Nuclear Import Factor Srp1 and Its Associated Protein Sts1 Couple Ribosome-bound Nascent Polypeptides to Proteasomes for Cotranslational Degradation*

Seung-Wook Ha, Donghong Ju, and Youming Xie

From the Karmanos Cancer Institute, Department of Oncology, and Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201

Background: The mechanism underlying cotranslational protein degradation remains poorly understood.
Results: The nuclear import factor Srp1 binds ribosome-bound nascent polypeptides. Sts1 mediates the interaction between Srp1 and the proteasome.
Conclusion: Srp1 and Sts1 couple proteasomes to nascent polypeptides emerging from the ribosome for cotranslational degradation.
Significance: This study unveils a novel role for Srp1 and Sts1 in cotranslational protein degradation.

Cotranslational protein degradation plays an important role in protein quality control and proteostasis. Although ubiquitylation has been suggested to signal cotranslational degradation of nascent polypeptides, cotranslational ubiquitylation occurs at a low level, suggesting the existence of an alternative route for delivery of nascent polypeptides to the proteasome. Here we report that the nuclear import factor Srp1 (also known as importin α or karyopherin α) is required for ubiquitin-independent cotranslational degradation of the transcription factor Rpn4. We further demonstrate that cotranslational protein degradation is generally impaired in the srp1–49 mutant. Srp1 binds nascent polypeptides emerging from the ribosome. The association of proteasomes with polysomes is weakened in srp1–49. The interaction between Srp1 and the proteasome is mediated by Sts1, a multicopy suppressor of srp1–49. The srp1–49 and sts1–2 mutants are hypersensitive to stressors that promote protein misfolding, underscoring the physiological function of Srp1 and Sts1 in degradation of misfolded nascent polypeptides. This study unveils a previously unknown role for Srp1 and Sts1 in cotranslational protein degradation and suggests a novel model whereby Srp1 and Sts1 cooperate to couple proteasomes to ribosome-bound nascent polypeptides.

Protein homeostasis is maintained by a complex quality control system that controls a delicate balance between protein synthesis, folding, and degradation (1–7). Remarkably, proteins are monitored by the quality control system at the moment they emerge from the ribosome. The N-terminal end of a nascent polypeptide is available for folding before the other end has been synthesized. Cotranslational folding helps to reduce aggregation of translational intermediates and promote accurate folding of newly made polypeptides. However, cotranslational folding is a complicated task that requires the participation of multiple chaperone proteins and is not always successful. To prevent the accumulation of misfolded, potentially toxic proteins arising from inefficient folding and translational errors, the cell must degrade the misfolded polypeptides immediately after their synthesis or even before reaching their mature size. This process is referred to as cotranslational protein degradation. It has been estimated that, under physiological conditions, as many as 30% of total nascent polypeptides are cotranslationally degraded by the proteasome in mammalian cells (8). This finding led to the elaboration of the so-called DRiP (defective ribosomal products) hypothesis by proposing that cotranslational degradation products serve as an important source of MHC class I peptides (8–13). Despite the physiological significance, the underlying mechanism of cotranslational protein degradation remains poorly understood. One of the central questions is how the proteasome targets ribosome-bound nascent chains destined for cotranslational degradation. Early studies suggested that ubiquitylation of nascent polypeptides provides a targeting signal for the proteasome (8, 14–16). This suggestion is supported by the identification of several ribosome-bound ubiquitin (Ub)2 ligases, including Not4 and Lin1 (17, 18). Interestingly, cotranslational ubiquitylation appears to occur at a low level. Two studies recently revealed that only 1–6% of nascent polypeptides are ubiquitylated in the budding yeast Saccharomyces cerevisiae (19, 20). This likely represents only a fraction of the nascent polypeptides that undergo cotranslational degradation (14). We speculate that there may be an alternative route for the handover of nascent polypeptides to the proteasome, particularly for those that are not modified by the Ub system.

The number of proteasomal substrates that are degraded without prior ubiquitylation continues to grow. One of the examples is the transcription factor Rpn4, which regulates the

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1 To whom correspondence should be addressed: Karmanos Cancer Institute, Dept. of Oncology, and Dept. of Pathology, Wayne State University School of Medicine, 110 E. Warren Ave., Detroit, MI 48201. Tel.: 313-578-4319; Fax: 313-831-7518; E-mail: xiey@karmanos.org.

