Yeast Importin-α (Srp1) Performs Distinct Roles in the Import of Nuclear Proteins and in Targeting Proteasomes to the Nucleus*

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Background: Srp1 imports proteins containing nuclear localization signals (NLS) into the nucleus.

Results: Srp1 can also target proteasomes to the nucleus.

Conclusion: Srp1 binds Sts1 to specifically target proteasomes to the nucleus.

Significance: The nuclear targeting of proteasomes by Srp1 is distinct from its well-established role in NLS-mediated import.

Srp1 (importin-α) can translocate proteins that contain a nuclear localization signal (NLS) into the nucleus. The loss of Srp1 is lethal, although several temperature-sensitive mutants have been described. Among these mutants, srp1-31 displays the characteristic nuclear import defect of importin-α mutants, whereas srp1-49 shows a defect in protein degradation. We characterized these and additional srp1 mutants to determine whether distinct mechanisms were required for intracellular proteolysis and the import of NLS-containing proteins. We determined that srp1 mutants that failed to import NLS-containing proteins (srp1-31 and srp1-55) successfully localized proteasomes to the nucleus. In contrast, srp1 mutants that did not target proteasomes to the nucleus (srp1-49 and srp1-E402Q) were able to import NLS-containing proteins. The proteasome targeting defect of specific srp1 mutants caused stabilization of nuclear substrates and overall accumulation of multibiquitylated proteins. Co-expression of a member of each class of srp1 mutants corrected both the proteasome localization defect and the import of NLS-containing proteins. These findings indicate that the targeting of proteasomes to the nucleus occurs by a mechanism distinct from the Srp1-mediated import of nuclear proteins.

Nucleocytoplasmic trafficking occurs by an evolutionarily conserved mechanism (1, 2). Small proteins may freely enter the nucleus, whereas most proteins are escorted to the nuclear pore by transport factors and subsequently into the interior of the nucleus (3, 4). Srp1 is a member of the importin-α family of nuclear transporters that bind proteins bearing a nuclear localization signal (NLS)² (5, 6). The yeast Saccharomyces cerevisiae encodes a single Srp1/importin-α protein that is essential for viability (7). Srp1 binds Kap95/importin-β, a member of a family of proteins that promotes the entry of diverse proteins into the nucleus (8). Other transporters that resemble Kap95/importin-β can import cargo independently of Srp1/importin-α (9). Nucleo-cytoplasmic trafficking is regulated by the Ran protein, which oscillates through a GTP/GDP cycle (2, 10). Srp1 is detected in the cytosol, in the nuclear pore fraction, and in punctate foci at the nuclear periphery (11), reflecting its reversible entry and exit from the nucleus.

SRP1 encodes a protein of 542 amino acid residues that comprise three distinct domains, including an amino-terminal importin-β binding (IBB) domain, a central armadillo repeat motif (ARM) (7), and a carboxyl-terminal Cse1-binding sequence (Fig. 1). The array of 10 ~40-residue ARMs in the central region of Srp1 forms the NLS-binding region. ARM-2 to -4 form a major NLS-binding domain, and ARM-7 to -8 generate a minor NLS-binding motif (12). The IBB contains a cryptic NLS motif that can bind the NLS-binding surface and exert an autoinhibitory effect (13). This interaction allows the IBB to regulate Srp1/substrate interaction and also promotes the release of cargo into the nucleus (14). Mutation of key basic residues reduces IBB interaction with the NLS binding surface and decreases the autoinhibitory effect. The carboxyl terminus of Srp1 interacts with Cse1 to promote substrate dissociation inside the nucleus and nuclear export of Srp1 (15).

SRP1 was first characterized as a suppressor of a polymerase I temperature-sensitive mutation (11). A number of recessive and dominant mutants of Srp1 were subsequently isolated and found to contribute to multiple nuclear activities (7). Intriguingly, the amino acid changes in these mutants occurred predominantly in the ARM repeats. One exception is srp1-31, in which the mutation (S116F) is present in an incomplete boundary ARM that does not contact residues in NLS. Despite its well-characterized requirement in nuclear import (8), srp1 mutants have disparate effects. Specific srp1 mutants were found to harbor defects in either nucleolar structure or RNA synthesis (7), suggesting functions that are unrelated to nuclear trafficking. Indeed, import-independent roles for importin-α have been described recently (16, 17).
Role for Srp1/Importin-α in Nuclear Targeting of Proteasomes

Targeted mutations were generated in SRP1 (18, 19) to investigate the role of nuclear import in cell cycle progression. Amino acid substitutions were engineered in the IBB domain (srp1-55) and in the bipartite NLS (srp1-E402Q) (19, 20). These studies showed that a defect in nuclear import involving both the autoinhibitory domain and the bipartite NLS caused cell cycle-specific defects.

The growth defects of srp1-31 and srp1-49 are suppressed by co-expressing both mutant proteins (21), providing compelling evidence that Srp1 has multiple functions. To investigate the divergent roles of Srp1, Tabb et al. (21) performed a genetic study that yielded Sts1 as a dosage suppressor of srp1-49. Significantly, Sts1 did not suppress the growth defect of srp1-31 (21). Sts1 is required for RNA polymerase I transcription (11), 3’ mRNA processing (22), endoplasmic reticulum/Golgi transport (23), and nuclear segregation and division (24).

Sts1 lacks distinctive structural features that could aid in understanding its biochemical role. Although it was unclear how Sts1 suppressed the proteolytic defect of srp1-49, it is thought to arise from a failure to import proteasomes into the nucleus (25). However, srp1-49 retained the ability to bind an NLS peptide (26), suggesting that it might be import-proficient. We characterized Sts1 and found that the level of nuclear proteasomes was severely reduced in sts1-2 (27).

