Ssts1 Plays a Key Role in Targeting Proteasomes to the Nucleus

Li Chen, Lizbeth Romero, Show-Mei Chuang, Vincent Tournier, Kishore Kumar Joshi, Jung Ah Lee, Gopala Kovvali, and Kiran Madura

From the Department of Biochemistry, Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

The evidence that nuclear proteins can be degraded by cytosolic proteasomes has received considerable experimental support. However, the presence of proteasome subunits in the nucleus also suggests that protein degradation could occur within this organelle. We determined that Ssts1 can target proteasomes to the nucleus and facilitate the degradation of a nuclear protein. Specific ssts1 mutants showed reduced nuclear proteasomes at the nonpermissive temperature. In contrast, high expression of Ssts1 increased the levels of nuclear proteasomes. Ssts1 targets proteasomes to the nucleus by interacting with Srp1, a nuclear import factor that binds nuclear localization signals. Deletion of the NLS in Ssts1 prevented its interaction with Srp1 and caused proteasome mislocalization. In agreement with this observation, a mutation in Srp1 that weakened its interaction with Ssts1 also reduced nuclear targeting of proteasomes. We reported that Ssts1 could suppress growth and proteolytic defects of rad23Δ rpn10Δ. We show here that Ssts1 suppresses a previously undetected proteasome localization defect in this mutant. Taken together, these findings explain the suppression of rad23Δ rpn10Δ by Ssts1 and suggest that the degradation of nuclear substrates requires efficient proteasome localization.

Many factors that regulate the cell cycle, DNA repair, transcription, and tumor suppression are nuclear proteins that are degraded by the ubiquitin/proteasome system (1). However, the mechanism that mediates their turnover and the subcellular location of degradation are often not known. Nuclear proteasomes (2, 3) may perform both proteolytic and nonproteolytic functions (4, 5). The evidence that the hydrolytic activities of proteasomes are present in the nucleus is limited. Although some proteins are degraded within the nucleus (4), others are exported from the nucleus and degraded by cytoplasmic proteasomes (6–8). It is unknown if nuclear degradation is restricted to specific proteins, while others are exported and degraded by cytoplasmic proteasomes.

Rad23 is a substrate shuttle factor that can transfer ubiquitinated proteins to the proteasome (9, 10), whereas Rpn10 is a major proteasome receptor for mult ubiquitinated proteins (11–15). The loss of both proteins in rad23Δ rpn10Δ caused severe growth and proteolytic defects, including sensitivity to drugs, stabilization of substrates, and accumulation of ubiquitinated proteins (16). Ssts1 is a dosage suppressor of these pleiotropic defects of rad23Δ rpn10Δ (17), indicating that it plays a role in the ubiquitin/proteasome system. In agreement, we found that Ssts1 protein can bind the proteasome, and an ssts1-2 mutant (C194Y) was defective in protein degradation and accumulated high levels of ubiquitinated proteins. Significantly, the interaction between mult ubiquitinated proteins and proteasomes was reduced in ssts1-2.

We reported here that Ssts1 is required for efficient translocation of proteasomes to the nucleus. We propose that the failure of proteasomes to bind mult ubiquitinated substrates in ssts1-2 (17) is most likely due to reduced levels of proteasomes at the nucleus. We determined that proteasomes are also mislocalized in rad23Δ rpn10Δ, and this defect is suppressed by Ssts1. The protein degradation deficiency of rad23Δ rpn10Δ is likely also caused by a failure to target proteasomes to the nucleus, which could interfere with the degradation of nuclear proteins.

Ssts1 binds Srp1 (importin-α), a nuclear transport protein (18), and also forms a distinct interaction with the proteasome subunit Rpn11. It was proposed that these interactions by Ssts1 mediated different functions (19). However, a specific srp1 mutant (srp1-49) harbors a defect in both protein degradation (19) and nuclear targeting of proteasomes (18). We determined that an srp1 mutant that is unable to bind Srp1 has reduced levels of nuclear proteasomes. As expected, a nuclear localization signal (NLS) in Ssts1 is required for binding Srp1 and promoting nuclear trafficking of proteasomes. In a reciprocal study, we found that a mutation in Srp1 that reduced its interaction with Ssts1 was also deficient in recruiting proteasomes to the nucleus. Thus, proteasome degradation appears to be affected by the level of nuclear proteasomes, which is a consequence of the interaction between Srp1 and Ssts1. Taken together, these genetic and biochemical studies offer insight into the mechanism of proteasome translocation to the nucleus and demonstrate that a failure causes cell death.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast strains harboring mutations in SRP1 were provided by Drs. P. Tongaonkar and M. Nomura (University of California, Irvine). DNA templates were sequenced, and the mutations were verified (srp1-31 S116P; srp1-49 E145K). Strains containing mutations in STS1 were also provided by Dr. F. Wyers. Plasmids for generating...
integrated derivatives of GFP-tagged proteasome subunits were generously provided by Dr. C. Enenkel (Humboldt University). All the amplified DNAs were verified by sequencing both strands. A list of yeast strains and plasmids is shown in Tables 1 and 2, respectively.

**Growth Assays and Sensitivity to Temperature**—Yeast cultures were grown in selective medium and normalized to an absorbance of ~1. 10-Fold serial dilutions were spotted on agar plates and incubated at 23 and 37 °C.

**Pulse-Chase Measurement of Protein Stability**—Protein stability measurements were performed as described previously (20). We used the EXPRE35S35S protein labeling reagent (PerkinElmer Life Sciences) to metabolically label STS1 and sts1-2 expressing FLAG-Sts1 and FLAG-sts1-2, respectively. Following incubation for 5 min at 30 °C, labeling was terminated by the addition of cycloheximide, and aliquots were withdrawn at 0, 10, 30, and 60 min. Equal amounts of trichloroacetic acid-insoluble material was incubated with anti-FLAG M2-agarose beads. The samples were resolved by SDS-PAGE and exposed to x-ray film.

**Native PAGE**—Measurement of peptidase activity of proteasomes was examined in a native polyacrylamide gel, as described previously (20). Protein lysates (50 μg) were separated in a native gel that was overlaid with buffer containing LLVY-AMC in the presence of 0.05% SDS. The fluorescence signal was detected with Kodak GelLogic Imager.

**Purification of Recombinant Proteins and in Vitro Binding Assay**—Expression of proteins from pGEX and PET28 vectors was achieved in *Escherichia coli* BL21S cells in the presence of 1 mM isopropyl-1-thio-β-D-galactopyranosidase. Cells were lysed (in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM Na-EDTA, 1% Triton X-100, and protease inhibitor mixture), and total protein lysate was applied to glutathione-Sepharose to purify GST-tagged proteins. Protein expression levels were determined before performing binding studies. Bacterial lysates that contained His₆-tagged proteins were mixed with purified GST proteins for 4 h at 4 °C. The unbound proteins were removed by four washes in lysis buffer. The bound proteins were released in SDS gel loading buffer, separated in 10 or 12% SDS-Tricine/PAGE, and examined by immunoblotting.

**Immunoprecipitation/Immunoblotting**—Yeast cells were suspended in buffer A (50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 10 mM β-glycerophosphate, 1 μM leupeptin, 1 μM aprotinin, 1 mM PMSF, 1 mM benzamidine, 10% glycerol) and lysed on ice. The cell lysate was centrifuged at 15,000 g for 15 min at 4 °C.