2 The abbreviations used are: Ub, ubiquitin; CHX, cycloheximide; IP, immunoprecipitation; RNC, ribosome-nascent chain complex; DTSSP, 3,3′-dithiobis(sulfosuccinimidyl propionate); ura, uracil; ade, adenine.
proteasome genes in *S. cerevisiae* (21, 22). Rpn4 is an extremely short-lived protein (t½ < 2 min) and is degraded by the proteasome, thereby forming a negative feedback circuit (23, 24). The degradation of Rpn4 can be mediated via the canonical Ub-dependent pathway, or it occurs in an Ub-independent manner (25–27). The Ub-dependent degron of Rpn4 is located in an acid domain including residues 211–229, whereas the Ub-independent degron is mapped to its N-terminal domain, consisting of the first 80 residues (28–30). Therefore, Rpn4Δ211–229 can serve as a model molecule for studying the mechanism underlying Ub-independent degradation of Rpn4.

In this study, we report that Rpn4Δ211–229 is degraded cotranslationally and that, unexpectedly, this process requires the nuclear import factor Srp1 (also known as importin α or karyopherin α). Moreover, cotranslational protein degradation is generally impaired in the *srp1–49* mutant expressing a defective version of Srp1. We show that Srp1 directly binds nascent polypeptides emerging from the ribosome and that the association of proteasomes with polyosomes is weakened in *srp1–49*. The interaction between Srp1 and the proteasome is mediated by Sts1, a multicopy suppressor of *srp1–49*. The *srp1–49* and *sts1–2* mutants are hypersensitive to conditions that increase protein misfolding. Our study unveils a new role for Srp1 and Sts1 in cotranslational protein degradation and suggests a model whereby Srp1 and Sts1 couple proteasomes to ribosome-bound nascent polypeptides.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Plasmids**—The yeast strains used in this study included W303-1A (MATα ura3-1 trp1-1 leu2-3,112 his3-11 ade2-1 can1-100), JLY555 (MATα ura3-1 trp1-1 leu2-3,112 his3-11 ade2-1 can1-100 srp1-49), provided by G. Fink and M. Nomura, LCY827 (MATα trp1-1 trp1-1 leu2-3,112 his3-11 ade2-1 sts1-2, a gift from K. Madura), EYJ140 (MATα trp1-1 Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 rpn4Δ:LEU2, see Ref. 31), and XYY206 (MATα trp1-1 Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 PRE1-Flag-6His::Yiplac211, see Ref. 23). Details of the plasmid constructs are available upon request. The replacement vector p304Rpn4Δ211–229-3HA for site-specific recombination to generate strains expressing Rpn4Δ211–229-3HA from the chromosomal locus has been described previously (32). For protein expression and/or purification from *Escherichia coli*, the pET11d vector was used to express C-terminally FLAG-tagged Rpn4, whereas N-terminally His-tagged Rpn4 were N-terminally fused to the GAL4 DNA binding domain (GAL4DB) to serve as bait. Rpn4Δ1–151-GAL4DB was expressed from the *CUP1* promoter on the vector pRS426. The two-hybrid DNA libraries and the host *S. cerevisiae* strain PJ69-4A were gifts from P. James and E. Craig (34).

**Pulse-Chase Analysis and Cotranslational Degradation Assay**—Exponentially growing cells in synthetic defined (SD) medium containing essential amino acids were harvested and resuspended in the same medium supplemented with 0.15 mM of [35S]Met/Cys for pulse labeling. Cells were then pelleted and resuspended in the same SD medium with CHX (0.2 mg/ml) and excess cold Met (2 mg/ml) and Cys (0.4 mg/ml) and chased for various time periods. An equal volume of sample was withdrawn at each time point. Labeled cells were lysed in 2× SDS buffer (2% SDS, 30 mM dithiothreitol, and 90 mM Na-HEPES (pH 7.5)) by incubation at 100 °C for 5 min. Supernatants were recovered by centrifugation and diluted 20-fold with buffer A (150 mM NaCl, 1 mM EDTA, 50 mM Na-HEPES (pH 7.5), and 1% Triton X-100) before being subjected to IP. The volumes of supernatants applied to IP were adjusted to equalize the amounts of [35S]incorporated into proteins using the TCA precipitation assay. To measure cotranslational degradation of ribosome-bound nascent polypeptides, cells were treated with 50 μM of MG-132 for 10 min before pulse labeling. Labeled cells were resuspended in buffer A and disrupted by vortex with glass beads. 100 μl of each cell extract was loaded onto a 25% sucrose cushion in buffer B (50 mM HEPES (pH 7.5), 140 mM NaCl, and 5 mM MgCl2) followed by ultracentrifugation at 85,000 rpm for 90 min at 4 °C using a TLA 100.2 rotor (Beckmann). Ribosome-nascent chain complexes (RNCs) were recovered and solubilized in buffer C (25 mM Na-HEPES, 80 mM KAOc, 1 mM MgOAc2) (pH 7.5) and applied to the TCA precipitation assay to quantify the remaining [35S]-labeled nascent polypeptides and to autoradiography after SDS-PAGE.

