suppression of *srp1-49*. This additional interaction may or may not be a direct interaction and most likely takes place within the nucleus.

Combination of the *sts1-11* and *srp1-49* mutations. The above *sts1* double mutation, *sts1-11,12*, showed good complementation of growth defects caused by an *sts1* deletion; no

difference was observed in growth rate from the wild-type control (data not shown). Thus, the interaction disrupted by the double mutation and required for suppression of *srp1-49* does not appear to be essential for normal growth in the context of the wild-type *SRP1*.

We also separated the two mutations, E43G and I162T, contained in the *sts1* double mutant *sts1-11,12* and examined them individually for the ability to interact with *SRP1* in the two-hybrid system and for the ability to suppress *srp1-49* by overexpression. Neither one of the mutations showed an interaction with *SRP1* in the two-hybrid system, and each partially abolished the suppression of *srp1-49* (data not shown). However, in the course of these experiments, we found that although the *sts1-11(E43G)* mutation itself did not show any growth defect phenotype, a strain which contained both *srp1-49* and *sts1-11* showed more severe temperature sensitivity than with *srp1-49* only (Fig. 6). This result supports the above-described conclusion that the interaction of Sts1p and Srp1p is functionally important; a point mutation that abolishes this interaction in the two-hybrid system affects cell growth at high temperatures in the context of the *srp1-49* mutation.

Interaction of *STSI* and *RPN11*. *RPN11*, the other high-dosage suppressor of *srp1-49*, did not show an interaction with *SRP1* when tested by the two-hybrid system. However, it did show a positive interaction with *STSI* in the two-hybrid system (Table 2). This result suggests that *STSI* and *RPN11* interact either directly or indirectly through other components and that this interaction might be related to *RPN11* suppression of *srp1-49*.

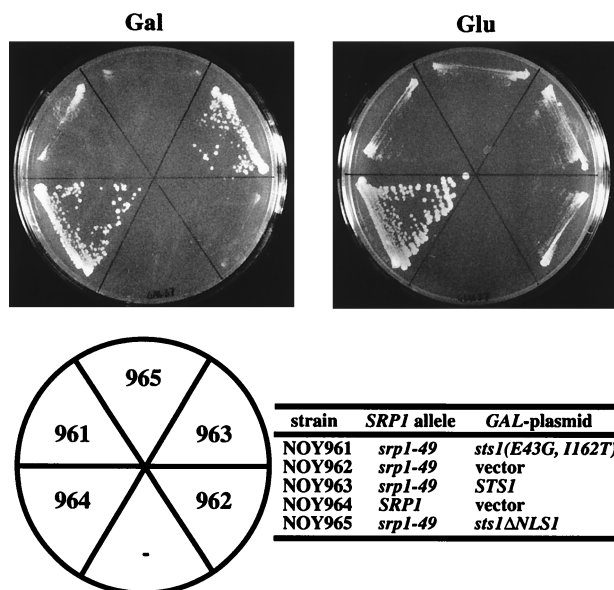


FIG. 5. Suppression of *srp1-49* by a high dosage of *STS1* and *sts1* mutants. Plasmids containing *STS1*, *sts1-11,12(E43G, I162T)*, and *sts1ΔNLS1* under control of the *GAL* promoter were transformed into the *srp1-49* strain. Resulting strains and a control *SRP1* strain were streaked onto synthetic complete galactose (Gal) or glucose (Glu) plates lacking uracil and incubated for 3 days at 38°C. Vector pRS314 was used as a plasmid control.

Degradation of Ub-P-βgal in *srp1-49* and *srp1-31*. The discovery that one of the high-dosage suppressors of *srp1-49* was *RPN11*, a subunit of the 19S proteasome regulatory particle, led us to examine ubiquitin-dependent protein degradation in *srp1-49* cells. For this purpose, we used Ub-P-βgal, a model substrate of the ubiquitin-proteasome pathway studied by previous investigators (3, 13). A plasmid carrying the gene for Ub-P-βgal fused to a *GAL* promoter was introduced into an *srp1-49* strain, an *srp1-31* strain, and a control wild-type *SRP1* strain, as well as *srp1-49* strains carrying an *RPN11* or *STS1* dosage suppressor plasmid. Degradation of Ub-P-βgal was then determined by pulse-chase and immunoprecipitation analysis (3, 13).

