Sts1 Can Overcome the Loss of Rad23 and Rpn10 and Represents a Novel Regulator of the Ubiquitin/Proteasome Pathway*

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A rad23Δ rpn10Δ double mutant accumulates multi-Ub proteins, is deficient in proteolysis, and displays sensitivity to drugs that generate damaged proteins. Overexpression of Sts1 restored normal growth in rad23Δ rpn10Δ but did not overcome the DNA repair defect of rad23Δ. To understand the nature of Sts1 suppression, we characterized stts1-2, a temperature-sensitive mutant. We determined that stts1-2 was sensitive to translation inhibitors, accumulated high levels of multi-Ub proteins, and caused stabilization of proteolytic substrates. Additionally, ubiquitinated proteins that were detected in proteasomes were inefficiently cleared in stts1-2. Despite these proteolytic defects, overall proteasome activity was increased in stts1-2. We propose that Sts1 is a new regulatory factor in the ubiquitin/proteasome pathway that controls the turnover of proteasome substrates.

A major pathway of intracellular protein degradation involves the attachment of a multi-ubiquitin (multi-Ub)3 chain to proteolytic substrates. Ubiquitinated proteins are degraded by the multicatalytic proteasome (8). The mechanism of substrate recognition, ubiquitination, and proteasome-specific hydrolysis is well described. However, the events that promote the recognition of multi-Ub substrates by the proteasome are not well defined. For instance, it is unclear whether multi-Ub substrates form unaided affinity-based interactions with the proteasome in vivo or whether regulatory proteins deliver substrates to the proteasome. It is also unclear whether compositionally distinct proteasomes promote the degradation of unique classes of substrates.

Rad23 and Rpn10 are multi-Ub chain-binding proteins that play an important role in the degradation of substrates by the proteasome (1–3, 5). Although a significant proportion of purified proteasomes contains Rpn10 (9), only a small fraction contains Rad23 (10). In an effort to understand the role of Rad23, we generated a yeast strain lacking both Rad23 and Rpn10 and discovered that the double mutant is severely growth impaired and displays significant proteolytic defects (3). Rad23 contains two autonomous sequences: UbL, which binds the proteasome, and UBA domains that interact with mult ubiquitin chains. Based on its ability to bind both ubiquitinated proteins and the proteasome, we proposed that Rad23 translocated proteolytic substrates to the proteasome (2). In this model, Rpn10 would represent an ideal proteasome receptor for Rad23-specific substrates. We also determined that expression of high levels of the Ubl domain caused stabilization of a reporter protein (2). A simple interpretation of this result is that Ubl/proteasome binding interferes with the normal docking of native Rad23 with the proteasome, consistent with a shuttle-factor model. In contrast, expression of a Rad23 derivative lacking the Ubl domain, but containing UBA sequences, retains efficient interaction with mult ubiquitinated proteins but not the proteasome (2). We conclude from these findings that Rad23 binds mult ubiquitin proteins, before it interacts with the proteasome. These two activities are functionally linked because single amino acid mutations in either Ubl or UBA domains cause a proteolytic defect. Further support for the shuttle-factor model was described recently (11). It has also been proposed that Rad23 and Rpn10 function as alternate multi-Ub chain-binding receptors in the proteasome (4, 7).

To understand the proteolytic defect of rad23Δ rpn10Δ, we sought high copy suppressors of its cold temperature growth defect. We isolated Sts1, a poorly characterized protein that lacks distinctive motifs that might offer insight into a biochemical function. However, previous studies showed that Sts1 is required for efficient chromosome segregation (12), maintaining rRNA stability, and suppressing an ER/Golgi secretion defect (13). Moreover, a mutation in STS1 suppressed the mRNA processing defect of an rna15 mutant (14). Sts1 also interacts with proteasome subunits Rpn11 and Srp1, a nuclear localization signal-binding protein (15). The effects of these seemingly unrelated genetic and biochemical interactions have not been resolved. However, the finding that high level expression of Sts1 restored the degradation of a proteolytic substrate in an srp1 mutant suggested an unexpected role in proteolysis. Additionally, a distantly related protein in Schizosaccharomyces pombe (Cut8) regulates the subcellular trafficking of proteasomes (16, 17), providing further evidence of a likely proteolytic function for Sts1.