TABLE 1
Plasmids used in this study

| Plasmid     | Description                       | Reference/Source |
|-------------|-----------------------------------|------------------|
| LEP37       | P<sub>3</sub>Cre-rad4D C-terminal fragment | Ref. 54          |
| LEP38       | P<sub>3</sub>Cre-rad4D N-terminal fragment | Ref. 54          |
| LEP796      | P<sub>gal</sub>-CLB2-HA           | This study       |
| PUL32       | P<sub>gal</sub>-MATA2-GFP         | Ref. 55          |
| pRS426      | pRS<sub>+</sub>-CDC13-3HA        | Ref. 56          |
| pSR849      | pRS806<sup>3</sup>-PIP1-Dimer-RFP::URA3 | Ref. 57          |
| LEP263      | pBSHU-RPT1-GFP-HA                 | Ref. 58          |
| pRS416      | p<sub>2</sub>-FLAG-SEC62-ProA    | Ref. 37          |
| pAC876      | pRS-SRP1::CEN::URA3              | Ref. 6           |
| pAC1104     | pRS-SRP1::CEN::LEU2              | Ref. 6           |
| pBAC126     | pRS-srp1-E402Q-CEN::LEU2         | Ref. 6           |
| LEP843      | pRS-srp1::CEN::LEU2              | Ref. 6           |
| pAC592      | pRS-SKL::2×Myc::TRP1             | Ref. 18          |
| pAC1293     | pRS-SRP1::CEN::URA3              | Ref. 18          |
| pAC1059     | p<sub>2</sub>-BSV403-NLS-GFP-GFP | Ref. 20          |
| pAC1065     | p<sub>2</sub>-SV40-NLS-GFP-GFP  | Ref. 20          |
| pNOY162     | pRS-SRP1::CEN::URA3              | Ref. 21          |
| pNOY163     | pRS-srp1::CEN::URA3              | Ref. 21          |
| pNOY166     | pRS-srp1-49::CEN::URA3           | Ref. 21          |
| DEP177      | p<sub>gal</sub>-STS1::2×μ         | Ref. 59          |

TABLE 2
Yeast strains used in this study

| Strain | Genotype                                      | Reference/Source |
|--------|-----------------------------------------------|------------------|
| NA10   | MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11 (STS1) | Ref. 22          |
| NA25   | MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11 (STS1) | Ref. 22          |
| NOY388 | MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11 (SRP1) | Ref. 21          |
| NOY162 | MATα ura3-1 trp1a63 ade2-1 leu2-3,112 his3-11 srp1-31 (S116F) | Ref. 21          |
| NOY163 | MATα ura3-1 trp1a63 ade2-1 leu2-3,112 his3-11 srp1-31 (E145K) | Ref. 21          |
| ACY641 | MATα ura3-2 srp1::Lα1 ade2 leu2Δ1 his3a200 srp1-55 (R55A) | Ref. 18          |
| ACY324 | MATα his3a200 leu2Δ1 ura3-52 lys2 srp1::HIS3 + SRP1::URA3 | Ref. 19          |
| LCY3084 | MATα his3a2000 leu2Δ1 ura3-52 lys2 srp1::HIS3 + SRP1::URA3 (ACY324 + Srp1) | This study       |
| LCY3085 | MATα his3a200 leu2Δ1 ura3-52 lys2 srp1::HIS3 + srp1-E402Q::URA3 | This study       |
| LCY2777 | NA10 (STS1) + RPN11-GFP::HIS3, PIP1-RFP::URA3 | This study       |
| LCY2778 | NA25 (sts1-2) + RPN11-GFP::HIS3, PIP1-RFP::URA3 | This study       |
| LCY3061 | NOY388 (SRP1) + RPN11-GFP::HIS3, PIP1-RFP::URA3 | This study       |
| LCY3063 | NOY613 (srp1-31) + RPN11-GFP::HIS3, PIP1-RFP::URA3 | This study       |
| LCY3065 | NOY613 (srp1-49) + RPN11-GFP::HIS3, PIP1-RFP::URA3 | This study       |
| LCY3067 | ACY641 (srp1-55) + RPT1-GFP::HIS3::URA3 | This study       |
| LCY3095 | LCY3084 (SRP1) + RPT1-GFP::HIS3::URA3 | This study       |
| LCY3098 | LCY3084 (srp1-E402Q) + RPT1-GFP::HIS3::URA3 | This study       |

FIGURE 1. Domain structure of Srp1/importin-α. A, the structure of the array of 10 ARM repeats in the central portion of Srp1 is shown (12), and the position of each repeat motif is indicated. The concave surface contains two NLS binding surfaces. The major NLS pocket is generated by ARM-3 and -4, and the minor NLS is formed by helix-3 residues in ARM-7 and -8. Small blue lines represent NLS peptides. The side chains of Ser-116, Glu-145, and Glu-402 are shown in yellow. The image in A was derived from crystallographic data of Conti et al. (12) (Protein Data Bank entry 18k6) and was prepared using PyMOL version 1.5,0,4, Schrodinger, LLC, New York). B, three key domains in Srp1 are shown in this schematic of the full-length Srp1. Solid lines demarcate the region of the protein that is represented in A. The amino-terminal IBB domain contains an engineered mutation, R55A, which causes an import defect. The ARM repeat motif are indicated by shaded ovals. The darker shaded ovals represent the major (ARM-3 and -4) and minor (ARM-7 and -8) NLS-binding pockets. The carboxy terminus interacts with Cse1. C, the growth defects associated with key mutants are shown. srp1-31 and srp1-49 are unable to grow at 37°C. srp1-55 showed a pronounced cold temperature growth defect (16°C). The srp1-E402Q mutant showed poor growth at both high and low temperatures.
We determined that nuclear targeting of proteasomes by Sts1 required an interaction with Srp1 (28). Sts1 formed a weak interaction with the srp1-49 mutant but efficient binding to both Srp1 and srp1-31 proteins (28), thus providing a straightforward explanation for the proteasome targeting defect of srp1-49. A protein distantly related to Sts1 in Schizosaccharomyces pombe (Cut8) was also found to target proteasome to the nucleus (32).