As shown in Fig. 7A and B, Ub-P-βgal was substantially more stable in *srp1-49* than in the control strain, but no such stabilization was observed for *srp1-31* (Fig. 7A). Significantly, both *srp1-49* strains carrying the suppressor *RPN11* and the suppressor *STS1* showed a faster degradation rate approaching that shown by the control strains (Fig. 7B), although the amounts of radioactive Ub-P-βgal synthesized during the pulse-labeling period were reduced in these suppressed strains. We conclude that the *srp1-49* mutation and its suppression by a high dosage of *RPN11* or *STS1* are correlated with a decrease in the rate of Ub-P-βgal degradation and its restoration, respectively. The results also demonstrate that the *srp1-49* and *srp1-31* mutations are clearly different in their effects on Ub-P-βgal degradation, confirming that the two mutations affect different functions of Srp1p. We note that the experiments shown in Fig. 7 were done at 30°C, which is a permissive temperature for these mutants. Similar results were also obtained when Ub-P-βgal degradation was assayed after a temperature shift from 30 to 38°C (data not shown). Thus, defects in the ability to degrade Ub-P-βgal observed in *srp1-49* do not affect growth at 30°C but appear to cause growth defects at higher temperatures.

Degradation of Ub-P-βgal involves the initial ubiquitination

steps of the ubiquitin fusion degradation (UFD) pathway which are different from other ubiquitination pathways, e.g., the N-end rule pathway (13, 14, 16). Therefore, we tested degradation of another model substrate, Leu-βgal, which is degraded through the N-end rule pathway (3). We observed that Leu-βgal degradation was also significantly slower in *srp1-49* than in the wild type (data not shown). Therefore, defects caused by the *srp1-49* mutation appear not to be limited to the UFD system, but to reside in a more general step(s) in protein degradation shared by these two model substrates.

DISCUSSION

Genetic evidence for separable functions of *SRP1*. The role of importin α (karyopherin α) and its yeast homolog Srp1p in NLS-dependent nuclear protein import has been well established. Genetic experiments described in this article demonstrate that Srp1p carries out at least one additional important function, unrelated to its NLS receptor function. The first evidence for this conclusion is the intragenic complementation of the *srp1-31* ts mutation and the *srp1-49* ts mutation. It should be noted that intragenic complementation could be observed for a protein which functions as a homodimer consisting of two identical subunits; one mutational alteration at a site disrupting the functional conformation of the dimer could conceivably be suppressed by another “compensatory” amino acid alteration at a different site of the same protein molecule. However, the NLS receptor function of Srp1p is carried out as a monomer (or as a heterodimer complexed with Kap95). It was demonstrated that, in the absence of NLS peptides, purified *Xenopus* importin α forms aggregates at higher concentrations, but that importin α with a bound SV40-type NLS peptide remains a monomer (32). Crystal structure analysis of a 50-kDa fragment of Srp1p with and without an NLS peptide also supports this conclusion (5). Thus, intragenic complementation of *srp1-31* and *srp1-49* cannot be explained by the model of formation of a functional homodimer with NLS receptor function.