The abbreviations used are: LLVY-AMC, Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; CP, catalytic (20 S) particle; GST, glutathione S-transferase; NLS, nuclear localization signal; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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**TABLE 1**

| Strain       | Description                                                                 | Source       |
|--------------|------------------------------------------------------------------------------|--------------|
| NA10         | MATa ura3-1, trp1-1, ade2-1, leu2-3, &his3-11 (STS1)                         | F. Wyers     |
| NA25         | MATa ura3-1, trp1-1, ade2-1, leu2-3, &his3-11, sts1-2 (C194Y)               | F. Wyers     |
| LRY171       | NAY10: PRE6-GFP::URA3::HIS3                                                  |              |
| LRY227       | NAY10: RPN1-GFP::URA3::HIS3                                                  |              |
| LRY159       | NAY10: RPN1-GFP::URA3::HIS3                                                  |              |
| LRY178       | LRY159, 5-FOAΔ                                                             |              |
| LRY241       | NAY10: RPN11-GFP::URA3::HIS3                                                |              |
| LRY161       | NAY25: RPN11-GFP::URA3::HIS3                                                |              |
| LCY1902      | LRY239 + Yeplac181 vector                                                   |              |
| LCY1933      | LRY239 + PcZIP-FLAG-Sts1                                                    |              |
| LCY1903      | LRY171 + PcZIP-FLAG-Sts1                                                    |              |
| LCY1927      | LRY227 + pRS vector                                                         |              |
| LCY1928      | LRY159 + pRS vector                                                         |              |
| LCY1929      | LRY159 + pRS-Sts1                                                           |              |
| LCY1930      | LRY159 + pRS-sts1ASNLS                                                      |              |
| LCY1951      | LRY178 + pRS-sts1-11                                                        |              |
| NOY388       | MATa, ade2-1, his3-11, trp1-1, ura3-1, leu2-3, −112 (SRP1)                  | M. Nomura    |
| NOY613       | MATa, ade2-1, his3-11, trp1-1, ura3-1, leu2-3, −112 (srp1-31: S116F)         | M. Nomura    |
| LRY223       | NOY388: RPN1-GFP::URA3::HIS3                                                | M. Nomura    |
| LRY224       | NOY612: RPN1-GFP::URA3::HIS3                                                | M. Nomura    |
| LRY225       | NOY613: RPN1-GFP::URA3::HIS3                                                | M. Nomura    |
| LRY203       | RAD23 RPN10:RPT1-GFP::URA3::HIS3, 5-FOAΔ                                    | 16           |
| LRY189       | rad23A rpn10Δ::RPT1-GFP::URA3::HIS3, 5-FOAΔ                                 | 16           |
| LCY2011      | LRY189 + PcZIP-STs1                                                        |              |
| LCY2087      | NA10: CLB2-HA::TRP1                                                         |              |
| LCY2088      | NA25: CLB2-HA::TRP1                                                         |              |
| MHY501       | MATa his3-200, leu2-2, 112, ura3-52, lys2-801, trp1-1                       | M. Hochstrasser |
| MHY553       | MATa his3-200, leu2-2, 112, ura3-52, lys2-801, trp1-1, ubc6Δ::HIS3 ubc7Δ::URA3 | M. Hochstrasser |

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**TABLE 2**

| Plasmid       | Description                  | Source  |
|---------------|------------------------------|---------|
| LEP164        | pCZIP-FLAG-STS1              | 9       |
| LEP297        | pCZIP-FLAG-sts1-2(C194Y)      | 9       |
| LEP177        | pGEX-STS1                    | 9       |
| pNOY470       | pRS-Sts1                     | M. Nomura      |
| pNOY480       | pRS-sts1ASNLS                | M. Nomura      |
| pNOY343       | phtst1-11 (E43G)             | M. Nomura      |
| LEP262        | pBSHU-Rpn1-GFP::HA          | C. Enenkel |
| LEP263        | pBSHU-Rpt1-GFP::HA          | C. Enenkel |
| LEP264        | pBSHU-Rpn11-GFP::HA         | C. Enenkel |
| LEP269        | pBSHU-Prep-GFP::HA          | C. Enenkel |
| LEP163        | pGEX-Sts1                    |         |
| LEP606        | pGEX-sts1-2                  |         |
| LEP633        | pGEX-sts1ASNLS               |         |
| LEP632        | pGEX-sts1-11,12              |         |
| LEP105        | pGEX-Rpn1                   |         |
| LEP601        | pET28-Rpn1                  |         |
| LEP603        | pET28-Sep1                  |         |
| LEP604        | pET28-39p1-31 (S116F)        |         |
| LEP605        | pET28-39p1-49 (E145K)        |         |
| LEP666        | pET28-Sts1                  |         |
| LEP667        | pET28-Sts1-2                |         |
| LEP668        | pET28-Sts1ASNLS             |         |
| LEP669        | pET28-Sts1-11,12             |         |
| LEP689        | pGEX-Sep1                   |         |
| LEP690        | pGEX-srp1-31                |         |
| LEP691        | pGEX-srp1-49                |         |
| LEP703        | pOC9 derivative of pCZIP-Ura3-HA-SL17 | R. Kulka and T. Ravid |
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5 mM EDTA, and 1% Triton X-100) containing protease inhibitors (Roche Applied Science) and lysed by glass bead disruption. Protein extracts were normalized using the Bradford method (Bio-Rad) and incubated with anti-FLAG-M2-agarose. The bound proteins were released in SDS gel loading buffer, separated in 10 or 12% SDS-Tricine/PAGE, and characterized by immunoblotting. The signals were quantified using Kodak GelLogic Imaging software.

Fluorescence Microscopy—500 μl of yeast cells were pelleted, washed with 1 ml of phosphate-buffered saline (PBS), and suspended for 10 min in 100 μl of PBS containing 10 ng of 4,6-diamidino-2-phenylindole (DAPI). DAPI was removed following several washes with PBS, and the cells were then suspended in 30 μl of PBS. Cells (3 μl) were spotted on Poly-Prep slides (Sigma) and examined using a Zeiss Imager M1 microscope. All exposures were for 600 ms for GFP (filter set 49) and 75 ms to detect DAPI fluorescence (filter set 49).

The scale bar represents 5 μm. Zeiss AxioVision software was used for quantifying the GFP fluorescence intensity. Between five and eight different fields of view were examined, and ~50–200 cells were analyzed. Standard mean value were collected by densitometry and plotted, and Student’s t test was applied for comparisons.

Ultrasonic Cell Disruption—Yeast were grown to exponential phase at 23 °C, suspended in medium that was prewarmed to 37 °C, and incubated for 5 h. Yeast cells were collected by centrifugation and washed with ice-cold buffer A (lacking Triton X-100), and 500 μl was placed in each of six microcentrifuge tubes. Each sample was exposed to varying durations of ultrasonication at 23 kHz using a horn tip diameter of 0.3 cm (Sonic Dismembrator, model 100, Fisher). The generator power setting (4) was identical for all treatments, and the duration of sonication is indicated in the figure. Following sonication, an aliquot was examined by microscopy to check the integrity of the cells and to visualize GFP localization. A 400-μl aliquot was centrifuged at 12,000 × g, and 50 μl of the supernatant was placed in a 96-well plate, in duplicate. GFP fluorescence was detected using a Tecan Infinite F-200 plate reader (using a filter set 38 HE) and 75 ms to detect DAPI fluorescence (filter set 49).