**In Vitro Transcription/Translation Reactions and Binding of Rpn4 Nascent Chains**—*In vitro* transcription/translation reactions were carried out using the TnT coupled transcription/translation system (Promega) according to the instructions of the manufacturer. In a typical reaction, a prerelaxed mixture containing [35S]Met/Cys was prepared without a DNA template. Aliquots of the mixture were added to reaction tubes and expressed three reporters from different inducible promoters: P**gal1**-HIS3, P**gal2**-ADE2, and P**gal7**-lacZ. The bait plasmid was transformed into PJ69-4A. Ura+ transformants were then transformed with the two-hybrid libraries, with selection directly on synthetic complete medium lacking ura and ade, SC (ura-, ade-), plates at 30 °C. Ade+ colonies were isolated, and library plasmids were rescued from the Ade+ transformants and retransformed into PJ69-4A expressing either the bait plasmid or a control vector. The plasmids that induced ADE2 in the presence but not in the absence of the bait plasmid were analyzed by sequencing. One clone thus identified encoded a 478-residue fragment (positions 65–542) of the SRP1 ORF.

**TCA Precipitation Assay**—A small volume (1.5 μl) of lysate prepared from [35S]methionine (Met)- and cysteine (Cys)-labeled cells was spotted on filter paper, which was then soaked in 10% TCA solution, air dried, rinsed in 100% ethanol, and air-dried. This process was repeated three times, and then the [35S]activity remaining on the filter paper was counted by a liquid scintillation counter. The TCA precipitation assay was used to adjust the input of cell extracts for immunoprecipitation and to measure bulk degradation of newly synthesized proteins and protein synthesis rates.
containing various DNA templates and were incubated for 90 min at 30 °C. The reactions were terminated by the addition of an equal volume of ice-cold buffer C supplemented with DNase I (5 µg/ml), CHX (200 µg/ml), and MG132 (50 µM). The coding sequences for Rpn4, Rpn4_1–79, and Srp1 were subcloned into the T7 promoter-based vector pET11d. To generate stop codon-less DNA templates, the Rpn4 and Rpn4_1–79 vectors were digested with the restriction enzyme EcoRI. RNPs were isolated by applying the reaction mixture to ultracentrifugation through a 0.5 M sucrose cushion. The pelleted RNPs containing 35S-labeled Rpn4_1–415 or Rpn4_480–415 and unloaded ribosomes were rinsed gently twice with buffer C, dissolved in sample buffer, and resolved by SDS-PAGE. For the RNC binding assay, RNPs containing Rpn4_1–415 and Rpn4_480–415 were prepared as above but without using [35S]Met/Cys. 35S-labeled Srp1 produced by the in vitro transcription/translation reaction was collected from the supernatant after ultracentrifugation of the reaction mixture at 100,000 rpm for 20 min. This step was able to get rid of the ribosome-bound Srp1. RNPs and unloaded ribosomes were resuspended in buffer C and incubated with 35S-labeled Srp1. The mixture was ultracentrifuged through a 0.5 M sucrose cushion as above to remove unbound Srp1. RNC-or ribosome-bound Srp1 was resolved by SDS-PAGE and visualized and quantified using a PhosphorImager.

Puromycin Labeling and Nascent Chain Binding Assay—RNPs were isolated through ultracentrifugation as described above. RNC pellets were dissolved in buffer D (10 mM HEPES (pH 7.5), 400 mM KCl, and 3 mM MgCl2) and incubated with 4 µM of puromycin for 1 h at 30 °C. The reversible cross-linker DTSSP (2 mM) was then added to the reaction and kept at room temperature for 30 min. The reaction mixture was heated at 95 °C for 5 min in the presence of 1% SDS. The denatured supernatant was then diluted 10-fold with buffer A and incubated with puromycin (KeraFAST, Boston, MA) or anti-Srp1 antibody (a gift from M. Nomura). The precipitates were eluted with sample buffer (50 mM Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, and 2 mM DTT), resolved by SDS-PAGE, and separated by SDS-PAGE, and examined by immunoblotting analysis with either anti-Srp1 or anti-puromycin antibody.