In this report, we show that overexpression of Sts1 suppressed the defects of rad23Δ rpn10Δ. However, the DNA repair defect associated with rad23Δ was not suppressed, and

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3 The abbreviations used are: Ub, ubiquitin; HA, hemagglutinin; β-gal, β-galactosidase.
sts1-2 is not sensitive to UV light. (We note that this is contrasted by the DNA damage sensitivity of an S. pombe cut8 mutant). We demonstrate that sts1-2 mutant accumulated high levels of multi-Ub proteins, displayed heightened sensitivity to drugs that generate damaged proteins, and failed to degrade test substrates of the ubiquitin/proteasome system. A physiological substrate (Sic1) is also stabilized in sts1-2. We also determined that multiquitinated proteins could bind the proteasome in sts1-2 but were inefficiently degraded. These compelling results indicate that Sts1 encodes a previously unrecognized factor that influences degradation by the ubiquitin/proteasome pathway.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids—Yeast rad23Δ rpn10Δ was transformed with a galactose-inducible yeast cDNA library (18), and STS1 was isolated as a high copy suppressor of the growth defect at 13 °C. FLAG-STS1 and FLAG-RPN10 were generated by PCR, and expression was induced from the P-clp1 promoter by the addition of 10 μM CuSO4 to the growth medium. SIC1 was amplified from yeast genomic DNA and cloned into YEplac181, containing two tandem hemagglutinin (HA) epitopes. The gene was expressed from the P-clp1 promoter. DNA encoding sts1-2 was amplified from NA25 (sts1-2A; Ref. 13), and all constructs were verified by DNA sequencing.

Growth Assays and Sensitivity to Translation Inhibitors—Yeast cells were grown in selective medium (~18 h), transferred to fresh medium, and grown to the exponential phase. The cultures were normalized to a density of 14,000/ml, and growth was measured by the number of colonies. A 10-fold serial dilution series was spotted on synthetic medium containing 2% glucose or galactose, and 1 mM paromomycin, or 1 mM neomycin. The plates were incubated at 30 °C, and 0.1-ml aliquots were withdrawn at the indicated times and frozen. Tri-chloroacetic acid precipitation and scintillation counting estimated incorporation of 35S into acid-insoluble proteins. Lysates containing equal 35S cpm were adjusted to equal volume and combined with anti-β-gal antibodies to immunoprecipitate β-gal proteolytic substrates (20). Autoradiograms were quantified using Kodak imaging software.

To examine Sic1-HA levels, we arrested growth of STS1 and sts1-2 in the G1 phase by growth into stationary phase. Yeast cells were then suspended for 30 min in medium containing 100 μM copper sulfate to induce expression of Sic1-HA. The cells were pelleted, washed, and resuspended in fresh medium containing 15 μM norethisterone to permit entry into mitotic growth but cause arrest in the G2 phase of the cell cycle. Aliquots of the cultures were withdrawn periodically and examined microscopically to gauge release from G1 arrest and to prepare protein extract. Equal amounts of protein were resolved by SDS/PAGE, and immunoblots were incubated with antibodies against the HA epitope and anti-Rad23 antibodies.

**Pulse-Chase Measurement of Protein Stability**—Protein stability measurements were described previously (19), using the EXPRE35S35S protein labeling reagent (Perkin-Elmer). Fifty milliliter cultures of exponentially growing cells were pelleted and resuspended in 0.4 ml of labeling buffer (50 mM sodium phosphate, pH 7.0, 2% glucose). EXPRES35S35S protein labeling mix (0.5 mCi) was added, and the suspension was incubated for 5 min at 30 °C. The cells were washed with 1 ml of water and resuspended in 0.4 ml of chase buffer (YPD-glucose medium containing unlabeled l-methionine and l-cysteine, and 0.5 mg/ml cycloheximide). An aliquot (0.1 ml) of the suspension was immediately withdrawn and frozen in liquid nitrogen. The rest of the suspension was incubated at 30 °C, and 0.1-ml aliquots were withdrawn at the indicated times and frozen. Tri-chloroacetic acid precipitation and scintillation counting estimated incorporation of 35S into acid-insoluble proteins. Lysates containing equal 35S cpm were adjusted to equal volume and combined with anti-β-gal antibodies to immunoprecipitate β-gal proteolytic substrates (20). Autoradiograms were quantified using Kodak imaging software.

**Microscopy**—Yeast strains were grown in synthetic medium and fixed in 5% ethanol, and the images were captured using a Zeiss microscope.