The intact 26 S proteasome contains a single catalytic 20 S particle (CP), which has a severe defect in proteasome assembly (20, 22). This large 23-MDa complex migrates slowly in the native gel (RP2CP), and its position can be distinguished from the free catalytic 20 S particle (CP), which is indicated in Fig. 1A, right margin. In the presence of low levels of detergent (0.05% SDS), the peptidase activity of both 26 S and 20 S proteasomes is detected using a fluorogenic assay. Fig. 1A shows that high levels of free 20 S CP, but low levels of intact proteasomes (RP2CP), are detected in rpn11-1 (compare ratio of RP2CP/CP in lanes 1 and 2). The levels of 26 S and 20 S complexes were similar in STS1 and sts1-2, at both permissive (Fig. 1A, lanes 3 and 4) and nonpermissive temperatures (lanes 5 and 6), demonstrating that proteasomes are assembled, and equally active in STS1 and sts1-2. However, this finding did not explain the cause of the growth and proteolytic defects of sts1-2 or the inability of proteasomes to efficiently bind polyubiquitinated proteins (17).

Defects of sts1-2 Are Linked to Instability of sts1-2 Protein—We examined the stability of Sts1 and sts1-2 proteins by in vivo labeling with [35S]methionine. FLAG-Sts1 was expressed cells were removed by centrifugation at 3,000 × g. The supernatant was centrifuged at 20,000 × g for 20 min to separate the cytoplasm from the enriched nuclear pellet. Protein concentration of the supernatant (representing cytosolic fraction) was determined, and an equal amount (15 μg) was examined by immunoblotting. The pellet, representing crude nuclei, was weighed and suspended in 30 μl/mg SDS-gel electrophoresis buffer. Equal amount of protein (45 μl) was resolved by SDS-PAGE.

Antibodies—Polyclonal antibodies against Rpt1, Rad23, Dsk2, and Cdc31 were generated at Pocono Rabbit Farm and Laboratory, Inc. (Canadensis, PA). Anti-Rpn12 was a gift from Dr. D. Skowyra (Washington University, St. Louis). Antibodies against ubiquitin, FLAG-HRP, and FLAG-M2 beads were from Sigma. Anti-His6 monoclonal antibody was purchased from Boston Biochem. Monoclonal anti-HA was obtained from Roche Applied Science. Glutathione-Sepharose 4B was from GE Healthcare.

Reagents—Suc-Leu-Leu-Val-Tyr-AMC (LLVY-AMC) was purchased from Boston Biochem. DAPI was purchased from Sigma. Enhanced chemiluminescent (ECL) reagents were from PerkinElmer Life Sciences, and the signals were detected using a Kodak GelLogic 1500 Imaging System.

RESULTS

Proteasomes Are Structurally Intact in sts1-2—We showed previously that proteasomes in sts1-2 failed to efficiently bind multiubiquitinylated proteins (17). We separated proteasomes in a native polyacrylamide gel to determine whether stability or function was altered. Separation using a native in-gel assay permits resolution of cellular proteasomes and its intermediates and can provide a qualitative assessment of both assembly and peptidase activity (20, 21). Protein extracts were characterized from STS1 and sts1-2 strains grown at the permissive (23 °C) and nonpermissive (37 °C) temperatures (Fig. 1A). We also separated extracts prepared from rpn11-1, which has a severe defect in proteasome assembly (20, 22).

The intact 26 S proteasome contains a single catalytic 20 S particle (CP) and two regulatory (19 S) particles (1). This large 2.3-MDa complex migrates slowly in the native gel (RP2CP), and its position can be distinguished from the free catalytic 20 S particle (CP), which is indicated in Fig. 1A, right margin. In the presence of low levels of detergent (0.05% SDS), the peptidase activity of both 26 S and 20 S proteasomes is detected using a fluorogenic assay. Fig. 1A shows that high levels of free 20 S CP, but low levels of intact proteasomes (RP2CP), are detected in rpn11-1 (compare ratio of RP2CP/CP in lanes 1 and 2). The levels of 26 S and 20 S complexes were similar in STS1 and sts1-2, at both permissive (Fig. 1A, lanes 3 and 4) and nonpermissive temperatures (lanes 5 and 6), demonstrating that proteasomes are assembled, and equally active in STS1 and sts1-2. However, this finding did not explain the cause of the growth and proteolytic defects of sts1-2 or the inability of proteasomes to efficiently bind polyubiquitinylated proteins (17).

Defects of sts1-2 Are Linked to Instability of sts1-2 Protein—We examined the stability of Sts1 and sts1-2 proteins by in vivo labeling with [35S]methionine. FLAG-Sts1 was expressed
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FIGURE 1. Proteasome are assembled and functional in sts1-2. A, protein lysates were resolved in a native polyacrylamide gel and incubated with proteasome substrate LLVY-AMC. The positions of intact 26 S proteasome (RP2CP) and the free CP are indicated on the right. In a control strain, increased proteasome dissociation in rpnl1-1 is indicated by the high levels of CP (lane 2). Lysates prepared from STS1 and sts1-2 cells that were grown at 23 and 37 °C were examined (lanes 3–6). B, incubation of cells with FLAG-Sts1 and FLAG-sts1-2 was determined by metabolic labeling with [35S]methionine. Cells were grown at 23 °C and then incubated at 37 °C for 4 h prior to labeling. Following 5 min of labeling, cells were transferred to chase medium, and samples were withdrawn at the times indicated in minutes. C, in vitro interaction of GST-Sts1 and GST-sts1-2 with His6-Rpn11 and His6-Srp1 was tested with control beads containing GST (lane 2). GST-Sts1 and GST-sts1-2 formed a strong interaction with His6-Srp1 and a weaker but reproducible interaction with His6-Rpn11. We conclude that the defect of sts1-2 in vivo is the result of its rapid degradation and is not due to its inability to bind physiological partners.

Proteasomes Are Mislocalized in sts1-2—Because Sts1 can bind a nuclear transport protein (Srp1), as well as a subunit in the proteasome (Rpn11) (Fig. 1C) (19), we investigated if it functioned as an adaptor that linked the proteasome to the nucleus. We integrated derivatives of several proteasome subunits as fusions to the green fluorescent protein (GFP) in STS1 and sts1-2. Rpn11-GFP and Rpn11-GFP are subunits in the regulatory (19 S) particle, and Pre6-GFP is a subunit in the catalytic (20 S) particle. Enenkel et al. (2, 23) previously showed that these chimeras were expressed at physiological levels and assembled into mature proteasomes. The GFP fusions proteins are fully functional, because they can replace essential proteasome subunits. In preliminary studies, yeast cells were grown at the semi-permissive temperature (30 °C) and examined by fluorescence microscopy. We determined that Rpn1, Rpn11, and Pre6 were co-localized with the nucleus in STS1 (data not shown). DAPI treatment showed that the GFP signal overlapped with the nucleus.

STS1 and sts1-2 cells were transferred to fresh medium and grown at the permissive temperature (23 °C) for 1.5 and 5 h. The subcellular distribution of Rpn1-GFP in STS1 and sts1-2 was investigated by fluorescence microscopy (Fig. 2A). Both STS1 and sts1-2 showed strong overlap of the GFP signal with nuclei at 23 °C. However, even at 23 °C, we observed higher cytosolic levels of Rpn1-GFP in sts1-2. Nonetheless, it is likely that adequate levels of proteasomes are present in the nucleus in sts1-2 to permit survival at 23 °C, the permissive temperature.