Sucrose Gradient Cosementation Assay—Yeast cells grown in 1 liter of YPD (yeast extract-peptone-dextrose) medium to A600 ~1.2 were harvested, and cell pellets were ground to a fine powder in the presence of liquid nitrogen. Cell extracts were prepared by dissolving the cell powder in buffer E (50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40) supplemented with 0.2 mg/ml of lysozyme and protease inhibitor mixture and lysed by ultrasonication. Cell extracts were recovered by centrifugation in a TLA-100.2 rotor (Beckman) for 1 h at 100,000 rpm at 4 °C. Input amounts of purified proteins or cell lysates were determined before the pulldowns assays. GST fusion proteins were applied to 1 µl of glutathione-agarose in 0.5 ml of buffer G and incubated for 1 h at 4 °C. Unbound proteins were removed by three washes in buffer A and equilibrated with buffer G. Cell extracts or purified proteins were applied to the equilibrated glutathione-agarose in 0.5 ml of buffer G and allowed to incubate for 3 h at 4 °C. After three washes in buffer G, bound proteins were eluted with sample buffer, separated by SDS-PAGE, and examined by immunoblotting analysis with anti-FLAG (Sigma) and anti-Srp1 antibodies, respectively. For the proteasome pulldown assay, GST-Srp1 or GST-Srp1E145K was loaded on glutathione-agarose in the presence or absence of his-Sts1 and equilibrated with buffer H (25 mM Na-HEPES (pH 7.8), 5 mM MgCl2, 25 mM KCl, and 2 mM ATP). 5 µg of purified yeast 26 S proteasomes bearing a His-tagged Pre1 subunit was applied to the pulldown assay. Retained proteasomes were detected by immunoblotting with an anti-His antibody (Santa Cruz Biotechnology). Yeast proteasomes were purified as described previously (28).

RESULTS

Srp1 Is Involved in Ub-independent Cotranslational Degradation of Rpn4—To understand the mechanism of Ub-independent degradation of Rpn4, we set out to identify proteins that interact with the N-terminal domain of Rpn4 by conducting a yeast two-hybrid screen. The first 151 amino acids of Rpn4 were N-terminally fused to the GAL4 DNA binding domain (GAL4DB) to serve as bait. One of the clones thus isolated encodes a large fragment of Srp1 including residues 65 to 542. Interestingly, the Srp1 fragment interacted with Rpn4_1–151+ GAL4DB but not Rpn4_11–151+GAL4DB (Fig. 1A), indicating that the N-terminal 10 residues of Rpn4 are essential for the interaction. This was further confirmed by pulldown assays in
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A Yeast two-hybrid assay

sc+ade sc-ade

GAL4DB + GAL4AD-Srp165-542
Rpn41-151-GAL4DB + GAL4AD-Srp165-542
Rpn411-151-GAL4DB + GAL4AD-Srp165-542
Rpn41-151-GAL4DB + GAL4AD

B Rpn4-Flag Rpn41-10-Flag

α-FLAG

1% input GST GST-Srp1 GST GST-Srp1 GST GST

GST 1-229 GST

GST-Srp1

GST-Srp1

C his-Srp1

α-Srp1

1% input GST GST-Srp1 GST GST-Srp1 GST GST

GST 1-229 GST

GST-Srp1

GST-Srp1

Coomassie staining

Coomassie staining

FIGURE 1. Srp1 binds to the N-terminal domain of Rpn4. A, yeast two-hybrid assay showing the interaction between Srp1 and Rpn4. The isolated Srp1 clone (GAL4AD-Srp165-542) from two-hybrid screening interacted with Rpn41-151-GAL4DB but not Rpn411-151-GAL4DB. B, Rpn4 but not Rpn41-10 was pulled down by GST-Srp1. C, Srp1 was retained by GST fusion with Rpn41-229 but not Rpn411-229 C-terminally FLAG-tagged Rpn4 and Rpn41-10 and N-terminally his-tagged Srp1 were expressed in E. coli cells. Input of GST fusion proteins in the pulldown assays was examined by Coomassie Blue staining (B and C, bottom panels).

which GST-Srp1 was able to retain Rpn4 but not Rpn41-10 (Fig. 1B). Vice versa, Srp1 was pulled down by Rpn41-229 GST but not Rpn411-229-GST (Fig. 1C). These results suggest that Srp1 binds the Ub-independent degron of Rpn4.