RESULTS

Sts1 Is a Dosage Suppressor of rad23Δ rpn10Δ—Loss of either Rad23 or Rpn10 does not cause a significant growth defect.
Sts1 Functions in the Ubiquitin/Proteasome System

However, loss of both proteins caused pleiotropic defects (3) that were suppressed by overexpressing Sts1 (PGAL1:STS1). Normal growth was restored in rad23Δ rpn10Δ at 13, 23, and 30 °C (Fig. 1A). Significantly, the growth improvement caused by overexpressing Sts1 was comparable with that observed by expressing Rad23 in rad23Δ rpn10Δ strains. The chase time points are in min. Significant stabilization was observed in the proteasome mutant pre1-1 pre2-2.

In Fig. 2, we speculated that a protein with similar binding properties might suppress rad23Δ rpn10Δ. Therefore, we investigated whether Sts1 possessed either of these biochemical properties of Rad23 and Rpn10.

Protein extracts were prepared from wild type cells expressing FLAG-Sts1, FLAG-Rad23, FLAG-Rpn10, or Pre1-FLAG, and applied to FLAG-agarose beads (Fig. 2A). The immunoprecipitated proteins were separated in SDS-PAGE and transferred to nitrocellulose. We note that the levels of FLAG-Rad23 and FLAG-Sts1 are quite similar. The immunoblot was incubated with antibodies against ubiquitin (Fig. 2A), and as expected, high levels of Ub cross-reacting material were co-purified with FLAG-Rad23 (lane 6; FLAG-IP) and FLAG-Rpn10 (lane 7). Lower levels of ubiquitinated protein were also co-purified with proteasomes (lane 5; Pre1-FLAG). However, no significant interaction with multi-Ub proteins was detected with FLAG-Sts1 (lane 8), demonstrating that, unlike Rad23 and Rpn10, it does not bind ubiquitin. The prominent ~70-kDa band detected in lane 8 is a nonspecific interaction and is also seen in the control immunoprecipitation from an extract lacking a FLAG-tagged protein (Ctrl). The expression of the FLAG-tagged proteins is shown in the lower panels following incubation of the nitrocellulose filter with anti-FLAG antibodies (left) and after staining the filter with Ponceau S (right). These findings suggest that Sts1 is not a substrate shuttle-factor or receptor for multi-Ub proteins. The expression of high levels of Sts1 resulted in a small increase in the overall levels of high molecular weight multi-Ub proteins in a wild type strain (Extracts;
**Sts1 Functions in the Ubiquitin/Proteasome System**

*Sts1 Can Bind the Proteasome*—Because Sts1/sts1-2 proteins are rapidly degraded (Fig. 1E), we surmised that their expression in a proteasome mutant would ease their biochemical characterization. Therefore, to determine whether Sts1 interacted with the proteasome, we expressed either FLAG-Sts1 or FLAG-sts1-2 in pre1-1 pre2-2 (Fig. 2B). Protein extracts were prepared and incubated with FLAG-agarose. Immunoblotting showed that a 19 S proteasome subunit, Rpt1, was co-purified with FLAG-Sts1 and FLAG-sts1-2 from cells grown at 23 and 37 °C (Fig. 2B; FLAG-IP). The same filter was also incubated with antibodies against Rpn12, and a similar interaction was observed. The expression of Rpt1 and Rpn12 was similar in *STS1* and *sts1-2*. These studies suggest that the defect of the *sts1-2* mutant protein is not caused by deficient interaction with the proteasome or altered proteasome abundance. Although the interaction between Sts1 and the proteasome is weak, we note that in a control reaction (Ctrl), we did not detect nonspecific purification of proteasome subunits on FLAG-agarose.