We determined that overexpression of Sts1 suppressed the proteasome localization defect of srp1-49 and restored protein degradation. However, Sts1 did not suppress the nuclear import defect of srp1-31, demonstrating that it contributes specifically to the proteasome targeting role of Srp1. In agreement, mutations in STS1 only inhibited proteasome targeting and did not affect nuclear import. We conclude that the proteasome targeting function of Sts1 embodies its involvement in multiple pathways, including cell cycle control, DNA repair, mating pheromone signaling, and DNA replication (28, 29).

The availability of well characterized yeast srp1 mutants and detailed information on importin-α function offered a unique opportunity to examine the role of Srp1 in proteasome targeting. We specifically investigated whether separate mechanisms were required for nuclear targeting of proteasomes and nuclear import of NLS-containing proteins. We determined that srp1 mutants that are import-deficient were able to target proteasomes to the nucleus. In contrast, srp1 mutants that mislocalized proteasomes continued to import nuclear proteins. These findings indicate that proteasome trafficking by Srp1 occurs by a mechanism that is distinct from its well characterized role in the import of nuclear proteins.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains and plasmids are described in Tables 1 and 2. ACY641 (srp1-55), ACY324 (srp1Δ::HIS3 + SRP1::LEU2), pAC1104 (SRP1::URA3), pAC1236 (srp1-E402Q::LEU2), and NLS-GFP were generously provided by Dr. A. Corbett (Emory University, Atlanta, GA) (18, 20). To make SRP1 and srp1-E402Q strains, plasmids pAC1104 and pAC1236 were transformed into ACY324. Transformants were plated on medium containing 5-fluoroorotic acid (5-FOA/LEU2), and uracil auxotrophy was confirmed. The growth of cells expressing srp1-E402Q was moderately impaired at 30 °C.

A plasmid expressing Matα2-GFP was obtained from Dr. Thomas Sommer (Max-Delbruck Center-MDC, Berlin). Deg1-FLAG-Sec62 was obtained from Dr. M. Hochstrasser (Yale University). Materials to integrate Pup1-RFP into the chromosome were provided by Dr. I. Sagot (Université Bordeaux, CNRS). Constructs to integrate Rpt1-GFP were provided by Dr. C. Enenkel (University of Toronto).

Yeast Growth—Wild type and mutant strains were grown at the permissive temperature (23 °C) in selective medium. A culture of exponential phase cells was diluted into selective medium and incubated for 2 h. For temperature shift studies, an aliquot of the culture was first withdrawn at 23 °C.
order of the culture was resuspended in medium containing cycloheximide (750 μg/ml) and incubated at the non-permissive temperature (either 37 or 18 °C). Samples were analyzed at the times indicated in the figure legends.

**Fluorescence Microscopy**—Cultures of 500 μl were pelleted, washed in 1 ml of PBS, and suspended in 10 μl of PBS. A 3-μl volume of the cell suspension was spotted on Poly-Prep Slides (Sigma). Fluorescence signal was captured with a Zeiss Imager M1 microscope, using filter set 38HE. All pairwise comparisons between WT and mutant strains were performed using identical exposure settings. The scale bars (see Figs. 4 and 10) represent 5 μm.

**Preparation of Yeast Lysate, Immunoprecipitation, and Immunoblotting**—Yeast cells were suspended in 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100 (containing protease inhibitors) and lysed by glass bead disruption (Thermo-Savant Fast Prep FP120). Protein extracts were normalized using the Bradford reagent (Bio-Rad). The lysate (50 μg) was resolved in a 12% polyacrylamide SDS-Tricine gel to detect Rad4-HA and Mata2-GFP. Clb2-HA was immunoprecipitated from 2 mg of lysate using anti-HA affinity matrix (Roche Applied Science). The proteins were resolved, transferred to nitrocellulose, and examined by immunoblotting using anti-HA-HRP and anti-GFP antibodies.

**Antibodies and Reagents**—Polyclonal anti-Rad23 antibody was prepared at Pocono Rabbit Farm and Laboratory, Inc. (Canadensis, PA). Anti-Rpn12 and Rpn10 antibodies were provided by Dr. D. Skowyra (St. Louis University). Monoclonal antibodies against HA was obtained from Roche Applied Science. Polyclonal anti-GFP and anti-ubiquitin antibodies, as well as monoclonal anti-FLAG-HRP antibodies were purchased from Sigma. Enhanced chemiluminescent (ECL) reagents were from PerkinElmer Life Sciences, and the signals were detected and quantified using an Eastman Kodak Co. GelLogic 1500 imaging system and software.

**RESULTS**

**srp1 Mutants Show Unique Allele-specific Defects**—We characterized srp1 mutants to understand how Srp1 targeted proteasomes to the nucleus. The effects of four engineered or genetically derived mutants were examined (Fig. 1, A and B). The mutation in srp1-55 resides in the IBB domain (R55A) (19),
Role for Srp1/Importin-α in Nuclear Targeting of Proteasomes

A SV40-NLS-GFP

23°C 37°C

SRP1

srp1-31
	srp1-49

B SV40-NLS-GFP

23°C 37°C 18°C

SRP1 srp1-E402Q srp1-55

C BPSV40T3-NLS-GFP

SRP1 srp1-31 srp1-49

FIGURE 4. An NLS-containing protein is successfully imported in srp1-49. A, SV40-NLS-GFP was detected in the nucleus of SRP1, srp1-31, and srp1-49 at the permissive temperature (23 °C). However, after transfer to the non-permissive temperature (37 °C), the level of nuclear SV40-NLS-GFP was significantly reduced in srp1-31 but not in SRP1 or srp1-49. B, the nuclear localization of SV40-NLS-GFP was also examined in srp1-E402Q and srp1-55. C, the nuclear import of a reporter protein bearing a bipartite NLS (BPSV40T3-NLS-GFP) was examined in srp1-31 and srp1-49, as described in A.