STS1 and sts1-2 cells were transferred to 37 °C and examined at the intervals shown (Fig. 2B). Significant mislocalization of proteasomes was evident in sts1-2 by 3 h, and specific nuclear staining was strongly reduced at 5 h (Fig. 2B, lower panels). GFP signal in the cytosol was ~2-fold higher in sts1-2. The GFP staining pattern in STS1 after transfer to 37 °C was essentially unchanged (1.5 h at 37 °C), although a significant loss in nuclear GFP was evident in sts1-2. After 5 h incubation at 37 °C, the GFP staining in nuclear and cytoplasmic regions in sts1-2 was indistinguishable, suggesting significant cytoplasmic localization of proteasomes. It is possible that low levels of proteasomes remain bound to nuclei at 37 °C in sts1-2 but cannot be distinguished from the background of mislocalized cytosolic Rpn1-GFP.

Nuclear Localization Defect of sts1-2 Is Reversible—STS1 and sts1-2 cultures that were examined in Fig. 2B were transferred from 37 to 23 °C to determine whether the proteasome targeting defect of sts1-2 was reversible. Fluorescence microscopy showed that proteasome targeting to the nucleus was restored in sts1-2 (Fig. 2C). This result demonstrates that the temperature-sensitive localization defect is reversible for at least 5 h. In contrast, continued incubation of sts1-2 at 37 °C resulted in the accumulation of large cells, which showed significant depletion of nuclear proteasomes. Because protea-
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A nuclear localization defect in \( \text{sts1-2} \). Rpn1-GFP was integrated in \( \text{STS1} \) and \( \text{sts1-2} \) at the chromosomal loci and expressed at physiological levels. A, actively growing cells were diluted into fresh medium and incubated at 23 °C and viewed by immunofluorescence at the times indicated. B, aliquot from the same cultures was transferred to medium at 37 °C, and samples were examined at the times indicated. C, following incubation for 5 h at 37 °C, \( \text{STS1} \) and \( \text{sts1-2} \) were diluted and incubated for −15 h in fresh medium at either 23 or 37 °C to test for reversibility of the defect. D, Zeiss imaging software was used to quantify the pixel density of the images seen in the microscopy images. A minimum of five independent viewing fields were examined, and well separated cells were quantified for both cytosolic (cyto) and nuclear compartment (NC). The numbers (\( n \)) represent the sum of individual cells that were viewed. The standard deviation is shown.

Proteasome Targeting Defect of \( \text{sts1-2} \) Is Suppressed by \( \text{Sts1} \)

To confirm that the reduced level of nuclear proteasomes was a result of a defect in the \( \text{sts1-2} \) protein, we transformed \( \text{sts1-2} \) (expressing Pre6-GFP) with a plasmid expressing wild type Sts1. As expected, proteasomes were inefficiently targeted to nuclei in \( \text{sts1-2} \) (Fig. 3A) after incubation at 37 °C for 4 h. However, expression of FLAG-Sts1 fully reversed this translocation defect, and the signals from GFP and DAPI were co-localized. These results confirmed that the proteasome
levels might be limiting. Moreover, Sts1 in Proteasomes cell. (Fig. 1 pombe (24) are both highly unstable proteins, raising the pos-
type (Fig. 3). Intriguingly, the GFP staining intensity in

**FIGURE 3. Proteasome translocation defect of sts1-2 is fully suppressed by plasmid-encoded Sts1.** A, high copy plasmid expressing FLAG-Sts1 was transformed into sts1-2, and the localization of integrated Pre6-GFP was examined at 37 °C. The localization defect is evident in sts1-2. However, this defect was completely rescued by expression of FLAG-Sts1. DAPI staining of the nuclei showed that the Pre6-GFP signal was predominantly co-localized to the nucleus. B, microscopy data were quantified (n, the number of cells quantified), and complete rescue of the trafficking defect of sts1-2 was confirmed.

translocation defect is specifically associated with the sts1-2 mutant. Rescue of the localization defect of sts1-2 by Sts1 was also verified by examining the subcellular distribution of other GFP-tagged proteasome subunits (data not shown). Because the temperature-sensitive growth defect of sts1-2 is overcome by FLAG-Sts1 (17), we conclude that cell growth is also verified by examining the subcellular distribution of other GFP-tagged proteasome subunits (data not shown). We immunoprecipitated FLAG-Sts1 from whole cell extracts and confirmed that the addition of CuSO4 increased the expression of Sts1. Sts1 was also higher in the absence of CuSO4 because the gene is expressed from a high copy plasmid. Higher levels of FLAG-Sts1 were detected when CuSO4 was added to the medium (Fig. 4C, 50 μM). Addition of 200 μM CuSO4 did not increase FLG-Sts1 levels significantly (beyond the level seen with 50 μM CuSO4), consistent with the nuclear targeting data (Fig. 4A). Sts1 interaction with the proteasome was confirmed by the co-precipitation of Rpt1. Yeast expressing only vector (V) showed no precipitation of Rpt1, although a faint cross-reaction with the immunoglobulin heavy chain is detected (Fig. 4C, asterisk). These results suggest that the nuclear targeting of proteasomes is influenced by the availability of Sts1, as its overexpression increased nuclear targeting and depleted proteasomes from the cytosol.

**Sts1 Overexpression Increases Nuclear Localization of Proteasomes**—The results shown in Fig. 3 suggested that Sts1 levels might be limiting. Moreover, Sts1 in Saccharomyces cerevisiae (Fig. 1B) (17) and Cut8 in Schizosaccharomyces pombe (24) are both highly unstable proteins, raising the possibility that Sts1 abundance might influence proteasome localization. To explore this idea, we overexpressed Sts1 in wild type (STS1) cells from a high copy plasmid, using the copper-inducible P_{CUP1} promoter. Expression was induced by adding CuSO4 directly to the growth medium. In comparison with “vector-only” control (that contained 200 μM CuSO4), we determined that by increasing the levels of Sts1 nuclear localization of Pre6-GFP was appreciably elevated, even without addition of CuSO4 (Fig. 4A). The addition of 50 μM CuSO4 to the culture medium further increased GFP localization to the nucleus. However, providing 200 μM CuSO4 had no additive effect on the levels of nuclear proteasomes. The quantified data (Fig. 4B) showed that the nuclear targeting of proteasomes was noticeably increased by higher levels of Sts1. Statistical analysis showed that the difference between vector-alone and plasmid-encoded Sts1 (0 μM CuSO4) was significant (p < 0.001). A further increase in nuclear localization of Pre6-GFP occurred in the presence of 50 μM CuSO4 (0–50 μM CuSO4, p < 0.007). Expressing higher levels of Sts1 (200 μM CuSO4) resulted in very low levels of GFP fluorescence in the cytosol, suggesting complete nuclear localization of proteasomes. Similar results were observed when we tracked a different proteasome subunit (Rpn1-GFP; data not shown). We immunoprecipitated FLAG-Sts1 from whole cell extracts and confirmed that the addition of CuSO4 increased the expression of Sts1. Sts1 was also higher in the absence of CuSO4 because the gene is expressed from a high copy plasmid. Higher levels of FLAG-Sts1 were detected when CuSO4 was added to the medium (Fig. 4C, 50 μM). Addition of 200 μM CuSO4 did not increase FLG-Sts1 levels significantly (beyond the level seen with 50 μM CuSO4), consistent with the nuclear targeting data (Fig. 4A). Sts1 interaction with the proteasome was confirmed by the co-precipitation of Rpt1. Yeast expressing only vector (V) showed no precipitation of Rpt1, although a faint cross-reaction with the immunoglobulin heavy chain is detected (Fig. 4C, asterisk). These results suggest that the nuclear targeting of proteasomes is influenced by the availability of Sts1, as its overexpression increased nuclear targeting and depleted proteasomes from the cytosol.