**Stabilization of Proteolytic Substrates**—The interaction between Sts1 and the proteasome led us to investigate whether it affected the degradation of proteolytic substrates. We transformed *STS1* and *sts1-2* with plasmids expressing the engineered substrates Arg-β-gal and Ub-Pro-β-gal. We used pulse-chase methods to compare the stabilities of these substrates to Met-β-gal, a stable reporter protein. Pioneering studies by Varshavsky and co-workers (20, 29) using these reagents uncovered the N-end rule pathway of substrate targeting, as well as the ubiquitin fusion degradation pathway. Yeast cells were grown to exponential phase at the semi-permissive temperature (30 °C) and then incubated in medium containing [35S]methionine. Following 5 min of labeling, aliquots were withdrawn, and protein extracts were examined, as described under “Experimental Procedures.” As expected, Ub-Pro-β-gal was rapidly degraded in a wild type strain. However, Ub-Pro-β-gal was strongly stabilized in *sts1-2* (Fig. 3A). The band intensities were quantified, and we determined that the half-life of Ub-Pro-β-gal over the initial 10 min of chase was 6 min in the wild type strain and 30 min in *sts1-2*. Arg-β-gal is an N-end rule substrate and was found to be similarly stabilized in *sts1-2* (Fig. 3B). In contrast, Met-β-gal was stable in both *STS1* and *sts1-2*. Collectively, these studies showed that substrates of the proteasome are inefficiently degraded in *sts1-2*.

To further investigate a role for Sts1 in protein degradation, we examined the stability of a physiological substrate, Sic1-HA. Sic1 is a key regulator of entry into the cell cycle, and its degradation by the ubiquitin/proteasome pathway is tightly regulated. We generated Sic1-HA and expressed the protein in *STS1* and *sts1-2* using the copper-inducible pCIP4 promoter. Because *sts1-2* showed no growth defect at low temperature, we grew *STS1* and *sts1-2* at 23 °C (Fig. 3C) to increase the efficiency of G1 arrest. We confirmed that ~90% of the cells were arrested in G1 following nutrient deprivation. To increase the level of Sic1-HA, the G1 arrested cells were incubated for 30 min with 100 μM CuSO4. To stimulate reentry of these G1 arrested cells containing higher levels of Sic1-HA into mitotic growth, they were transferred to fresh prewarmed medium (30 °C) containing 15 μg/ml nocodazole. This approach permitted cells to exit the G1

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**FIGURE 2. Sts1 binds the proteasome, but not mult ubiquitinated proteins.** A, a wild type strain was transformed with plasmids expressing Pre1-FLAG (lanes 1 and 5), FLAG-Rad23 (lanes 2 and 6), FLAG-Rpn10 (lanes 3 and 7), or FLAG-Sts1 (lanes 4 and 8). Equal amounts of total protein extract were prepared from these strains, as well as a control (Ctrl) strain lacking a FLAG-tagged protein. Protein extracts were examined by immunoblotting to determine the levels of polyubiquitinated proteins (lanes 1–4). Similarly, equal amounts of extract were applied to FLAG-agarose to immunoprecipitate the FLAG-tagged proteins and measure the interaction with polyubiquitinated proteins (lanes 5–8). The nitrocellulose filter described in A above was stained with Ponceau S (lower right panel) and probed with antibodies against FLAG (lower left panel) to determine the expression level and purification of the FLAG-tagged proteins. FLAG-Rad23 migrates close to the immunoglobulin heavy chain and is therefore not readily detected by Ponceau S staining. B, because of the rapid degradation of Sts1 and sts1-2, we expressed both FLAG-tagged proteins in the proteasome mutant pre1-1 pre2-2. Protein extracts were prepared from cells incubated at either 23 °C (lanes 1 and 2) or 37 °C (lanes 3 and 4), incubated with FLAG-agarose, and examined by immunoblotting with antibodies against proteasome subunits Rpt1 and Rpn12. The expression of these proteasome subunits was not affected significantly by temperature (Extract), or by expression of FLAG-Sts1 (lanes 1 and 3), or FLAG-sts1-2 (lanes 2 and 4). Furthermore, similar amounts of Rpt1 and Rpn12 were co-precipitated with FLAG-Sts1 and FLAG-sts1-2 (FLAG-IP). Ponceau S staining (lower panel) showed equivalent expression levels of the FLAG-tagged proteins. Asterisks indicate the positions of the immunoglobulin heavy and light chains. mw, molecular weight.
Sic1-HA was detectable for up to 240 min (Fig. 3). The studies described in Fig. 3 showed that the rapid elimination of Sic1-HA following exit from G1 (in both STS1 and sts1-2) under semi-permissive conditions (30°C) obscured any proteolytic defect of sts1-2. Therefore, in the study described in Fig. 3D, we grew STS1 and sts1-2 to a stationary phase at 30°C to cause partial defect in sts1-2. The cultures were then transferred to 37°C and Sic1-HA levels examined. In contrast to the study described in Fig. 3C, Sic1-HA levels remained elevated in sts1-2 at 37°C for at least 60 min. Sic1-HA was detectable for up to 240 min (Fig. 3D). The filters were subsequently incubated with antibodies against Rad23, and similar levels were detected in all of the samples that were examined. We are aware that the stabilization of Sic1-HA might be a consequence of a cell cycle progression delay in sts1-2, and further study will be required to resolve this possibility. Nonetheless, the stabilization of β-gal substrates (as well as Sic1-HA) is consistent with our hypothesis that Sts1 is a previously uncharacterized regulatory factor in the ubiquitin/proteasome system. Because Sts1 affects the stability of substrates of distinct targeting mechanisms, we propose that it functions after proteolytic substrates have been targeted by the ubiquitin-conjugating apparatus.