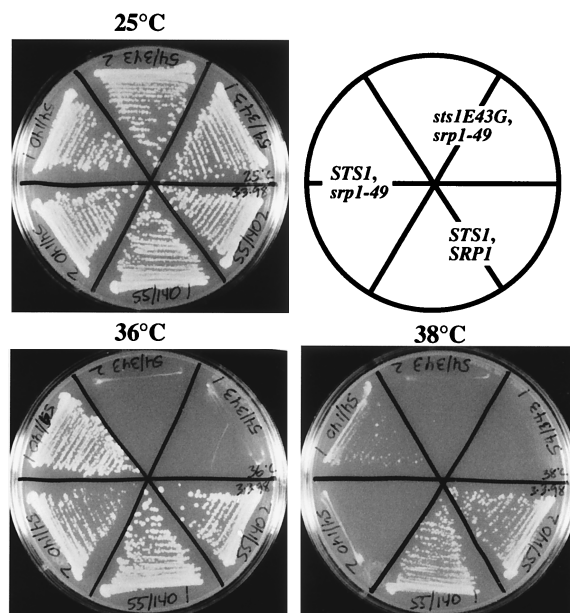


FIG. 6. Temperature sensitivity of the *sts1-11(E43G) srp1-49* double mutant. Strains containing combinations of wild-type *STS1* or *sts1(E43G)* and wild-type *SRP1* or *srp1-49* were streaked onto synthetic complete glucose plates lacking tryptophan and uracil and grown for 3 days at 25, 36, or 38°C.