which plays a key role in regulating access to the NLS-binding pocket and also facilitates release of the bound substrate after import. The import defect in srp1-55 arises from a failure to release cargo proteins following entry into the nucleus (20). The mutation in srp1-31 also causes a nuclear import defect. The residue that is mutated (S116F) does not contribute directly to binding NLS residues in cargo proteins but is believed to alter the structure of the major NLS-binding pocket (12). The mutation in srp1-49 is present on the opposite side of the NLS-binding surface. In contrast, srp1-E402Q is present on the concave surface and specifically inhibits interaction with the bipartite NLS (12, 20). We confirmed the temperature-sensitive growth defects of srp1-31 and srp1-49 mutants at 37 °C (Fig. 1C) (11, 33) and the cold temperature growth defect of srp1-55. The srp1-E402Q mutant showed poor growth at all temperatures tested.

The distribution of a 19 S proteasome subunit (Rpn11-GFP) was examined in SRP1, srp1-31, and srp1-49 by fluorescence microscopy (Fig. 2A). The localization of Pup1-RFP (a 20 S core subunit) was similarly tested. Both Rpn11-GFP and Pup1-RFP were efficiently localized to the nucleus in SRP1, srp1-31, and srp1-49 at the permissive temperature (23 °C). DAPI staining confirmed that the proteasome subunits were co-localized with the nucleus. The same cultures were transferred to 37 °C and examined after 4.5 h (Fig. 2B). Proteasome subunits Rpn11-GFP and Pup1-RFP were both efficiently localized to the nucleus in SRP1 and srp1-31. However, neither proteasome subunit was enriched in the nucleus in srp1-49. Protein extracts were examined by immunoblotting to determine whether the lack of fluorescence in srp1-49 reflected proteasome mislocalization and not degradation (Fig. 2C). Protein extracts were prepared from the same cells examined microscopically and resolved by SDS-PAGE. An immunoblot was incubated with antibodies against GFP, RFP, Rpn12, and Rad23. The abundance of all four proteins was similar at 23 °C. Similar levels were also observed at 37 °C. The moderately lower level of Pup1-RFP protein in srp1-31 is consistent with the lower fluorescence in this strain. However, Pup1-RFP was efficiently nucleus-localized in srp1-31. More importantly, the levels of three proteasome subunits (Rpn11-GFP, Pup1-RFP, and Rpn12) were similar in srp1-49. The moderately lower level of Rpn11-GFP, Pup1-RFP, and Rpn12 were similar in SRP1 and srp1-49 at 37 °C, confirming that the loss of nuclear fluorescence in srp1-49 was caused by mislocalization and not degradation.

Fluorescence intensity was quantified in the three strains at both 23 °C and 37 °C (Fig. 2D). Fluorescence pixel density was determined in the cytosol and the nucleus from multiple fields of view. The ratio of nuclear/cytosolic distribution of Pup1-RFP and Rpn11-GFP approached equivalence in srp1-49 at 37 °C, consistent with predominant localization in the cytosol.

To extend these observations, we examined srp1-E402Q and srp1-55 mutants, which harbor distinct defects in nuclear import (20). srp1-E402Q shows defective binding to a bipartite nuclear localization signal, and srp1-55 is unable to efficiently release NLS-containing substrates in the nucleus (20). We expressed the proteasome subunit Rpt1-GFP at physiological levels in srp1-E402Q and srp1-55 and examined its subcellular distribution (Fig. 3A). srp1-55 is a cold-sensitive mutant, and nuclear targeting of proteasomes was compared at 23 and 18 °C (non-permissive condition). Rpt1-GFP was targeted efficiently to the nucleus in srp1-55 (at 18 °C), similar to SRP1 and srp1-31. In contrast, Rpt1-GFP was significantly mislocalized in srp1-E402Q at 37 °C, similar to srp1-49. Fluorescence intensity was measured in the cytosol and nucleus (Fig. 3B, top). We confirmed that the levels of Rpt1-GFP, Rpn12, and Rad23 were essentially unchanged at 37 °C (Fig. 3B, bottom). A modest decrease in the levels of these proteins in srp1-E402Q may be due to the higher fraction of inviable cells. These studies confirmed that the loss of fluorescence signal in the nucleus was due to mislocalization and not protein degradation.

Distinct Import Defects in srp1 Mutants—The failure of srp1-49 and srp1-E402Q to target proteasomes to the nucleus led us to investigate their proficiency in nuclear import. SV40-
NLS-GFP is an engineered protein whose entry into the nucleus is readily observed. SV40-NLS-GFP was enriched in the nucleus in wild type (SRP1), srp1-31, and srp1-49 mutants at 23 °C (Fig. 4A). As expected, after transfer to 37 °C, nuclear import of SV40-NLS-GFP ceased in srp1-31. Surprisingly, nuclear import of SV40-NLS-GFP was not affected in srp1-49. NLS-mediated import was also examined in srp1-55 and srp1-E402Q (Fig. 4B). Despite the severe proteasome targeting defect in srp1-E402Q (Fig. 3A), SV40-NLS-GFP was efficiently imported. We also examined the import of BPSV40-NLS-GFP, a reporter bearing the bipartite nuclear targeting sequence (Fig. 4C). BPSV40-NLS-GFP was imported into the nucleus in SRP1 and srp1-49 at both permissive and non-permissive temperatures, whereas a moderate import deficiency was seen in srp1-31 (Fig. 4C).