SRP1, encoding a 542-amino acid polypeptide, was originally identified as a suppressor of mutations in two RNA polymerase I subunits, Rpa190 and Rpa135 (35). Subsequent work firmly established Srp1 as a nuclear import factor, and for this reason, Srp1 is also known as importin-α (35). Subsequent work firmly established Srp1 as a nuclear import factor, and for this reason, Srp1 is also known as importin-α (35). Subsequent work firmly established Srp1 as a nuclear import factor, and for this reason, Srp1 is also known as importin-α (35).

We suspected that Rpn41211-229-3HA could be degraded during pulse labeling and that the cotranslational degradation of Rpn41211-229-3HA could be impaired in srp1−49, resulting in the zero time effect (e.g. more protein products are detected at time 0). To test this possibility, we shortened the pulse labeling time to 1 min. This experimental design was on the basis of the observation that the average rate of translation in eukaryotic cells is 2–10 residues/s (42). Assuming a rate of 6 residues/s in S. cerevisiae, the complete synthesis of Rpn41211-229-3HA containing more than 500 residues needs at least 80 s. Therefore, the decline of 35S-labeled Rpn41211-229-3HA shortly after 1-min pulse labeling (in early chase) is largely caused by cotranslational degradation. We found that the degradation of Rpn41211-229-3HA was substantially slower in YXY470 than in YXY468 during a 5-min chase period following 1-min pulse labeling (Fig. 2, F and G). Approximately 70% Rpn41211-229-3HA was degraded in YXY468, whereas less than 30% was degraded in YXY470. The Rpn41211-229-3HA level at zero time was relatively higher (∼15%) in YXY470 than in YXY468, indicating that the degradation readily occurs during the 1-min pulse labeling. Taken together, these results demonstrate that Srp1 is required for cotranslational degradation of Rpn41211-229-3HA.

A Role of Srp1 in Global Cotranslational Protein Degradation—We next examined whether Srp1 plays a broad role in cotranslational protein degradation. We first compared the bulk degradation of newly synthesized proteins in srp1−49 and its WT counterpart. Cells were labeled with [35S]Met/Cys for 1 min, followed by a chase for up to 40 min in the presence of CHX and cold Met/Cys. Cells were harvested at different intervals, and the remaining 35S-labeled polypeptides were measured by TCA precipitation assay. As shown in Fig. 3A, the degradation of newly made proteins was substantially slower in srp1−49 than in the WT strain. This result suggests that Srp1 is involved in global cotranslational protein degradation. Of note, the outcome of the pulse-chase analysis is the sum of degradation of nascent polypeptides being synthesized on the ribo-

independently of ubiquitylation (25, 29). srp1−49 is a temperature-sensitive mutant and does not grow at 37 °C. At the semi-permissive temperature (30 °C), there is no complete block but rather a slowdown of growth, indicating that the activity of Srp1 is impaired at 30 °C. To avoid unexpected, perhaps complicated, phenotypes associated with the restrictive temperature, we decided to perform a pulse-chase analysis to compare the degradation of Rpn41211-229-3HA in YXY468 and YXY470 at 30 °C. Cells were labeled with [35S]Met/Cys for 5 min and chased for 0, 5, and 15 min in the presence of CHX and excess cold Met/Cys. The kinetics of Rpn41211-229-3HA turnover were not much different between these two strains during the chase period (Fig. 2, A and B). However, we repeatedly detected a stronger (>2-fold) Rpn41211-229-3HA signal at 0 min (zero time) in YXY470 than in YXY468. Similar results were obtained when the expression of Rpn41211-229-3HA was driven by the copper-induced CUP1 promoter from a low-copy plasmid in WT and srp1−49 cells (Fig. 2, C and D). We noticed that protein synthesis was modestly slower in srp1−49 than in WT cells (Fig. 2E). These results indicate that the higher level of Rpn41211-229-3HA at zero time in srp1−49 is not caused by up-regulation of transcription and/or translation.
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some; by definition, the \textit{bona fide} cotranslational degradation and the completed, newly made proteins already released from the ribosome. To further assess the role of Srp1 in cotranslational protein degradation, we wanted to quantify the degradation of nascent polypeptides emerging from the ribosome during pulse labeling in WT and \textit{srp1–49} cells. To this end, we deleted the \textit{PDR5} gene from the WT and \textit{srp1–49} strains to increase the permeability of proteasome inhibitor MG132 so that we could use MG132 to gauge proteasome-dependent cotranslational degradation. MG132, controlled by DMSO, was added to exponentially growing cell cultures to shut down the proteasome activity 10 min before pulse labeling with [\textsuperscript{35}S] Met/Cys. It has been shown that the addition of MG132 shortly prior to pulse labeling does not affect labeling efficiency or translation rates (19). After a short (1-min) pulse labeling, cells were immediately frozen in liquid nitrogen. Cell lysates were prepared in the presence of CHX and cold Met/Cys. RNCs were separated from the supernatant by ultracentrifugation through a 25% sucrose cushion. [\textsuperscript{35}S]-labeled proteins in the RNC and supernatant fractions were quantified by TCA precipitation assay. Data are mean ± S.D. of three independent experiments. C, autoradiogram of RNCs following SDS-PAGE. Equal amounts of RNCs prepared as in B were applied to SDS-PAGE.