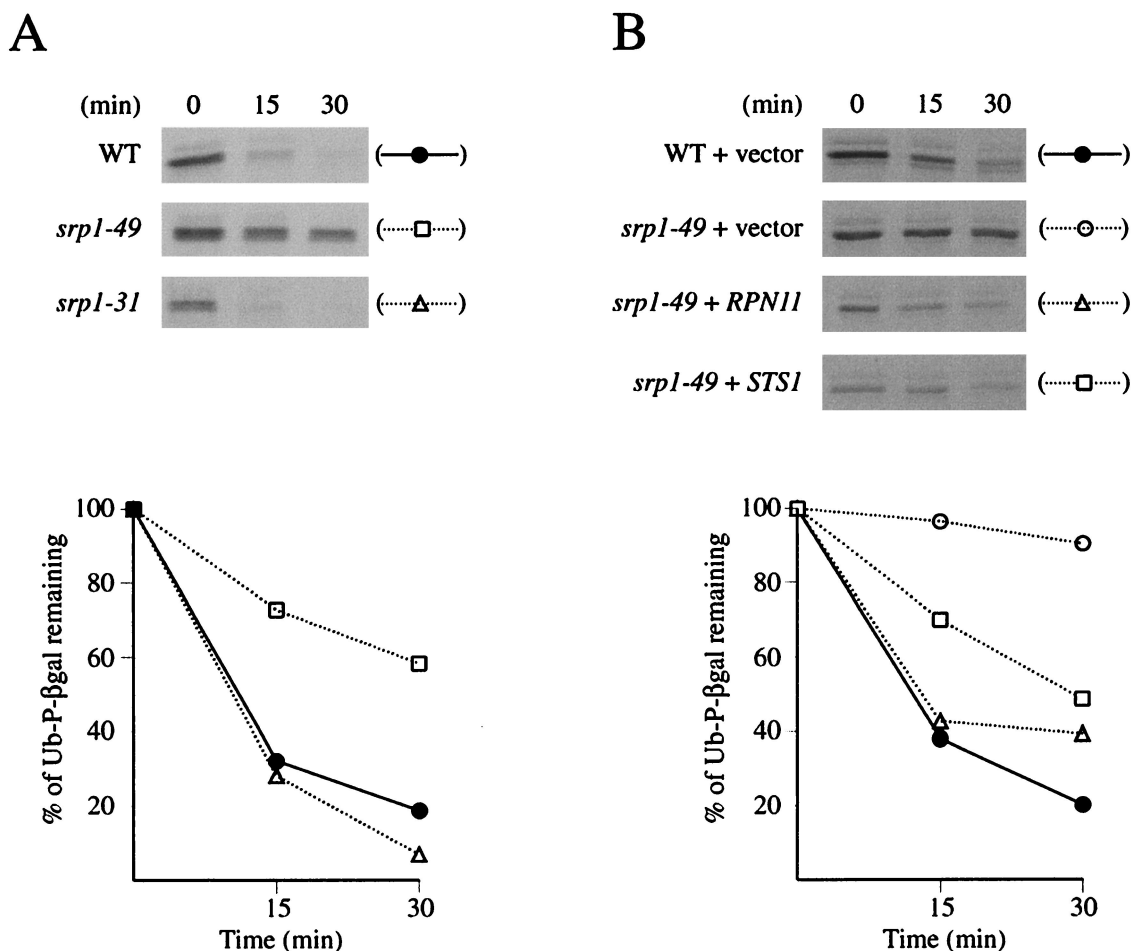


FIG. 7. Degradation of Ub-P-βgal. Cells were grown overnight in SGal minimal medium at 30°C and labeled with ³⁵S labeling mix for 5 min. The cells were resuspended in cycloheximide-containing chase mix, and aliquots were removed at the indicated times. The amount of labeled Ub-P-βgal in the extracts was analyzed by immunoprecipitation with anti-β-galactosidase antibodies followed by SDS-PAGE and quantification by phosphorimager analysis. (A) Degradation of Ub-P-βgal in wild-type (WT), *srp1-49*, and *srp1-31* mutants (strains NOY934, NOY937, and NOY940, respectively). (B) Degradation of Ub-P-βgal in *srp1-49* mutant in the presence of the suppressors *RPN11* (NOY938) and *STS1* (NOY939). Degradation of Ub-P-βgal was also assayed simultaneously in *srp1-49* in the presence of vector (NOY937) and in wild-type cells (NOY934) as a control. The quantification of Ub-P-βgal is shown in the graphs below the autoradiograms.

Instead, this complementation demonstrates that the function affected by the *srp1-49* mutation is separate from the NLS receptor function affected by the *srp1-31* mutation. This intragenic complementation also shows that the mutational defect caused by *srp1-31* is specific rather than a nonspecific heat inactivation of the protein; the mutant protein Srp1-31p is defective in the NLS protein transport function at nonpermissive temperatures but is able to carry out another function that is affected by the *srp1-49* mutation at the same nonpermissive temperatures. We also note that both Srp1-31 and Srp1-49 mutant proteins are stable at 38°C for at least 9 h (unpublished experiments; 48a). This observation excludes the (unlikely) possibility that the observed intragenic complementation is a result of stabilization of a mutant protein(s) by some kind of interaction between the two proteins.

The second evidence is our finding that a high dosage of either *STS1* or *RPN11* suppresses *srp1-49* but not *srp1-31*. This suppression in *srp1-49* cells is correlated with suppression of decreased ubiquitin-dependent protein degradation, as assayed by the use of a model substrate, Ub-P-βgal. No such stabilization of Ub-P-βgal degradation was observed for *srp1-31*. It would be difficult to explain defects in Ub-P-βgal degra-

dation observed in *srp1-49* as a consequence of a defect in the NLS receptor-nuclear protein import function, as further discussed below.

The amino acid residue serine 116 affected by *srp1-31* (S116F) (55) is an invariant residue among all published Srp1p homologs from a variety of organisms. The S116F mutation at this residue likely disrupts the structural integrity of the NLS binding site, as discussed by Conti and coworkers (5), based on the crystal structure of an Srp1p-NLS peptide complex, and this is indeed consistent with the experimental observations (44). The amino acid residue glutamic acid 145 altered by the *srp1-49* mutation (E145K) (55) is located on the opposite, convex face of the Srp1p structure, away from the concave face where the known NLS binding sites are located. This is also consistent with our observations that mutation *srp1-49* shows no defects in NLS binding or in the import of any protein substrate tested so far at nonpermissive temperatures (44; our unpublished experiments). As mentioned above, in vivo complementation of *srp1-31* with *srp1-49* implies the absence of any significant effect of this mutational alteration on the NLS receptor function of Srp1p.