We confirmed the temperature-sensitive growth defect of sts1-2 at 37°C (Fig. 4A, lower left panel) and verified that this defect could be overcome by expressing FLAG-Sts1 from a plasmid (Fig. 4A, lower right panel). In contrast, growth of sts1-2 at 37°C was not restored by the FLAG-sts1-2 mutant protein (lower right panel).

**Sensitivity to Translation Inhibitors**—Mutations in the Ub/proteasome pathway frequently result in poor growth on medium containing translation inhibitors. One interpretation of this observation is that the Ub/proteasome pathway is required for the degradation of nascent, misfolded proteins that are generated by the ribosome. To determine whether Sts1 played a general role in the Ub/proteasome system, we examined growth in the presence of drugs that induce protein damage. 10-fold serial dilutions of exponential phase cultures of...
STS1 and sts1-2 were grown (at 23 °C) on medium containing cycloheximide or paromomycin (Fig. 4B). We found that sts1-2 is exceedingly sensitive to cycloheximide and also displayed poor growth on medium containing paromomycin (data not shown). We speculate that this defect will be more severe at the semi-permissive temperature.

Sts1 Cannot be Functionally Replaced by Rad23 and Rpn10—To determine whether Sts1 and Rad23/Rpn10 operated in a functionally redundant manner, we simultaneously overexpressed both 
P_{GAL1}:RAD23 and \(P_{GAL1}:RPN10\) in the temperature-sensitive \(sts1-2\) mutant (14). However, the growth defect of \(sts1-2\) at 37 °C was not suppressed (Fig. 4C), indicating that Sts1 cannot be replaced by either Rad23 or Rpn10. This finding also suggests that the mechanism of suppression achieved by Sts1 occurred by an alternate mechanism.

Sts1 Does Not Contribute to the DNA Repair Function of Rad23—Because high level expression of Sts1 did not overcome the UV sensitivity or \(rad23\Delta\ rpn10\Delta\) (Fig. 1C), we anticipated that it would not be required for the cellular response to DNA damage. To verify this conjecture, we measured the sensitivity of \(sts1-2\) to 254-nm UV light, at both permissive and nonpermissive temperatures. Wild type and \(sts1-2\) cells were grown at 23 °C, incubated for 2 h at either 23 or 37 °C, plated on selective agar medium, and exposed to UV light (at 1 J/m²/s). (The viability of \(sts1-2\) is not affected by incubation at 37 °C for this duration.) UV-irradiated cells were allowed to recover in the dark at 23 °C. We found that the survival of \(sts1-2\) was indistinguishable from the wild type strain following UV light treatment (Fig. 4D), demonstrating that Sts1 contributes to the growth and proteolytic functions of Rad23 but not its DNA repair activities. We recognize, however, that because \(STS1\) is essential for viability, its apparent dispensability in DNA repair cannot be verified unambiguously.

Increased Levels of Multi-Ub Proteins in \(sts1-2\)—The sensitivity of \(sts1-2\) to translational drugs, and stabilization of proteolytic substrates are consistent with a role in protein degradation. To investigate whether the targeting of proteolytic substrates was altered in \(sts1-2\), we examined the levels of total multi-Ub proteins. Wild type and \(sts1-2\) cells were pregrown at 23 °C and then transferred to 37 °C. Aliquots were withdrawn periodically, and total proteins were separated in SDS/PAGE and transferred to nitrocellulose. An immunoblot was incubated with antibodies against Ub. At 23 °C the levels of multi-Ub proteins in \(sts1-2\) were similar to the wild type strain (Fig. 5A, 0 h, lanes 1 and 2). However, after transfer to 37 °C the levels of high molecular weight multi-Ub proteins increased progressively in \(sts1-2\) (even-numbered lanes marked ts). The increased levels of multi-Ub proteins in \(sts1-2\) is evident by 1 h (lane 4), and marked elevation was observed by 4 h (lane 8). In contrast, the levels of multi-Ub proteins in \(STS1\) decreased in the corresponding time points at 37 °C (odd-numbered lanes marked WT). Note for instance that at 6 h, only lower molecular weight ubiquitinated species were detected in \(STS1\) (lane 9).