**Sts1 Forms Independent Interactions with Srp1 and Rpn11**—Sts1 performs an important role in the ubiquitin/proteasome pathway (17). We speculate that its interaction with the proteasome subunit Rpn11 is related to its function in the ubiquitin/proteasome system (Fig. 1C). Sts1 interaction with Srp1 was proposed to function in nucleocytoplasmic trafficking (19). We note that srp1-49 has a defect in protein degradation that is suppressed by high levels of Sts1 (19). This finding suggested to us that Srp1 might be functionally linked to Rpn11, through their mutual interactions with Sts1. We speculated that Srp1/Sts1 and Sts1/Rpn11 interactions might guide the translocation of proteasomes to the nucleus. Therefore, the protein degradation defect of srp1-49 could be due to reduced levels of nuclear proteasomes (18). To investigate if Srp1 and Rpn11 competed for binding to Sts1, we simultaneously added both His6-Srp1 and His6-Rpn11 to immobilized GST-Sts1. The binding reactions contained a fixed amount of His6-Srp1 and increasing amounts of His6-Rpn11. We found that GST-Sts1 interaction with His6-Srp1 was unaffected by the amount of His6-Rpn11 that was present (Fig. 5A, compare lane 1 with lane 4). These results are consistent with the interpre-
tation that Srp1 and Rpn11 bind distinct regions in the Sts1 protein. Furthermore, prior binding of Sts1 to Rpn11 did not improve or interfere with subsequent interaction with Srp1 (data not shown). Similarly, the Sts1/Srp1 interaction did not affect Sts1 binding to Rpn11. We recognize that these data do not exclude the possibility that the interactions might represent separate complexes with Sts1. No binding was detected with GST control beads (Fig. 5A, lane 5).

**NLS in Sts1 Is Required for Interaction with Srp1**—To further characterize these interactions, we immobilized GST-Sts1 and mutant derivatives on glutathione-Sepharose beads and tested their interaction with purified His$_6$-tagged Srp1 and Rpn11 (Fig. 5B). Following a 3-h incubation at 4 °C, the unbound proteins were removed, and the bound proteins were released into SDS-containing buffer and resolved by SDS-PAGE. Consistent with earlier results, no binding was detected with GST (Fig. 5B, lane 2), and Sts1 and sts1-2 formed comparable interactions with both His$_6$-Srp1 and His$_6$-Rpn11 (Fig. 5B, lanes 3 and 4). However, because sts1-2 is highly unstable in vivo (Fig. 1B), its interaction with Srp1 and Rpn11 is expected to be significantly reduced under physiological conditions.

We investigated if the NLS in Sts1 was required for its interaction with Srp1. We incubated recombinant His$_6$-Srp1 and His$_6$-Rpn11 with immobilized sts1 mutants, including GST-sts1$^{H9004}$NLS (Fig. 5B, lane 5). We confirmed that an Sts1 mutant lacking the NLS (sts1$^{H9004}$NLS) does not bind Srp1 (lane 5) (19). In contrast, another Sts1 mutant (sts1-11,12) formed efficient interactions with both Srp1 and Rpn11 (Fig. 5B, lane 6). Although sts1$^{ΔNLS}$ also failed to bind Rpn11 (Fig. 5B, lane 5), we suspected this interaction was adversely affected by the presence of GST. Moreover, Tabb et al. (19) had previously described a direct interaction between Sts1 and Rpn11.
Further evidence of a role for the Sts1 NLS is shown in Fig. 5C. GST-Srp1 was immobilized and incubated with recombinant His<sub>c</sub>-Sts1 and various mutant derivatives. As expected, His<sub>c</sub>-sts1<sup>A NLS</sup> failed to bind GST-Srp1 (Fig. 5C, lane 3). His<sub>c</sub>-sts1-2 and His<sub>c</sub>-sts1-11,12 formed weak interactions with GST-Srp1 (Fig. 5C, lanes 2 and 4), proportional to their lower expression in E. coli (Fig. 5E). The interactions were specific because incubation of His<sub>c</sub>-Sts1 with GST showed no binding. Collectively, these studies show that the nuclear localization of proteasomes is reduced by preventing Srp1 interaction with Sts1 (sts1<sup>A NLS</sup>).

We performed a reciprocal experiment in which recombinant GST-Rpn11 was immobilized on glutathione-Sepharose and incubated with His<sub>c</sub>-Sts1, His<sub>c</sub>-sts1-2, His<sub>c</sub>-sts1<sup>A NLS</sup>, and His<sub>c</sub>-sts1-11,12. All four proteins formed interactions with GST-Rpn11 (Fig. 5D, lanes 2–5), demonstrating that the NLS in Sts1 is not required for binding Rpn11. The expression level of the various His<sub>c</sub>-tagged proteins indicates that the interaction with Rpn11 is proportional to the amount of the Sts1 proteins available in E. coli extracts (Fig. 5E).

**NLS in Sts1 Is Required for Targeting Proteasomes to the Nucleus**—The ability of mutant sts1 proteins to suppress the temperature-sensitive growth defect of sts1-2 was assessed at 23 and 37 °C. Sts1 and sts1-11 (19) suppressed the growth defect of sts1-2 at 37 °C (Fig. 6A, lower panels). However, sts1<sup>A NLS</sup> was unable to support growth of sts1-2 at 37 °C. Because stts1<sup>A NLS</sup> is unable to bind Srp1, we speculate that its inability to suppress sts1-2 is due to a proteasome translocation defect. We therefore examined the distribution of Pre6-GFP (Fig. 6B) and found that the proteasome localization defect of stts1-2 was not suppressed by stts1<sup>A NLS</sup>. In contrast, Sts1 fully suppressed this proteasome translocation defect. The quantified data (Fig. 6C) showed that STS1 containing vector and stts1-2 expressing Sts1 from a low copy (CEN) plasmid had equivalent levels of nuclear proteasomes. In contrast, expression of stts1<sup>A NLS</sup> was distinguishable from stts1-2 containing only vector. We conclude that the failure of stts1<sup>A NLS</sup> to bind Srp1 underlies the inefficient nuclear localization of proteasomes and demonstrates the importance of Sts1/Srp1 interaction for cell viability.
Rapid Release of Cytoplasmic Proteasome from *sts1-2* Cells—