One possible model to explain our observations is that the

srp1-49 mutation is specifically defective in the transport of some proteasome components but is not defective in the transport of many other proteins, whereas the *srp1-31* mutation is opposite, i.e., is defective in the transport of many NLS-containing proteins but not in the transport of the proteasome components. According to this model, Srp1p functions in protein transport using two entirely different mechanisms: one is the well-established mechanisms, i.e., by binding the classical SV40-nucleoplasmin-type NLS peptides at the concave face of the Srp1p protein structure; and the second is an ad hoc and undefined mechanism without using the classical NLS-Srp1p interaction. This second mechanism is not affected by the *srp1-31* mutation but is somehow affected by the *srp1-49* mutation; that is, this mechanism assumes binding of cargo proteins perhaps to the convex face of the protein rather than to the well-established NLS-interacting concave face. We originally considered the possibility that high-dosage suppression of *srp1-49* by *STS1* or *RPN11* might reflect a possible failure of transport of Sts1p or Rpn11p or related proteins to the nucleus catalyzed by Srp1p. However, no defect in Sts1p transport to the nucleus was observed in *srp1-49* mutant cells, as described in this paper, and no interaction of Srp1p with Rpn11p was detected in the two-hybrid system or in vitro. Thus, while we cannot exclude the above-mentioned model completely, we think that it is probably unlikely.

Ubiquitin-dependent protein degradation is affected by the *srp1-49* mutation. An important clue to the nature of the Srp1p function affected by the *srp1-49* mutation came from the discovery of *RPN11* as a high-dosage suppressor of the *srp1-49* mutation. *RPN11* is a component of the 19S regulatory particle of the proteasome and shows 65% identity to its human counterpart Poh1 (8, 47). Rpn11p contains a highly conserved sequence with similarity to the active-site Cys box seen in many deubiquitinating enzymes, and its possible function within the proteasome was speculated on in this connection (8). However, deubiquitination activity of isolated Rpn11p itself has not been demonstrated, and the significance of this similarity is not clear.

The *Schizosaccharomyces pombe* homolog of *RPN11*, *pad1*⁺, was originally isolated as a gene which confers resistance to staurosporine and other drugs when expressed in high dosage (43). The increased resistance to the drugs was explained by suggesting that overexpression of *RPN11* caused stabilization of unstable transcription factors, such as Pap1, required for the expression of genes responsible for the drug resistance (31, 47). However, no experimental tests have been carried out to examine the validity of this explanation. Using the model substrate Ub-P- β gal for the ubiquitin-proteasome system, we have found that this model substrate is significantly more stable in the *srp1-49* cells than in wild-type cells or the *srp1-31* mutant cells. Overexpression of *RPN11* in *srp1-49* cells decreased the stability of this model substrate close to the wild-type level, although no such effects, i.e., further decrease in the stability, were observed in the control wild-type cells (Fig. 7 and unpublished experiments). It appears that the *srp1-49* mutation causes some defects, directly or indirectly, in the activity of the ubiquitin-proteasome system and that overexpression of *RPN11* somehow restores its activity. A similar mutational defect, increased stability of protein, was also observed for another model substrate, Leu- β gal. It is known that initial steps of degradation of Ub-P- β gal are through the UFD pathway, whereas those of Leu- β gal degradation take place through the N-end rule pathway (13, 14, 16). These two pathways do not share ubiquitination enzymes E2 or E3, but share later steps involving proteasome activity. Thus, the defect caused by the *srp1-49* mutation might be in a step(s) involving

the proteasome. However, mutations in proteasome subunits were often reported to cause accumulation of polyubiquitinated protein substrates. No increased accumulation of polyubiquitinated forms of Ub-Pro- β gal or Leu- β gal in the *srp1-49* mutant was observed in our experiments. Therefore, it is possible that a ubiquitination-polyubiquitination step(s) of these model substrates is somehow affected by the mutation regardless of the kinds of E2-E3 ubiquitination enzyme systems.