\textbf{FIGURE 3.} Cotranslational protein degradation is impaired in \textit{srp1–49}. A, bulk degradation of newly synthesized proteins is slower in \textit{srp1–49} than in WT cells. Cells were labeled with [\textsuperscript{35}S]Met/Cys for 5 min and chased for 0, 5, 10, 20, and 40 min. Remaining [\textsuperscript{35}S]-labeled proteins were measured by TCA precipitation assay. Shown are the results of three independent experiments.

\textbf{FIGURE 2.} Ub-independent cotranslational degradation of Rpn4 is impaired in \textit{srp1–49}. A, pulse-chase analysis for the degradation of newly synthesized Rpn4\textsubscript{4211–229}-3HA expressed from the native \textit{RPN4} locus in WT and \textit{srp1–49} cells. Cells were labeled with [\textsuperscript{35}S]Met/Cys for 5 min and chased for different intervals as indicated. Cell extracts were subjected to IP with an anti-ha antibody, followed by SDS-PAGE and autoradiography. Rpn4\textsubscript{4211–229}-3HA was marked by an arrow. B, decay curves of Rpn4\textsubscript{4211–229}-3HA. [\textsuperscript{35}S]-labeled Rpn4\textsubscript{4211–229}-3HA from C was quantified by a Phosphorimager. Remaining [\textsuperscript{35}S]-labeled Rpn4\textsubscript{4211–229}-3HA at each time point was plotted as a percentage of that at time 0. Data are mean ± S.D. of three independent experiments. C, pulse-chase analysis was carried out as in A, except that Rpn4\textsubscript{4211–229}-3HA was expressed from the copper-induced \textit{CUP1} promoter on a low-copy vector. D, quantification of [\textsuperscript{35}S]-labeled Rpn4\textsubscript{4211–229}-3HA from C to show the decay curves. E, comparison of protein synthesis in WT and \textit{srp1–49} strains. Aliquots of cells were withdrawn at different time points after addition of [\textsuperscript{35}S]Met/Cys and used to prepare extracts. The incorporation of [\textsuperscript{35}S] into polypeptides was measured by TCA precipitation assay. Shown are the results of three independent experiments. F, pulse-chase analysis was performed as in A, with the pulse labeling time shortened to 1 min, followed by a chase for 0 and 5 min. G, quantification of [\textsuperscript{35}S]-labeled Rpn4\textsubscript{4211–229}-3HA from F.
some, we first used Rpn4 as a model molecule to test this possibility. In addition, we wanted to examine whether the N-terminal sequence of Rpn4 is targeted by Srp1. The TNT coupled transcription/translation system was applied to produce ribosome-bound Rpn4 polypeptides. To overcome the constraint of analysis of the interaction between nascent chains and their binding proteins, i.e. the heterogeneous nature of the elongating nascent chains, we took advantage of the fact that the translation products of mRNAs lacking a stop codon remain ribosome-bound as peptidyl-tRNA and are homogeneous in length (43). Specifically, we digested the DNA templates encoding full-some-bound as peptidyl-tRNA and are homogeneous in lengthing nascent chains, we took advantage of the fact that the trans-