To quantify the higher levels of proteasomes in the cytosol of *sts1-2*, we rapidly lysed cells by sonication (30). This approach can permit rupture of the cell wall and membrane, without disrupting nuclear integrity. We co-expressed Rpn1-GFP in *sts1-2* with either an empty plasmid or the same plasmid expressing FLAG-Sts1. We also expressed the nuclear pore protein Nup49-GFP, and the cytosolic reporter GFP in *STS1*, to provide a way to monitor the efficiency of cell lysis. The localization of both these proteins was predicted to be unaffected in *sts1-2*. Yeast cells were grown to exponential phase and transferred to pre-warmed medium at 37 °C. Following 5 h of incubation at the nonpermissive temperature (37 °C), the cells were pelleted and lysed by brief pulses of ultrasonication. Examination of the cells post-sonication showed that overall morphology and nuclear integrity remained intact (Fig. 7A).
After 20 s of sonication, proteasomes were still localized to the nuclei in *sts1-2* expressing FLAG-Sts1, and similarly, Nup49-GFP was detected in the nuclear envelope in *STS1*. Following each ultrasonic pulse, cells were pelleted, and the supernatant was examined for the release of GFP-Rpn1 (Fig. 7B). We found that in wild type cells (*sts1-2* expressing FLAG-Sts1), Rpn1-GFP was detected in the medium only after 20 s of continuous sonication. Similarly, nuclear integrity was unaffected following shorter pulses of treatment (as determined by microscopy and the retention of Nup49-GFP in the cells). The release of Nup49-GFP into the medium was not observed until the cells had been treated for >30 s. In striking contrast, higher levels of GFP-Rpn1 were released from *sts1-2* into the medium following 5 s of treatment. As expected, cytosolic GFP was also rapidly released from *STS1*, providing strong support for the hypothesis that mislocalized proteasomes in *sts1-2* accumulate in the cytosol. It is also significant that the values measured from four independent experiments offer very robust statistical confirmation of this hypothesis. Cell fractionation studies also lend support for this model (supplemental Fig. 1). Whole cell extracts were partitioned into cytosolic and crude nuclei fractions and characterized by immunoblotting. Higher levels of proteasome subunits were detected in the cytosol of *sts1-2*.

**srp1 Mutant Is Defective in Nuclear Translocation of Proteasomes**—Of two *srp1* mutants characterized by Tabb et al. (19) (*srp1-31* S116F; *srp1-49* E145K), only *srp1-49* had a defect in protein degradation. This mutant was subsequently reported to mislocalize proteasomes at the nonpermissive temperature (18). We predicted that if Sts1/Srp1 interaction promoted nuclear translocation of proteasomes, then an *srp1* mutant protein that is deficient in binding Sts1 would also have lower levels of nuclear proteasomes. We therefore examined *srp1-31* and *srp1-49* interactions with Sts1. GST-Srp1, GST-*srp1-31*, and GST-*srp1-49* were bound to glutathione-Sepharose and incubated with His<sub>6</sub>-Sts1 (Fig. 8A). The bound protein was identified by immunoblotting and quantified (Fig. 8B). Although higher levels of GST-*srp1-49* were present on the Sepharose beads, ~3-fold lower amounts of His<sub>6</sub>-Sts1 were co-purified (Fig. 8B, lane 5). His<sub>6</sub>-Sts1 formed similar interactions with GST-Srp1 and GST-*srp1-31* (Fig. 8A, lanes 3 and 4). In a reciprocal experiment, recombinant His<sub>6</sub>-Srp1, His<sub>6</sub>-*srp1-31*, and His<sub>6</sub>-*srp1-49* were incubated with immobilized GST-Sts1 (Fig. 8C). Although equal amounts of His<sub>6</sub>-tagged Srp1, *srp1-31*, and *srp1-49* were present on the matrix, His<sub>6</sub>-*srp1-49* showed reproducibly reduced interaction with GST-Sts1 that was confirmed by densitometry (Fig. 8D). The proteolytic defect of *srp1-49* could be caused by reduced proteasome integrity and function or by poor nuclear localization. To address this, we examined total protein extracts in a native in-gel assay and found that proteasomes were fully active in *srp1-49*, and the assembly resembled the wild type strain (Fig. 8E).

The distribution of proteasomes was examined in *SRP1*, *srp1-49*, and *srp1-31* using Rpn1-GFP. The level of nuclear proteasomes was reduced in *srp1-49* but not as significantly in *srp1-31* (Fig. 9A), consistent with the previously reported protein degradation defect of *srp1-49* (19). It is significant in this regard that *srp1-49*, but not *srp1-31*, showed reduced binding to Sts1 (Fig. 8A). The fluorescence data were quantified (Fig. 9B) and showed reduced nuclear staining intensity in *srp1-49*. Nuclear GFP staining in *SRP1* was ~2-fold higher than in *srp1-49*, consistent with reduced nuclear localization in the mutant. A trend toward lower nuclear localization was also
evident in srp1-31, although the data were not statistically robust. In contrast, proteasome mislocalization in srp1-49 was highly significant \((p < 0.001)\). We propose that Srp1 stimulates protein degradation by binding Sts1 and recruiting proteasomes to the nucleus.

**Sts1 Restores Nuclear Targeting of Proteasomes in rad23Δ/rpn10Δ**—Rad23 translocates ubiquitinated proteins to the proteasome (9). Rpn10 represents a major proteasome receptor for multiubiquitinated proteins (11). We reported previously that the loss of both Rad23 and Rpn10 \((rad23Δ/rpn10Δ)\) caused pleiotropic growth and proteolytic defects (16), revealing a functional link between these proteins (16). The defects of rad23Δ/rpn10Δ were suppressed by high levels of Sts1 (17). Although Rad23 and Rpn10 can bind proteasomes and multiubiquitinated proteins (25, 26), we determined that Sts1 only interacts with the proteasome (17). This finding suggested that Sts1 did not suppress rad23Δ/rpn10Δ by functionally replacing either Rad23 or Rpn10.

We investigated the subcellular distribution of proteasomes in rad23Δ/rpn10Δ. A DNA construct expressing Rpt1-GFP was integrated in rad23Δ/rpn10Δ, and the subcellular distribution of the GFP signal was examined in actively growing cells at 18 °C (which is a semi-permissive temperature for rad23Δ/rpn10Δ). Proteasomes were significantly mislocalized (Fig. 10A). However, expression of Sts1 from the galactose-inducible P\(_{GAL1}\) promoter restored localization of protea-

**FIGURE 9. srp1 mutant displays defective proteasome localization.**

A, Rpn1-GFP was detected by immunofluorescence, and defective nuclear targeting of proteasomes was observed in srp1-49. The defect was less severe in srp1-31. B, fluorescence signals were quantified, and significant decreases in nuclear staining \((p < 0.001)\) were evident in srp1-49 compared with the SRP1 wild type strain.
re-established normal levels of multiubiquitinated proteins. We prepared protein extracts from wild type (RAD23 RPN10) and rad23Δ rpn10Δ (Fig. 10B) and examined the effect of overexpressing Sts1. Equal amounts of protein were separated and immunoblotting showed that normal levels of multiubiquitinated proteins were detected when Sts1 was overexpressed in rad23Δ rpn10Δ. The abundance of the proteasome (indicated by Rpn12) was unaffected. One interpretation of this result is that by successfully restoring proteasome targeting to the nucleus, Sts1 re-established the efficient turnover of nuclear substrates in rad23Δ rpn10Δ. Further study will be required to test the idea that defective proteasome targeting in rad23Δ rpn10Δ interferes with the degradation of nuclear proteins and accounts for the pleiotropic defects of this double mutant.