Possible significance of an Srp1p-Sts1p complex in ubiquitin-dependent protein degradation. The function of Srp1p affected by *srp1-49*, i.e., the function related to protein degradation, as discussed above, may be carried out by a complex containing Srp1p and Sts1p, and a direct interaction of these two proteins may be important for this function. First, Srp1p and Sts1p interact in vivo, as revealed in the two-hybrid system, and interact directly in vitro. Second, a protein complex containing these two proteins exists in cell extracts, as shown by coimmunoprecipitation experiments. Third, the ts phenotype of *srp1-49* can be suppressed by a high dosage of *STS1*, and this suppression is abolished by an *sts1* mutation that disrupts the Srp1p-Sts1p interaction detected by the two-hybrid system. Fourth, the same *sts1* mutation, which does not cause growth defects by itself, causes a synthetic growth defect when combined with *srp1-49*, i.e., a more severe ts phenotype. In addition, *RPN11* and *STS1* interact in the two-hybrid system. Therefore, the main function of *STS1* may also be related to protein degradation. In fact, we observed that overexpression of *STS1* restores the decay rate of Ub-P- β gal in *srp1-49* cells close to the wild-type level, and this may be the basis of suppression of the *srp1-49* mutation by a high dosage of *STS1*. Several previous observations can be explained on the basis of the proposed new function (related to protein degradation) for both *STS1* and *SRP1*, as discussed below.

First, *SRP1* was originally identified as a suppressor of certain ts mutations in the A190 subunit of Pol I (54). Several point mutations of *SRP1*, such as *SRP1-1* and *SRP1-2*, were isolated as suppressors. These mutations, when separated from the *rpa190* mutations, did not show any phenotype by themselves, but the suppressor activity was dominant over the wild-type *SRP1* (54, 55). The *rpa190* ts mutations, suppressed by these *SRP1* suppressors, such as *rpa190-1* and *rpa190-5*, caused a large decrease in the amount of A190 at nonpermissive temperatures, apparently due to an instability of mutant A190 protein (26). One possibility that we considered based on the NLS receptor function of Srp1p was an increase in the efficiency of binding of A190 (or a complex containing A190, e.g., Pol I) to Srp1p, leading to an increased supply of A190 (or a Pol I precursor or Pol I) to the nucleus, counteracting the instability of the mutant protein. We tested this possibility by constructing a *CEN* plasmid carrying the *srp1* gene (*srp1-2,31*) containing both the *SRP1-2* suppressor mutation and the *srp1-31* mutation. We found that the Srp1p carrying these two mutations was more severely ts in NLS binding in vitro than that carrying the *srp1-31* mutation only, and yet the plasmid introduced into the *rpa190-1* mutant suppressed the ts phenotype (our unpublished experiments). Thus, the suppressor activity was not abolished by inactivation of the NLS binding-NLS protein transport function. Therefore, we suggest that the suppressor mutations such as *SRP1-2* affect another function of Srp1p which is related to protein degradation and counteract the degradation of the unstable mutant Pol I subunit protein.

A similar interpretation can be applied to the reported suppression of the *ma15-2* mutation by a mutation in *STS1* (1). Rna15p is an essential component of cleavage factor I that, together with Rna14p, is involved in cleavage and polyadenylation of mRNAs (27). For *ma15-2*, the observed defect in

3'-end mRNA processing was due to unstable Rna15-2 protein at high temperature. A mutation in *STSI* partially corrects this protein instability and so partially rescues 3'-end mRNA processing (1). Since Srp1p and Sts1p appear to carry out a function as a complex, as shown in this paper, we suggest that this suppressor mutation in *STSI* may involve stabilization of the unstable nuclear protein Rna15-2p. Increased amounts of the protein rescue the defect of *ma15-2* at high temperature.