Nuclear Localization of a Proteasome Subunit (Rpt1) Is Restored by Intragenic Complementation—The temperature-sensitive growth defect of srp1-31 and srp1-49 was confirmed (Fig. 5A). Wild type Srp1 restored growth of srp1-31 and srp1-49 at 37 °C. The temperature-sensitive growth defects of srp1-31 and srp1-49 were overcome when both mutant proteins were co-expressed (Fig. 5A) (21). Specifically, the growth of srp1-49 at 37 °C was restored by expression of all derivatives of Srp1, except srp1-49. Similarly, the growth defect of srp1-31 was restored by each srp1 mutant protein except srp1-31. However, the mechanism underlying intragenic complementation had not been investigated.

FIGURE 5. Intragenic complementation by srp1 alleles. SRP1, srp1-31, and srp1-49 were transformed with plasmids expressing Srp1, srp1-31, srp1-49, srp1-E402Q, and srp1-55. A, yeast cells were plated on agar medium and incubated at either 23 or 37 °C. The growth defect of srp1-31 and srp1-49 (37 °C; second panel) was suppressed by wild type Srp1. The expression of srp1-31 protein restored growth in all mutants except for srp1-31. Similarly, only srp1-49 failed to suppress the growth defect of srp1-49. The expression of srp1-E402Q and srp1-55 suppressed the temperature-sensitive growth defects of both srp1-31 and srp1-49. B, the inability of srp1-31 to import SV40-NLS-GFP was suppressed by all srp1 mutant proteins except for srp1-31. C, the proteasome targeting defect of srp1-49 was restored by all srp1 mutant proteins except for srp1-49. As expected, wild type Srp1 suppressed the NLS import and proteasome targeting defects of srp1-31 and srp1-49, respectively.

FIGURE 6. Proteasome mislocalization in srp1-49 stabilizes Mat2-GFP. A, Mat2-GFP was examined in SRP1, srp1-31, and srp1-49 at 23 and 37 °C. High level of Mat2-GFP was detected in srp1-49 at 23 °C. Mat2-GFP levels increased significantly at 37 °C. B, extracts were prepared from the cultures examined in A and characterized by immunoblotting. Anti-GFP antibodies confirmed elevated levels of Mat2-GFP in srp1-49. The levels of proteasome subunits Pre10 and Rpn12 were unaffected.
We determined that all srp1 mutant proteins, except srp1-31, could restore nuclear import of SV40-NLS-GFP in srp1-31 (Fig. 5B). In a reciprocal study, we found that all srp1 mutants restored nuclear targeting of proteasome subunit Rpt1-GFP in srp1-49 except srp1-49 (Fig. 5C). These trans-complementation studies indicate that NLS-mediated import occurs by a mechanism distinct from the targeting of proteasomes to the nucleus.

The Stabilization of a Nuclear Substrate in srp1 Mutants Is Allele-specific—Matα2 is a well studied transcription regulator that is degraded by the ubiquitin/proteasome system (34). At the permissive temperature (23 °C) Matα2-GFP was detected in the nucleus in SRP1, srp1-31, and srp1-49 (Fig. 6A). However, markedly higher levels were present in the nucleus in srp1-49, suggesting that proteasome mislocalization occurs even at the permissive temperature. The nuclear accumulation of Matα2-GFP increased dramatically at 37 °C in srp1-49. We measured Matα2-GFP protein levels and confirmed higher levels in srp1-49 at both 23 °C (Fig. 6B, lane 3) and 37 °C (lane 6). In contrast, the levels of proteasome subunits Pre10 and Rpn12 as well as the shuttle-factor Rad23 were essentially unchanged. We note that at 37 °C, Matα2-GFP is not detected in the nucleus of srp1-31 because of its severe import defect (Fig. 6A). However, the abundance of Matα2-GFP was similar to that of the wild type strain, indicating that it was present in the cytosol.

A time-based assay was used to examine Matα2-GFP levels. We blocked protein synthesis and observed rapid depletion of Matα2-GFP in SRP1 and srp1-31 (Fig. 7A). In contrast, Matα2-GFP fluorescence was high in srp1-49 at 23 °C and remained elevated for 75 min at 37 °C. A reduced exposure (Fig. 7A, 1/2 exposure) confirmed the striking accumulation of Matα2-GFP in the nucleus of srp1-49. Matα2-GFP fluorescence also dimin-
These results suggest that the failure to translocate proteasomes to the nucleus in either srp1-31 or srp1-49 (Fig. 8B), as noted by the loss of nuclear fluorescence (0-, 25-, 45-, and 75-min chase). In contrast, Matα2-GFP was expressed in srp1-49, and its subcellular distribution was examined after cells were transferred from 23 to 37 °C. Cycloheximide was added to the medium to inhibit further synthesis of Matα2-GFP. Initial levels of Matα2-GFP were noticeably higher in srp1-2 at the permissive temperature (Fig. 9A, panel 6) and are consistent with our findings in srp1-49 (Fig. 7A). After transfer to 37 °C, Matα2-GFP was rapidly eliminated in STS1, as noted by the loss of nuclear fluorescence (0-, 25-, 45-, and 75-min chase). In contrast, Matα2-GFP levels were essentially unchanged in srp1-2. After 75 min, Matα2-GFP was undetectable in STS1, but nuclear levels were still present in srp1-2. The fluorescence intensity was quantified (Fig. 9B). We examined extracts by immunoblotting and confirmed that Matα2-GFP was rapidly degraded in STS1 but was stable in srp1-2 (Fig. 9C, top). These data were quantified by densitometry (Fig. 9C, bottom).