**Turnover of a Nuclear Protein Is Reduced in sts1-2**—A simple prediction of our findings is that nuclear substrates should be stabilized if proteasomes are inefficiently targeted to the nucleus in sts1-2. We tested this hypothesis by expressing Clb2-HA at physiological levels in STS1 and sts1-2. Protein extracts were prepared from cells grown at 23, 30, and 37 °C (Fig. 11A). Equal amount of protein was resolved by SDS-PAGE and examined by immunoblotting. We found that Clb2-HA levels were increased in sts1-2, at all three temperatures. The levels of Clb2-HA were standardized to the abundance of Rpn12 (Fig. 11B). The quantified results indicated that Clb2-HA levels increased 3-fold in sts1-2 at 37 °C. The same extracts were also incubated with antibodies against the HA epitope to immunoprecipitate Clb2-HA. Higher levels were recovered from sts1-2 at all three temperatures (Fig. 11C). Fig. 11C, lane 6, showed evidence for higher molecular weight derivatives of Clb2-HA, consistent with the accumulation of multiubiquitinated Clb2 in sts1-2. A longer exposure of this filter showed clear evidence for higher molecular weight forms of Clb2-HA (Fig. 11D, arrow). The filter shown in Fig. 11D was subsequently probed with antibodies against ubiquitin. In agreement with the aforementioned results, the high molecular weight species detected with anti-HA antibodies appear to represent multiubiquitinated forms of Clb2-HA (Fig. 11E, arrow). Total extracts were also examined, and as we showed previously (17), the levels of multiubiquitinated proteins increased in sts1-2 at all temperatures examined (supplemental Fig. 2).

**Increased Turnover of a Cytosolic Substrate in sts1-2**—The prediction that nuclear proteins should be stabilized if proteasomes are inefficiently targeted to the nucleus in sts1-2 would affect the turnover of cytosolic substrates. Testing this idea is not straightforward because few proteins have been clearly shown to be degraded by the proteasome in the cytosol. We avoided examining proteins linked to endoplasmic reticulum-associated degradation because in S. cerevisiae the endoplasmic reticulum membrane is closely associated with the nuclear envelope. We therefore examined the turnover of an engineered substrate, Ura3-SL17 (28). This substrate contains Ura3-HA with a carboxyl-terminal extension that promotes rapid turnover, without involvement of the endoplasmic reticulum. Ura3-SL17 was expressed in STS1 and sts1-2, and growth on synthetic medium lacking uracil

![FIGURE 10. Sts1 can suppress the proteasome localization defect of rad23Δ rpn10Δ.](image)

We showed previously that high levels of multiubiquitinated proteins accumulated in sts1-2 (17). This defect intensified when the cells were incubated at the nonpermissive temperature. Similarly, the levels of multiubiquitinated proteins increased in rad23Δ rpn10Δ (16). Based on our findings that proteasomes are mislocalized in rad23Δ rpn10Δ and sts1-2, we questioned if restoring nuclear targeting of proteasomes to the nucleus in rad23Δ rpn10Δ. Quantification of these data is difficult because of the aberrant cell morphology and the high levels of nuclear fragmentation and cell death. As seen in Fig. 10A, DAPI staining was poor in rad23Δ rpn10Δ.

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was examined (Fig. 12A). No growth was observed in the wild type strain (STS1), consistent with rapid degradation of this reporter protein. Significantly, no growth was detected in sts1-2, demonstrating that the cytosolic degradation of Ura3-SL17 was unaffected. In contrast, growth was observed in pre1-2 pre2-2, which expresses a peptidase-deficient proteasome. These results demonstrate that although Ura3-SL17 degradation requires the proteasome, it does not involve Sts1.

We also confirmed previous studies that showed that Ura3-SL17 degradation requires ubiquitination by the Ubc6 and Ubc7 ubiquitin-conjugating enzymes (28).

Based on these findings, we examined the steady-state abundance of Ura3-SL17 (Fig. 12B). Total extracts were examined by immunoblotting, and we detected very low levels of Ura3-SL17 in STS1 and sts1-2 (Fig. 12B, lanes 1 and 2), as well as in pre1-1 pre2-2 (lane 3). Stabilization of Ura3-SL17 was more easily observed in ubc6Δ ubc7Δ, consistent with previous reports (Fig. 12B, compare lanes 4 and 5). The higher basal level in these strains (Fig. 12B, lanes 4 and 5) is due to a different genetic background. The expression of Rad23 and Rpn12 is also shown, and their levels are similar in STS1 and sts1-2. Total extracts were also examined for the levels of multoubiquitinated proteins, and high levels were detected in pre1-1 pre2-2 (Fig. 12B, lower panel, lane 3), consistent with its defect in proteasome function. Higher levels were also observed in sts1-2 (lane 2; and see supplemental Fig. 2). The reduced levels of ubiquitinated species in ubc6Δ ubc7Δ were not observed consistently (Fig. 12B, lane 4). (As noted earlier, the strains shown in Fig. 12B, lanes 1–3 are in a different genetic background than strains in lanes 4 and 5).

Ura3-SL17 contains an HA epitope and was immunoprecipitated from the strains described. The purified proteins were examined by immunoblotting, using antibodies against the HA epitope (Fig. 12C). Consistent with the results in Fig. 12B, we detected very low levels of Ura3-SL17 in sts1-2, indicating significantly accelerated degradation. Stabilization of this substrate was observed in both pre1-1 pre2-2 (Fig. 12B, lane 3) and in ubc6Δ ubc7Δ (lane 4). The same filter was incubated with antibodies against ubiquitin. Evidence for higher molecular weight derivatives of Ura3-SL17 is evident in the proteasome mutant (Fig. 12C, lower panel, lane 3). These results agree with previous reports that showed that Ura3-SL17 is degraded by the ubiquitin/proteasome system. Our findings demonstrate that the turnover of this cytosolic substrate is not prevented in sts1-2. Remarkedly, the abundance of Ura3-SL17 actually decreased, suggesting that the higher levels of cytosolic proteasomes in sts1-2 can accelerate turnover of cytosolic substrates.

**DISCUSSION**

The failure to localize proteasomes to the nucleus underlies the temperature-sensitive growth defect of sts1-2. The target-

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**FIGURE 11. Stabilization of a ubiquitinated protein in sts1-2.** A, gene expressing Clb2-HA was integrated in STS1 and sts1-2, and extracts were prepared at 23, 30, and 37 °C. Total protein extract was examined by immunoblotting. Clb2-HA levels were elevated in sts1-2 at all three temperatures. In contrast, the abundance of proteasome subunit Rpn12 was essentially unchanged. B, abundance of Clb2-HA was standardized to the level of Rpn12 by densitometry and plotted. The most significant increase was observed at the nonpermissive temperature (37 °C), consistent with the proteasome mislocalization defect of this mutant. C, extracts described above were incubated with antibodies against the HA epitope to purify Clb2-HA. The purified protein was examined by immunoblotting and incubation with anti-HA antibodies. Consistent with the results in A, we detected higher levels of Clb2-HA in sts1-2 (lanes 2, 4, and 6). D, longer exposure of the image shown in C reveals higher molecular weight forms of Clb2-HA, consistent with multoubiquitination. A temperature-specific accumulation is evident, because extracts prepared from cultures grown at 37 °C showed the highest levels (compare lanes 5 and 6). E, filter shown in D was stripped and reprobed with antibodies against ubiquitin, and a strong reaction was detected. As noted above, the intensity of this modification was higher at 37 °C (lane 6), when the proteasome trafficking defect of sts1-2 is most severe.
Proteasome Targeting to the Nucleus

We isolated Sts1 as a dosage suppressor of the proteolytic defects of rad23Δ rpn10Δ (17). This mutant lacks the Rad23 shuttle factor that delivers ubiquitinated proteins to the proteasome, as well as Rpn10, a proteasome receptor for ubiquitinated proteins. Proteasome integrity and function were unaffected in rad23Δ rpn10Δ, suggesting that the proteolytic defects were caused by some other deficiency. We show here that rad23Δ rpn10Δ has reduced levels of nuclear proteasomes. We determined that Sts1 could restore the levels of nuclear proteasomes in rad23Δ rpn10Δ.