Liang et al. (22) originally discovered that a high dosage of *STSI* suppresses the ts phenotype of *sec23-11* but not the ts phenotype of *sec23-1*. These authors reported that a shift to the nonpermissive temperature leads to accumulation of the ER form of a secreted protein, invertase. Overexpression of *STSI* alleviated this accumulation of invertase, which led them to suggest that *STSI* corrects the secretion defects of this mutant. However, they have not followed the fate of the ER form of invertase, and so it is not clear if overexpression of *STSI* leads to transport of the ER form of invertase to the cell surface. The ubiquitin-proteasome system is also involved in the degradation of mutant secretory proteins that are accumulated in the ER (reviewed in reference 34). It is therefore likely that a high dosage of *STSI* stimulates the degradation of proteins accumulated in the ER, in the same way as it stimulates degradation of Ub-P- β gal in *srp1-49* cells, leading to suppression of the ts phenotype of *sec23-11*. In fact, we found that like *STSI*, *RPN11* can also suppress the ts defect of *sec23-11* (our unpublished experiments) (48a), which supports our interpretation of the results published by Liang et al. (22).

Role of *SRP1* and *STSI* in mitosis. Although the function of Srp1p as the NLS receptor in protein import has been well established, we have argued that Srp1p may also carry out an additional essential function(s) concerned with the regulation of protein degradation. It has been well established that regulated protein degradation through the ubiquitin-proteasome system is essential for progression of the cell cycle (reviewed in references 33 and 50). Mutations in proteasome components or in the anaphase-promoting complex (a ubiquitin protein ligase E3) are known to cause mitotic arrest. In fact, some mutations in *RPN11*, which encodes a proteasome regulatory subunit and is a high-dosage suppressor of *srp1-49*, have recently been shown to cause a mitotic arrest (38; see reference 31 for mutations in the *S. pombe* homolog *pad1*⁺). The basis of mitotic arrest observed in these instances were shown or interpreted to be due to mutational defects in the regulated proteolysis of certain key proteins, such as mitotic cyclins and Pds1p (Cut2), which were shown to be essential for progression of mitosis (33, 50, 52). Thus, the mitotic arrest phenotype evident in the *srp1-49* mutant at nonpermissive temperatures (55) or after depletion of Srp1p (20) can now be explained on the basis of defects in protein degradation, as observed in the present work using model substrates for the ubiquitin-proteasome system. However, we have not determined the identity of the natural protein substrates whose stability is affected by the *srp1-49* mutation. In this connection, we note that the *srp1-31* mutation was reported to cause a clean mitotic arrest at nonpermissive temperatures and degradation of Clb2p is defective under these conditions (24). This observation was interpreted on the basis of defects in nuclear import of some cell cycle regulators (24). In our hands, however, mitotic arrests were clearly evident for *srp1-49* but not for *srp1-31* (our unpublished experiments). The reason for this discrepancy is not clear. As already discussed, we favor our interpretation that the mitotic arrest observed for *srp1-49* is a consequence of defects in the function related to protein degradation and not a consequence of defects in nuclear import of hypothetical cell cycle regulatory proteins.

STSI, which was also called *DBF8*, was independently identified as a gene essential for nuclear division and segregation. Strains carrying *dbf8* mutations show a mitotic arrest phenotype at nonpermissive temperatures similar to that seen for *srp1-49* (12). This phenotype is consistent with our conclusion that Sts1p, like Rpn11p, plays a role in the regulation of protein degradation through the ubiquitin-proteasome system.