A failure to target proteasomes to the nucleus in either sts1 or srp1 mutants inhibits the degradation of nuclear proteins. We therefore questioned whether defects in proteasome function would also cause nuclear accumulation of proteolytic substrates. We expressed Matα2-GFP in RPN11 and rpn11-1 and examined protein levels at permissive (23 °C) and non-permis-

FIGURE 8. Proteasome mislocalization stabilizes DNA repair and cell-cycle factors. A, the turnover of the nucleotide excision repair factor Rad4-HA was determined in SRP1, srp1-31, and srp1-49, following the addition of cycloheximide. The reactions to antibodies against the HA epitope, ubiquitin (Ub), Rad23, and proteasome subunit Rpn12 are shown (Fig. 8A). Rad4-HA levels were quantified by densitometry, and the level in each strain was compared with its individual zero time point. C, Cib2-HA was expressed from a galactose-inducible promoter (P GAL1 ) in SRP1, srp1-31, and srp1-49. Protein levels were examined after the cells were transferred from inducing (Gal) to repressive (Glu) medium at 37 °C and quantified (D) as described above.

ished rapidly in srp1-55, reflecting efficient nuclear targeting of proteasomes in this mutant. However, Matα2-GFP levels increased dramatically in srp1-402Q, causing difficulty in obtaining an exposure within the dynamic range.

We verified that the GFP fluorescence seen in Fig. 7A reflected higher Matα2-GFP protein abundance (Fig. 7B). Immunoblotting showed that Matα2-GFP was stabilized in both srp1-49 and srp1-402Q. We measured the levels of multiubiquitylated proteins and detected higher levels in srp1-49 and srp1-402Q (Fig. 7B, bottom). The stabilization of Matα2-GFP in Fig. 7B was quantified by densitometry (Fig. 7D). The fluorescence signal in Fig. 7A was also quantified (Fig. 7C). These results suggest that the failure to translocate proteasomes to the nucleus in srp1-49 and srp1-402Q will cause stabilization of nuclear substrates.

We measured the turnover of additional nuclear proteins, including the DNA repair protein Rad4 (29, 35) and the mitotic cyclin Cib2 (29, 36). Rad4-HA was degraded in both SRP1 and srp1-31 (Fig. 8A) but was strongly stabilized in srp1-49. Significant accumulation of high molecular weight ubiquitylated proteins (Fig. 8B, Ub) was confirmed in srp1-49, whereas the levels of Rad23 and proteasome subunit Rpn12 were unchanged. Rad4-HA levels were quantified by densitometry, and data compiled from three independent experiments are shown (Fig. 8B). The stability of Cib2-HA was similarly examined in SRP1, srp1-31, and srp1-49, and significant stabilization was detected in srp1-49 (Fig. 8C) and quantified (Fig. 8D).

Failure to Degradate Matα2-GFP Causes Nuclear Accumulation—We reported previously that Sts1 binds Srp1 to target proteasomes to the nucleus (28). Because Matα2-GFP is stabilized when proteasomes are mislocalized in srp1-49 and srp1-402Q, a similar effect was predicted to occur in sts1-2. Matα2-GFP was expressed in sts1-2, and its subcellular distribution was examined after cells were transferred from 23 to 37 °C. Cycloheximide was added to the medium to inhibit further synthesis of Matα2-GFP. Initial levels of Matα2-GFP were noticeably higher in sts1-2 at the permissive temperature (Fig. 9A, panel 6) and are consistent with our findings in srp1-49 (Fig. 7A). After transfer to 37 °C, Matα2-GFP was rapidly eliminated in STS1, as noted by the loss of nuclear fluorescence (0-, 25-, 45-, and 75-min chase). In contrast, Matα2-GFP levels were essentially unchanged in sts1-2. After 75 min, Matα2-GFP was undetectable in STS1, but nuclear levels were still present in sts1-2. The fluorescence intensity was quantified (Fig. 9B). We examined extracts by immunoblotting and confirmed that Matα2-GFP was rapidly degraded in STS1 but was stable in sts1-2 (Fig. 9C, top). These data were quantified by densitometry (Fig. 9C, bottom).

A failure to target proteasomes to the nucleus in either sts1 or srp1 mutants inhibits the degradation of nuclear proteins. We therefore questioned whether defects in proteasome function would also cause nuclear accumulation of proteolytic substrates. We expressed Matα2-GFP in RPN11 and rpn11-1 and examined protein levels at permissive (23 °C) and non-permis-
sive (37 °C) temperatures (Fig. 10A). Similar levels of Matα2-GFP were detected at 23 °C. However, Matα2-GFP levels increased dramatically in rpn11-1 at 37 °C.

Proteasomes in sts1-2 are mislocalized within 30 min after cells are transferred to 37 °C. We investigated whether Matα2-GFP would be rapidly stabilized in rpn11-1 after transfer to 37 °C. Higher levels of Matα2-GFP were seen in rpn11-1 at 23 °C (Fig. 10B). GFP fluorescence increased rapidly in rpn11-1 after transfer to 37 °C, and the accumulation of Matα2-GFP protein and multiubiquitylated proteins was confirmed (Fig. 10C). Matα2-GFP also accumulated in proteasome mutants rpt1/cim5-1 and pre1-1 pre2-2 (Fig. 10D). pre1-1 pre2-2 is not temperature-sensitive (ts) for growth, and higher levels of Matα2-GFP were seen at both 23 and 37 °C. rpt1 has a temperature-sensitive growth defect, and higher levels of Matα2-GFP were seen predominantly at 37 °C.

**Srp1 Specifically Affects the Turnover of Nuclear Proteins—**
FLAG-Deg1-Sec62 is an engineered, endoplasmic reticulum-associated protein that is degraded by cytosolic proteasomes (37). FLAG-Deg1-Sec62 was efficiently degraded in SRP1, srp1-31, srp1-49, srp1-E402Q, and srp1-55 mutants (Fig. 11). However, FLAG-Deg1-Sec62 was strongly stabilized in pre1-1 pre2-2, confirming its turnover by cytosolic proteasomes and demonstrating that Srp1 only affects the turnover of nuclear proteins.