FIGURE 4. The binding of Srp1 to ribosome-bound Rpn4 nascent chains. A, the production of ribosome-bound Rpn41–415 and Rpn480–415 polypeptides. Stop codon-less DNA templates encoding Rpn41–415 (long temp) and Rpn480–415 (short temp) were applied to the TNT coupled transcription/translation system supplemented with [35S]Met/Cys. A reaction without template DNA (no temp) was used as a control. After the reactions were terminated, the mixtures were ultracentrifuged through a 0.5 M sucrose cushion. Pelleted RNCs containing [35S]-labeled Rpn41–415 or Rpn480–415 and unloaded ribosomes were dissolved in sample buffer and resolved by SDS-PAGE. B, measurement of the binding of Srp1 to ribosome-bound Rpn4 nascent chains. [35S]-labeled Srp1 was incubated with non-labeled RNCs bearing Rpn41–415 (lane 2), Rpn480–415 (lane 3), or unloaded ribosomes (lane 1). Free [35S]Srp1 was removed by ultracentrifugation. Retained [35S]Srp1 was resolved by SDS-PAGE and quantified by a PhosphorImager. C, quantification of the results from B. Data are mean ± S.D. of three independent experiments. The amount of Srp1 binding to Rpn41–415 RNC is set at 100%.

much more Srp1 associated with the RNCs than with the unloaded ribosomes, and Srp1 had a higher affinity for RNCs bearing Rpn41–415 than Rpn480–415 (Fig. 4, B and C). These results indicate that Srp1 binds the ribosome-bound Rpn4 nascent chains and that the N-terminal domain of Rpn4 is important for recognition by Srp1. Note that a small but noticeable amount of Srp1 was associated with the ribosomes bearing no nascent chains. This observation suggests that Srp1 may also bind to the ribosome.

We went on to examine the binding of Srp1 to general nascent chains emerging from the ribosome using a method outlined in Fig. 5A. In addition, we wanted to test whether this activity is impaired by the E145K mutation in srp1–49. Polysomes were collected from exponentially growing WT and srp1–49 cells by ultracentrifugation through a sucrose cushion. The nascent polypeptides being translated on the polysomes were labeled with puromycin, which forms a covalent bond with the carboxyl terminus of nascent polypeptides and blocks further translation (44). To stabilize the interaction between nascent polypeptides and Srp1, the thiol-cleavable cross-linker DTSSP was added to the reaction after puromycin labeling. Anti-puromycin immunoblotting analysis confirmed that the nascent polypeptides were labeled with puromycin and that DTSSP was able to cross-link the nascent polypeptides (Fig. 5B). The puromycin-labeled nascent polypeptides appeared as a high molecular weight smear under non-reducing conditions but returned to regular size bands after being treated with DTT. The association of endogenous Srp1 (and Srp1E145K) with RNCs was also demonstrated by cross-linking and anti-Srp1 immunoblotting analysis (Fig. 5C). The detection of cross-linked Srp1 by immunoblotting under nonreducing conditions was somewhat inefficient. To illustrate the binding of Srp1 to nascent polypeptides, we disassembled the RNCs by heating the reaction mixture at 95 °C in the presence of 1% SDS. The samples were then diluted to reduce the SDS concentration and subjected to IP with anti-Srp1 and anti-puromycin antibodies, respectively. The precipitates were resolved by SDS-PAGE under reducing conditions and detected by immunoblotting analysis with anti-puromycin or anti-Srp1 antibody. We found that Srp1 was precipitated by the anti-puromycin antibody (Fig. 5D, lanes 3 and 6). Vice versa, puromycin-labeled nascent polypeptides were brought down by the anti-Srp1 antibody (Fig. 5E, lanes 3 and 6). These results indicated that Srp1 was indeed cross-linked to the nascent polypeptides. This analysis also showed that Srp1E145K had a similar affinity for ribosome-bound nascent chains as for wild-type Srp1.

Coupling of Proteasomes to Ribosome-bound Nascent Chains via Srp1 and Sts1—It is of interest to note that, although cotranslational protein degradation is impaired in srp1–49, Srp1E145K binds ribosome-bound nascent chains as efficiently as wild-type Srp1 (Fig. 5). These observations suggest that targeting ribosome-bound nascent chains is probably not the only function of Srp1 in cotranslational degradation. We speculated that Srp1 might be involved in coupling proteasomes to ribosome-bound nascent chains and that this critical activity for cotranslational degradation might be impaired in srp1–49. To test this hypothesis, cell extracts prepared from WT and srp1–49 strains were fractionated by sucrose gradient ultracen-
Srp1 and Sts1 in Cotranslational Protein Degradation

A flow chart of experiments to show the binding of Srp1 to ribosome-bound nascent chains. 