We also investigated whether normal levels of multi-Ub proteins would be restored if cells were returned to 23 °C following 6 h of incubation at 37 °C. We found that ubiquitin levels remained high in \(sts1-2\) following transfer to 23 °C for 2 h (lanes 13 and 14), although a perceptible increase in the amount of higher molecular weight species was observed during this recovery period (compare lanes 10 and 14). Prolonged incubation of the cultures at 23 °C (untreated; lanes 11 and 12) had no effect on the levels of multi-Ub proteins, suggesting that the accumulation of ubiquitinated proteins at 37 °C is related to the temperature-sensitive growth defect of \(sts1-2\). The same filter...
was probed with antibodies against Pab1 (loading control), and similar levels were detected in each set of paired extracts (Fig. 5A, compare WT to ts at each time point).

**Increased Proteasome Activity in sts1-2**—Because both Sts1 and sts1-2 proteins formed equivalent interactions with the proteasome, we questioned whether the underlying defect of *sts1-2* was linked to proteasome function. For instance, altered proteasome peptidase activity or reduced binding to multi-Ub proteins could underlie the defects of *sts1-2*. To address this question, we examined the hydrolytic activity of proteasomes. We prepared total protein extracts from yeast cells grown at 23 °C. Proteasome activity was determined using a fluorogenic substrate (SUC-LLVY-AMC), which measures chymotryptic activity. We detected >2-fold increased activity in *sts1-2* (*p* < 0.05) that was grown at 23 °C (Fig. 5B). Because the abundance of proteasomes was similar in *STS1* and *sts1-2* at 23 °C (Fig. 2B, lanes 1 and 3, and see below), the increased chymotryptic levels in *sts1-2* might reflect a compensatory response to a defect in protein degradation. For instance, higher chymotryptic activity might enable the removal of an increased level of substrates, following their interaction with the proteasome. Wise comparisons suggest increasing accumulation of multi-Ub proteins in *sts1-2* at high temperature.

**Proteasome Composition and Interaction with Sts1 Is Not Affected in sts1-2**—Based on the accumulation of multi-Ub proteins, as well as higher proteasome activity in *sts1-2*, we questioned whether proteasome integrity was affected in the mutant strain. We purified proteasomes (using Pre1-FLAG) from *STS1* and *sts1-2* at both 23 and 37 °C and examined the interaction between the 20 and 19 S particles. The immunoprecipitates were characterized by immunoblotting, using antibodies against the 19 S subunits Rpt1, Rpn12, and Rpn10. Rpt1 is present in the base complex, whereas Rpn12 is present in the lid complex. Previous studies suggested that Rpn10 functioned as a hinge that linked the lid to the base (30). All three subunits were co-purified with Pre1-FLAG in both *STS1* and *sts1-2*. Equivalent expression of Pre1-FLAG was also observed in these strains. Collectively, these studies suggest that the assembly of the proteasome is likely to be unaffected in *sts1-2*. These studies also showed that the interaction between the Rpn10 and proteasome was unaffected in the *sts1-2* mutant. Similarly, Rad23/proteasome interaction was unaffected in *sts1-2* (data not shown).