**Overexpression of Sts1 Restored Normal Growth and Protein Degradation in srp1-49—** Tabb et al. (21) found that Sts1 could suppress the growth defect of srp1-49 but not srp1-31, reinforc-
ing the idea that Srp1 has two biochemically distinct functions. To determine whether an interaction between Sts1 and Srp1 was required for suppressing the growth defect of srp1−49, we examined NLSsts1, which lacks an Srp1-binding motif (28). The ΔNLSsts1 mutant failed to suppress the growth defect of srp1−49 (Fig. 12A), demonstrating that the Srp1/STS1 interaction is required for growth at 37 °C. In contrast, overexpression of Sts1 in srp1−49 re-established nuclear localization of proteasomes (Fig. 12B, Rpt1-GFP) but did not suppress the import deficiency of srp1−31 (data not shown). Overexpression of Sts1 had no adverse effect on the targeting of Rpt1-GFP in srp1−31 and SRP1. By restoring proteasome targeting to the nucleus, Sts1 overexpression also restored Matα2-GFP degradation in srp1−49 (Fig. 12C). We conclude that the targeting of proteasomes to the nucleus by Srp1 and Sts1 involves a mechanism that is distinct from the import of NLS-containing proteins.

**DISCUSSION**

Multiple pathways promote nuclear import, and a well-characterized mechanism involves the importin-α/β heterodimer. In yeast, importin-α is encoded by SRP1, and its role in NLS-mediated protein import has been characterized extensively (20, 38, 39). Srp1 also performs a poorly understood role in protein degradation that is generally believed to involve its nuclear import activity (21, 25).

We found that the protein degradation function of Srp1 requires its interaction with Sts1 (28). Sts1 performs a key role in targeting proteasomes to the nucleus (28). Mutations in both Srp1 and Sts1 can cause proteasome mislocalization, which inhibits protein degradation. A distantly related protein in *S. pombe* (Cut8) can also guide proteasomes to the nuclear surface (32, 40). Blm10 was recently reported to traffic proteasome core particles to the nucleus (41). However, Blm10 operates independently of Srp1 and is not required for viability. In contrast, Sts1 is essential for viability, suggesting that it mediates a major pathway for proteasome localization.
The presence of NLS motifs in certain proteasome subunits suggests that they may be imported directly through an interaction with Srp1 (29). A bipartite NLS in Rpn2 was shown to target a reporter protein to the nucleus (25). We note that proteasome subcomplexes can operate in the nucleus (31, 42), although they perform non-proteolytic roles (43, 44). It is significant that peptidase activity has not been convincingly demonstrated in the nucleus. The import of proteasome subcomplexes that perform non-proteolytic functions could be different from the localization of proteasomes by Sts1 that promotes degradation. None of the proteasome subunits that were mislocalized in sts1-2 contained identifiable NLS motifs.

We identified two types of srp1 mutants that are either unable to import NLS-containing proteins (srp1-31 and srp1-55) or fail to target proteasomes to the nucleus (srp1-49 and srp1-E402Q). These are distinct and non-overlapping functions because co-expression of srp1-31 and srp1-49 mutants resulted in intragenic complementation. Specifically, the expression of srp1-31 protein in srp1-49 restored nuclear targeting of proteasomes and Mata2 degradation. Similarly, nuclear import resumed in srp1-31 when srp1-49 was co-expressed (Fig. 5). Overexpression of Sts1 suppressed the growth defect of srp1-49 (21) but not srp1-31, reinforcing the view that Srp1 has multiple roles.

The biochemical basis for proteasome mislocalization in srp1-49 is probably due to its weak interaction with Sts1 (28). In contrast, Sts1 formed an efficient interaction with srp1-31, and nuclear localization of proteasomes is proficient in srp1-49. Intra-allelic complementation can be explained by the formation of a dimer. Although Tabb et al. (21) had speculated that Srp1 functioned as a monomer, we note that the structure of Srp1 revealed a homodimer in the crystal (12). The formation of a Srp1/Srp1 dimer is also suggested by the complementation observed by co-expressing srp1-49 and srp1-E402Q, which are both defective in proteasome targeting (Fig. 5C). Further study will be required to confirm the existence and functional relevance of an Srp1/Srp1 dimer.

Several srp1 mutants that we examined have been well characterized (20, 21, 25). To strengthen our hypothesis that Srp1 performs two different roles, we characterized srp1-55 and srp1-E402Q, which have unique defects in nuclear import (18, 19, 20). The srp1-55 mutant contains an alanine substitution of Arg-55 (see Fig. 1), which inhibits the release of NLS cargo (20). SV40-GFP-NLS was localized to the nucleus in srp1-55, because its defect occurs after nuclear entry. Proteasomes were efficiently targeted to the nucleus in srp1-55, and the turnover of a nuclear protein was not affected. In contrast, srp1-E402Q failed to target proteasomes to the nucleus (Fig. 3B) and caused striking stabilization of a nuclear substrate (Fig. 7A).

The mutations in Srp1 that cause proteasome mislocalization (E145K and E402Q) are spatially distant. Glu-145 is present in ARM-2 but lies on the opposite side of the NLS binding surface (12). Therefore, it is not surprising that srp1-49 successfully imported NLS-containing proteins. However, srp1-49 formed a weak interaction with Sts1, suggesting that this region of Srp1 might be important for binding regulatory factors such as Sts1. Glu-402 is located in the middle of helix-3 of ARM-8 and is present in the concave NLS-binding surface of Srp1. Pulliam et al. (20) showed that srp1-E402Q is defective in binding a bipartite NLS motif.