Another observation relevant to our conclusion on the new function of Srp1p separate from its NLS receptor function is a recent work by Matsusaka et al. (25) on the identification of *S. pombe* gene *cut15*⁺ as a homolog of *SRP1*. These investigators found that a ts mutation in *cut15*⁺ shows a mitotic arrest at nonpermissive temperatures, presumably due to defects in chromosome condensation. Surprisingly, the mutation caused a defect in NLS binding activity of the protein in vitro, and yet no defects in protein import were observed in vivo. They concluded that *cut15*⁺, a homolog of *SRP1* is essential for mitotic chromosome condensation, but its role in nuclear protein import is dispensable (25). Their results support our conclusion that Srp1p has a function which is separable from the NLS receptor function. We note that chromosome condensation appears to be defective in *srp1-49* mutants at nonpermissive temperatures (55) and in Srp1p-depleted cells (20, 55). It will be interesting to examine whether there is any defect in activities of the ubiquitin-proteasome system in the *cut15* mutant analyzed.

We also note that a ts mutation (*cse1-2*) in *CSE1*, which encodes the yeast homolog of mammalian CAS, shows a mitotic arrest phenotype at nonpermissive temperatures which is very similar to *srp1-49* (55), including a significant percentage of arrested cells with spindle orientation defects (41). Both yeast Cse1p and mammalian CAS have been demonstrated to function in recycling Srp1p back to the cytoplasm after completion of nuclear protein import (11, 18, 46). Consequently, the phenotypes of *cse1* mutations and various genetic interactions between *CSE1* and *SRP1* have been explained solely on the basis of the functions of these two genes in nuclear protein transport. Schroeder et al. (41) reported suppression of *srp1-49* but not *srp1-31* by a high dosage of *CSE1*. Since *srp1-49* does not show any defect in nuclear protein import, this observed suppression is not easy to explain on the basis of the Srp1p-recycling function of Cse1p. It is known that the CAS protein is associated with microtubules, including mitotic spindles (40), and its depletion by the use of antisense RNA leads to mitotic arrest in mammalian cells (29). The *S. pombe* Srp1p homolog, *cut15* protein, was also shown to be clearly associated with the mitotic spindle, in addition to other intranuclear sites, during late mitosis (25). It is known that degradation of a microtubule-associated protein, Ase1p, is mediated by the anaphase-promoting complex-proteasome system in *S. cerevisiae* and that inappropriate expression of nondegradable Ase1p during G₁ caused mitotic arrest (15). Thus, it is possible that Cse1p, like Srp1p, has functions important for mitosis that are independent of its role in nuclear transport and that suppression of defects in *srp1-49* cells by a high dosage of *CSE1* is related to the proposed function of regulation of protein degradation.

The proposed role of Srp1p in mitosis is also consistent with earlier observations that pendulin, one of the *Drosophila* homologs of Srp1p, rapidly translocates from the cytoplasm to the nucleus at the transition between G₂ and M phase and is found associated with condensed metaphase chromosomes (19, 49). Such observations suggest that an important function of pendulin is related to mitosis. Since there are several distinct Srp1p homologs in higher eukaryotes (17 and references therein), there might be a functional differentiation among these homologs. In the yeast *S. cerevisiae*, only a single protein spe-

cies, Srp1p, exists as an importin α homolog. Thus, one can speculate that the yeast Srp1p participates in a variety of nuclear functions through regulation of degradation of various regulatory proteins and that these regulatory functions might be divided among different Srp1p homologs in higher eukaryotes. One can also speculate that during an earlier time in evolution, Srp1p was a nuclear protein interacting with several other nuclear proteins, such as Sts1p, and carrying out a specific function(s) such as those discussed above, and that one of the importin β family members, that corresponding to the present-day Kap95p, was the import machinery for Srp1p. Therefore, the protein import function of Srp1p as a piggyback receptor might be a result of more recent evolution and be limited to only certain kinds of nuclear proteins. This speculative notion is supported by the fact that several importin β family members are engaged in nuclear import of a variety of proteins without using Srp1p (for review, see references 9, 28, and 30).

In conclusion, the work described in this paper strongly indicates that Srp1p carries out a function, almost certainly as a complex with Sts1p, separate from its function as the NLS receptor in nuclear protein import, and that this function is related to regulation of protein degradation through the ubiquitin-proteasome system. Elucidation of the molecular details of the proposed function is a subject for future studies.

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