**The Interaction between Proteasome and Multiubiquitinated Proteins Is Altered in sts1-2**—To determine whether Sts1 influenced the translocation of substrates to the proteasome, we compared the levels of multi-Ub proteins in total extract (as described in Fig. 5A) with that present in proteasomes. We expressed Pre1-FLAG in *STS1* and *sts1-2* and transferred the cells from 23 °C to 37 °C, and protein extracts were prepared and characterized by immunoblotting (as described in Fig. 5). Consistent with earlier findings (Fig. 5A), we observed significant accumulation of high molecular weight multi-Ub species in *sts1-2* at 37 °C (Fig. 5A). The same protein extracts were also applied to FLAG-agarose to specifically measure the levels of ubiquitinated substrates in association with proteasomes (Fig. 6B). Low levels of multiubiquitinated proteins were detected in proteasomes purified at 23 °C from both *STS1* and *sts1-2* (lanes 1 and 2). After correcting for the level of Pre1-FLAG that was purified (Fig. 6C), we estimated that higher levels were already present in proteasomes purified from *sts1-2* at 23 °C (Fig. 6B, compare lanes 1 and 2). The levels of multi-Ub proteins copurified with proteasomes from *STS1* increased initially after transfer from 23 to 37 °C (compare lanes 1 and 3) but remained constant thereafter, based on the level of Pre1-FLAG that was purified (Fig. 6C). In striking contrast, the level of multiubiquitinated proteins purified with proteasomes from *sts1-2* increased progressively after transfer from 23 to 37 °C (lanes 4, 6, 8, and 10), consistent with the higher levels in total extract (Fig. 6A). Based on these results, we propose that proteasomes purified from *sts1-2* may be deficient in the clearance of multiubiquitinated substrates, following their interaction with the proteasome.

**DISCUSSION**

The ability of Sts1 to suppress the defects of *rad23Δ rpn10Δ* suggested a link to protein degradation. Our characterization of
the Sts1 protein and a temperature-sensitive \textit{sts1} mutant demonstrate that Sts1 performs a role in the Ub/proteasome system. Specifically, we found that Sts1 could bind the proteasome and affected proteasome activity. Furthermore, we determined that \textit{sts1}-2 is highly sensitive to drugs that cause protein damage and is unable to efficiently degrade proteolytic substrates. Strikingly, \textit{sts1}-2 accumulated high levels of multiubiquitinated proteins at the nonpermissive temperature, and proteasomes showed a reduced ability to clear these substrates. Pulse-chase studies confirmed the stabilization of test substrates. Moreover, the regulated turnover of Sic1-HA, which is degraded following the exit of cells from G\textsubscript{1}, was significantly impaired in \textit{sts1}-2. Although the stabilization of Sic1-HA in \textit{sts1}-2 might be caused by defective entry into mitotic growth, it could also be the result of the aforementioned proteolytic defects.

The accumulation of multi-Ub proteins in \textit{sts1}-2 does not appear to be caused by deficient proteasome activity, because chymotryptic activity was increased. However, one interpretation of our results is that an increased load of proteolytic substrates could contribute to the higher levels of multi-Ub proteins in \textit{sts1}-2 (Fig. 5A). Because a major fraction of newly synthesized proteins are degraded before they mature (31, 32), it is possible that the acute sensitivity of \textit{sts1}-2 to translation inhibitors is caused by inefficient elimination of nascent damaged proteins.

Despite higher proteasome activity, the levels of multi-Ub proteins increased rapidly in total extracts at 37°C. However, the accumulation of these substrates in proteasomes in the \textit{sts1}-2 mutant was delayed. One interpretation of this finding is that substrate delivery to the proteasome is normal, although their degradation is defective. The increased proteasome activity in \textit{sts1}-2 could reflect a compensatory response to a defect at a different step in degradation. It is evident that substrate ubiquitination is unaffected in \textit{sts1}-2 and that ubiquitinated proteins could successfully bind the proteasome. However, the interaction of ubiquitinated substrates to proteasomes is poorly understood, and it is uncertain whether these proteins interact accurately with proteasomes in \textit{sts1}-2. Moreover, other steps in degradation by the proteasome, such as deubiquitination, substrate unfolding, or substrate translocation into the catalytic particle, could be affected in \textit{sts1}-2.

We explored a plausible mechanism for Sts1-mediated suppression. Rad23 and Rpn10 can bind multi-Ub proteins and the proteasome. Based on these properties, we proposed that Rad23 might function as a \textit{shuttle-factor} that delivered multi-Ub proteins to the proteasome. Genetic studies suggest that multi-Ub substrates may be transferred from Rad23 to Rpn10, a multiubiquitin chain-binding protein in the proteasome. Other models have also been proposed to explain the function of Rad23 and Rpn10 in promoting the degradation of substrates by the proteasome (4, 7). Sts1 does not bind either Rad23 or Rpn10 or affect their interactions with proteasomes (Fig. 5C and data not shown). This is not surprising because high level expression of Sts1 suppressed the loss of Rad23 and Rpn10, indicating that Sts1 operates through an independent and functionally distinct mechanism. The ability of Sts1 to bind the proteasome, but not multi-Ub proteins, suggested that it might exert its effect on the proteasome. In agreement with this conjecture, we found that proteasome activity increased in \textit{sts1}-2, and multiubiquitinated substrates were inefficiently cleared.