Tabb et al. (21) identified two clusters of closely juxtaposed basis residues in Sts1. Disruption of the first cluster partially reduced nuclear localization of Sts1, suggesting a requirement for an additional targeting motif. We characterized the Sts1 protein sequence using cNLS-Mapper, a tool designed to search for NLS motifs (45). cNLS-Mapper identified a bipartite NLS that included both clusters of basic residues detected previously by Tabb et al. (21). The proteasome targeting defect of srp1-E402Q might be due to its inability to bind a putative bipartite NLS in Sts1.
The mutation in \textit{srp1-31} replaces serine with phenylalanine (S116F). Serine 116 is located in ARM-1, which lies at the boundary of the NLS-binding pocket. ARM-1 does not contribute directly to binding NLS but is likely to affect the structural integrity of the importin-\(\alpha\) NLS-binding surface (12). \textit{srp1-31} showed a severe defect in importing a monopartite NLS (SV40-GFP-NLS). However, \textit{srp1-31} only showed a moderate defect in importing bipartite NLS (Fig. 4; BPSV40T3-GFP-NLS). We showed previously that \textit{srp1-31} forms an efficient interaction with Sts1 (28), consistent with the idea that a bipartite NLS in Sts1 binds the minor NLS motif in Srp1. However, further study will be required to determine whether Sts1 contains a bipartite NLS.

A requirement for importin-\(\alpha\) in stress response was reported to be distinct from its function in importing NLS-containing proteins (17). Importin-\(\alpha\) was also found to restrict...

\textbf{FIGURE 13. A model for Srp1 function.} \textit{A}, we propose that in wild type cells, Sts1 binds Srp1 to translocate proteasomes to the nucleus. Proteasomes at the nuclear surface may receive nuclear substrates that are exported. A separate activity of Srp1 is to import NLS-containing proteins (cargo), which we presume occurs concurrently with the nuclear targeting of proteasomes. NLS-mediated import requires Srp1 interactions with other import factors (such as Kap85/importin-\(\beta\)). \textit{B}, a mutation in Sts1 that prevents interaction with Srp1 (\textit{Sts1-\textit{NLS}}) prevents the translocation of proteasomes to the nucleus and causes stabilization and nuclear accumulation of proteins (28). However, the nuclear import of other NLS-containing proteins is not affected by Sts1. \textit{C}, certain mutations in \textit{Srp1} (\textit{srp1-49} and \textit{srp1-E402Q}) prevent nuclear targeting of proteasomes. However, \textit{srp1-49} and \textit{srp1-E402Q} mutants are proficient in importing cNLS-containing proteins. Proteolytic substrates accumulate inside the nucleus in these mutants. One interpretation of this result is that nuclear substrates are not exported unless proteasomes are available at the nuclear surface. \textit{D}, the prototypical \textit{srp1} mutants, embodied by \textit{srp1-31}, failed to import NLS-containing proteins. However, these mutants could target proteasomes to the nucleus, allowing successful degradation of nuclear substrates.
the import of specific transcription factors (16), underscoring unique and unexpected functions for this nuclear transporter. Similarly, we report that Srp1 function in targeting proteasomes to the nucleus is exclusive from its action in importing NLS-containing proteins. We speculate that Sts1 diverts Srp1 from its nuclear import function to promote nuclear localization of proteasomes. There is evidence that proteasome subunits are enriched in the nuclear envelope (11, 31, 46, 47).

Both regulatory and misfolded nuclear proteins are ubiquitylated and degraded by the proteasome (48–50). Whereas the ligation of ubiquitin to nuclear proteins has been described, the site of their degradation has not been examined systematically. Indeed, there is no compelling evidence that intact 26 S proteasomes and peptidase activity are present in the nucleus. The degradation of nuclear proteins is inhibited in both srp1-49 and sts1-2 (Fig. 7B), consistent with the proteasome-targeting defect in these mutants (25, 28). However, the rapid stabilization of nuclear substrates in srp1-49, srp1-E402Q, and sts1-2 was surprising because nuclear substrates should have been degraded if proteasomes had entered prior to the shift to non-permissive temperature. This was not observed, suggesting a scarcity of catalytically active proteasomes inside the nucleus. In agreement with this view, we recently reported that many nuclear proteins are degraded by cytosolic proteasomes after export, in agreement with other studies (29, 51, 52). The degradation of cytoplasmic proteins was not affected in either srp1-49 or sts1-2 (Fig. 11), demonstrating that the accumulation of multiubiquitylated proteins in these mutants (29) represent nuclear proteins.

We propose that Srp1 is engaged concurrently in importing nuclear proteins and localizing proteasomes to the nucleus (see model in Fig. 13A). However, a mutation that disrupts Srp1/Sts1 interaction (srp1-49; sts1ANLS) specifically inhibits proteasome targeting without affecting NLS-mediated import. Nuclear substrates that are stabilized in srp1-49, srp1-E402Q, and sts1-2 accumulate inside the nucleus (Fig. 13B). In contrast, mutations in Srp1 that inhibit delivery of NLS cargo (srp1-31 and srp1-55) do not prevent the nuclear targeting of proteasomes (Fig. 13D).

It is presently not known whether Srp1/Sts1 import proteasomes into the nucleus or merely guide them to the nuclear surface. We speculate that proteasomes are located on the nuclear surface because the export pathway is required for degrading multiple nuclear substrates (29, 53). Our studies do not rule out the degradation of specific proteins within the nucleus, although the significant accumulation of multiubiquitylated proteins in export mutants (28) indicates that the export pathway plays an important role in nuclear protein turnover. Further study will be required to establish the location of catalytically active proteasomes, the site of nuclear protein degradation, and the specific involvement of the export mechanism.

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