Our studies showed that proteasomes are assembled and catalytically active in sts1 mutants. The protein degradation defect of sts1-2 (17) is closely linked to its failure to target proteasomes to the nucleus. Overexpression of Sts1 noticeably increased the levels of nuclear proteasomes (Fig. 4) while depleting proteasome levels in the cytoplasm. Overexpression of Sts1 had no effect on proteasome abundance or function, consistent with a specific role in subcellular trafficking. We propose that Sts1 is rate-limiting in this targeting mechanism, because Sts1 in S. cerevisiae and a related protein in S. pombe (Cut8) are both highly unstable (18). Sts1-2 mutant protein can form normal interactions with its cellular partners (Srp1; Rpn11), but due to its rapid degradation, the levels of nuclear proteasomes are significantly diminished. The failure of sts1ANLS to target proteasomes is mechanistically different from sts1-2, but the effect is similar. Overexpression of Sts1 increased the level of nuclear proteasomes, consistent with a previously reported role for Sts1 and Cut8 in this process (24, 27).

The significance of Srp1/Sts1 and Sts1/Rpn11 interactions was not fully understood, and it was proposed that these interactions might have different biochemical effects (19). The yeast Srp1 protein binds nuclear localization signals to translocate proteins into the nucleus. Strikingly, a mutant form of Srp1 (srp1-49) showed reduced binding to Sts1 (Fig. 8) (19) and reduced levels of nuclear proteasomes (Fig. 9) (18). We note that the nuclear translocation defect of srp1-49 was weaker than that observed in sts1-2. We speculate this occurs because srp1-49 can form a weak interaction with Sts1, whereas sts1ANLS failed to bind Srp1. Collectively, these results suggest that Sts1, Srp1, and Rpn11 represent essential components of a pathway that target proteasomes to the nucleus. The Srp1 protein (importin-α) has been characterized extensively as a central component of the nuclear import machinery. In contrast to the many factors that compose the nuclear pore, Srp1 is a soluble protein. The interaction between Srp1 and Sts1, and the resulting trafficking of proteasomes to the nucleus, raises interesting questions regarding the ultimate destination of nuclear proteasomes. Although subunits of the proteasome have been detected inside the nucleus, it is far less clear if intact and proteolytically active proteasomes exist in the nucleus. Our findings describe a key initiator step leading to the trafficking of proteasomes to the nucleus. Although our findings support the idea that proteasomes reside on the nuclear periphery, we cannot discount alternative locations, including the inner surface of the nuclear envelope.

Another line of evidence that links Sts1 to protein degradation is the effect of overexpression. High levels of Sts1 increased the levels of nuclear proteasomes in wild type, sts1-1, and rad23Δ rpn10Δ cells. This can explain why the proteasome localization defect of rad23Δ rpn10Δ was suppressed by overexpressing Sts1. A nuclear targeting defect is expected to cause accumulation of multiubiquitinated proteins, ultimately leading to cell death. In agreement, the levels of multiubiquitinated proteins increased in sts1-2, although they were not associated with proteasomes at their correspondingly high levels (17). This observation suggests that nuclear ubiquitinated proteins may accumulate when there are insufficient levels of proteasomes at the nucleus.

We showed previously that the nuclear protein Sic1 is stabilized in sts1-2 (9). We show here that another nuclear protein, Clb2, is also stabilized in sts1-2 and, significantly, was conjugated to multiubiquitin chains. This effect was exacerbated at 37 °C when the nuclear localization defect of sts1-2 is most severe. Based on these findings, we conclude that nu-
clear proteins are successfully ubiquitinated in sts1-2 but fail to bind proteasomes, because they are not targeted to the nucleus. Further study will be required to determine whether the failure to localize proteasomes to the nucleus is the immediate cause of nuclear substrate stabilization. A previous report that defective proteasomes (cin5-1; cin3-1) can be successfully delivered to the nucleus suggests that catalytic function is not required for nuclear localization (18).

Previous studies from Enenkel et al. (2) showed that a major fraction of proteasomes in yeast (~80%) are targeted to the nucleus. Proteasomes can be co-purified with nuclear/endoplasmic reticulum membranes, demonstrating that they are localized to the nuclear envelope (29). As noted earlier, the present studies do not ascertain if proteasomes targeted by Sts1 enter the nucleus or remain tethered to the cytoplasmic surface of nuclei.

Multiple mechanisms may co-exist to traffic proteasomes to the nucleus. Several proteasome subunits contain NLS that can promote nuclear targeting (18). Consequently, Sts1/Rpn11 interaction might not represent the sole mechanism for trafficking proteasomes. Because Srp1 is intact in cells expressing either sts1-2 or sts1 ΔNLS, its interaction with the NLS in other proteasome subunits should be unaffected (18). However, removal of the NLS in Sts1 (sts1 ΔNLS) caused a complete loss of nuclear proteasomes, indicating that Sts1 plays a central role in this mechanism. We propose that Sts1 either participates in a major mechanism for mobilizing proteasomes or is an essential component in multiple targeting pathways.

Although the Sts1/Rpn11 binding is weak, this interaction is reproducible, in agreement with previous two-hybrid studies (18). Sts1 interaction with Rpn11 is specific because they are not targeted to the nucleus. Several proteasome subunits contain NLS that can promote nuclear targeting (18). However, removal of the NLS in Sts1 (sts1 ΔNLS) caused a complete loss of nuclear proteasomes, indicating that Sts1 plays a central role in this mechanism. We propose that Sts1 either participates in a major mechanism for mobilizing proteasomes or is an essential component in multiple targeting pathways.

Although the Sts1/Rpn11 binding is weak, this interaction is reproducible, in agreement with previous two-hybrid studies (18). Sts1 interaction with Rpn11 is specific because no binding was detected with other recombinant proteasome subunits (Rpn1, Rpn8, and Rpn10; data not shown). We propose that by binding both Rpn11 and Srp1, Sts1 guides the interaction of proteasomes with the nucleus. Previous studies indicated that subcomplexes of the proteasome might be independently targeted to the proteasome (18). However, our finding shows that components of both 19S and 20S particles are mislocalized in sts1-2, suggesting that intact proteasomes are targeted to the nucleus by Sts1. Whether Sts1 binds the proteasome first (via Rpn11), or Srp1 (via NLS), remains to be determined.

These studies provide insight into the mechanism for nuclear targeting of proteasomes. We propose that a tripartite interaction involving Srp1, Sts1, and Rpn11 promotes this mechanism. We reported previously that the interaction between proteasomes and multiquitinated proteins was significantly reduced in sts1-2 (18). We suggest that if proteasomes are not successfully translocated to the nucleus, nuclear substrates are ubiquitinated but cannot be degraded. This idea is supported by our finding that the nuclear subunit Clb2-HA is ubiquitinated but stabilized in sts1-2. However, a cytoplasmic reporter protein, Ura3-SL17, was degraded more rapidly, suggesting that the availability of increased amounts of cytosolic proteasomes can accelerate the turnover of cytosolic substrates. Taken together, these findings suggest that efficient protein degradation requires adequate levels of nuclear proteasomes.

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