To suppress the pleiotropic defects of \textit{rad23Δ rpn10Δ}, Sts1 might improve proteasome function to restore the degradation of Rad23- and Rpn10-specific substrates. Alternatively, Sts1 might improve the targeting of proteolytic substrates to the proteasome by recruiting other shuttle factors. This model would support the hypothesis that Sts1 bypasses the defects of \textit{rad23Δ rpn10Δ} by using an alternate pathway. Further study will be required to determine whether other \textit{shuttle-factors} provide a mechanism to bypass the defects of \textit{rad23Δ rpn10Δ}.

The proteolytic defects of \textit{sts1}-2 are similar to those caused by mutations in genes encoding components of the ubiquitin/proteasome pathway. However, Sts1 is unique because proteasome activity is increased in \textit{sts1}-2, despite its failure to degrade proteolytic substrates efficiently. Dual overexpression of Rad23 and Rpn10 did not suppress the temperature-sensitive growth defect of \textit{sts1}-2. This nonredundancy supports the hypothesis that multiple mechanisms contribute to the removal of proteolytic substrates.

Yanagida and co-workers (16, 17) reported that Cut8, the \textit{S. pombe} counterpart of Sts1, could tether proteasomes to the nuclear membrane. It was proposed that multi-ubiquitination of Cut8 promoted its interaction with the proteasome and also initiated its eventual degradation. Similarly, we report that Sts1 is highly unstable in a wild type strain and is further destabilized in \textit{rad23Δ rpn10Δ}. We speculate that the dosage suppression of \textit{rad23Δ rpn10Δ} by Sts1 might reflect restoring normal levels of this otherwise unstable protein. Despite some functional similarity, Cut8 and Sts1 may operate through distinct mechanisms, because \textit{STSI} is an essential gene, whereas Cut8 is dispensable for viability in \textit{S. pombe}. Moreover, a \textit{cut8} null mutant is sensitive to DNA damage, whereas \textit{sts1}-2 displayed no apparent sensitivity to UV light. At the nonpermissive temperature, the levels of multiubiquitinated proteins increased dramatically in \textit{sts1}-2 but not in \textit{cut8} (17).

Additionally, Cut8/proteasome interaction required multiubiquitination of Cut8, whereas proteasomes purified from \textit{S. cerevisiae} contained unconjugated Sts1. (However, it is possible that the multi-Ub chain is removed from Sts1, following its interaction with the proteasome.)

Srpl is a nuclear localization signal-binding factor that can bind karyophilic proteins and facilitate their translocation into the nucleus. Specific mutations disrupted nucleocytoplasmic transport and also caused proteolytic defects. Tabb \textit{et al.} (15) isolated Sts1 as a high copy dosage suppressor of \textit{srp1-49} and found that it suppressed the protein degradation defect of this mutant. Characterization of Sts1 revealed predominant localization to the nucleus (15). Studies described by Enenkel \textit{et al.} (33, 34) showed that a major fraction of cellular proteasomes co-localized with nuclei. Collectively, these studies are consistent with the findings of Tatebe and Yanagida (17), who reported that Cut8 was required for the nuclear targeting of proteasomes. We isolated Sts1 as a dosage suppressor of the growth and proteolytic defects of \textit{rad23Δ rpn10Δ}, a well characterized mutant in the Ub/proteasome pathway (3). Although the underlying mechanism of suppression is not known, the studies
described here provide compelling evidence of a role for Sts1 in the Ub/proteasome system. Furthermore, because Sts1 can bind proteasomes but not multi-Ub chains, it is distinct from both Rad23 and Rpn10. Because both Sts1 and sts1-2 can bind the proteasome, the defect in sts1-2 might occur after Sts1/proteasome binding. Collectively, these and other findings (16, 33, 35) suggest that Sts1 contributes to the ubiquitin/proteasome pathway of protein degradation. We propose that Sts1 is a novel regulatory factor that affects the activity of the proteasome.

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