**FIGURE 5. Srp1 binds ribosome-bound nascent polypeptides.** A, flow chart of experiments to show the binding of Srp1 to ribosome-bound nascent chains. Puro, puromycin. B, immunoblot (IB) analysis to show puromycin labeling and cross-linking of nascent chains. RNCs isolated from WT and srp1–49 cells were incubated with puromycin prior to the addition of the cross-linker DTSSP. The reaction mixture was heated at 95 °C for 5 min in sample buffer with or without DTT, followed by SDS-PAGE and immunoblotting with an anti-puromycin antibody. Puro-NCs, puromycin-labeled nascent chains; M, molecular markers. C, endogenous Srp1 and Srp1E145K are physically associated with RNCs. The same samples as in B were analyzed by immunoblotting with an anti-Srp1 antibody. D and E, Srp1 binds ribosome-bound nascent polypeptides. Puromycin-labeled, DTSSP-cross-linked RNCs were disassembled by incubation with 1% SDS at 95 °C for 5 min. The samples were then diluted to reduce the SDS concentration and subjected to IP with anti-puromycin (D) or anti-Srp1 (E) antibody. The precipitates were resolved by SDS-PAGE under reducing conditions and analyzed by immunoblotting with anti-Srp1 (D) or anti-puromycin (E) antibody.

precipitation. Fractions were collected from the top to the bottom of the gradient. The bottom fractions containing polysomes or translating ribosomes were analyzed by immunoblotting with antibodies against proteasome subunit Rpn12, ribosome component L3, and Srp1 (Fig. 6, A–C). Although comparable amounts of Srp1 and Srp1E145K were associated with the polysomes, more than 2-fold proteasomes cosedimented with polysomes in the WT strain than in srp1–49. Similar results were obtained when an anti-20 S proteasome antibody was used to measure the cosedimented proteasomes (data not shown). Note that the proteasome abundance was slightly higher in srp1–49 than in WT cells, likely because of the relatively higher steady-state level of Rpn4 in srp1–49 (data not shown). Thus, Srp1 is involved in the coupling of proteasomes to ribosome-bound nascent chains, and this activity is compromised in Srp1E145K.

It has been suggested that the interaction between Srp1 and the proteasome may be mediated or facilitated by Sts1, a multicyclic suppressor of srp1–49 (45, 46). We found that the cosedimentation of proteasomes with polysomes significantly decreased in the sts1–2 mutant (Fig. 6, A–C, and data not shown). Using GST pulldown assays, we showed that Sts1 directly interacted with Srp1 (Fig. 6D). Moreover, GST-Srp1 pulled down purified proteasomes in the presence but not in the absence of Sts1 (Fig. 6E, compare lanes 2 and 6). These results indicate that Sts1 links the proteasome to Srp1. It is of interest to note that Sts1 had much lower affinity for Srp1E145K than Srp1 (Fig. 6D, compare lanes 2 and 3). In addition, proteasomes were only weakly retained by GST-Srp1E145K, even in the presence of Sts1 (Fig. 6, lane 7). These observations explain why the coupling of proteasomes to polysomes is less efficient in srp1–49. Taken together, these results show that Srp1 and Sts1 recruit proteasomes to ribosome-bound nascent chains.

srp1–49 and sts1–2 Are Hypersensitive to Conditions That Increase Protein Misfolding—We have shown that cotranslational protein degradation is defective in srp1–49 (Figs. 3 and 4). Using a short pulse labeling chase assay, we demonstrated that the bulk degradation of newly synthesized proteins was significantly slower in sts1–2 than in the WT strain (data not shown). This result suggests that Sts1 also plays a general role in cotranslational protein degradation. We next examined whether srp1–49 and sts1–2 are hypersensitive to conditions that enhance the production of misfolded nascent polypeptides. Specifically, we compared the sensitivity of WT, srp1–49, and sts1–2 cells to CHX, hygromycin B, and L-azetidine-2-carboxylic acid. CHX and hygromycin B are translation inhibitors that enhance the production of misfolded nascent polypeptides. Specifically, we compared the sensitivity of WT, srp1–49, and sts1–2 cells to CHX, hygromycin B, and L-azetidine-2-carboxylic acid. CHX and hygromycin B are translation inhibitors that promote the premature release of nascent chains. L-azetidine-2-carboxylic acid is a proline analog that induces protein misfolding after incorporation into nascent polypeptides. Indeed, we found that the srp1–49 and sts1–2 mutants were much more sensitive to these stressors than the WT strain (Fig. 7). These results underscore the physiological function of Srp1 and Sts1 in cotranslational degradation of misfolded nascent polypeptides.
DISCUSSION

Cotranslational protein degradation plays a critical role in preventing the accumulation of misfolded, potentially toxic proteins in the cell. However, the molecular details of cotranslational protein degradation remain poorly understood. One important unanswered question is how the proteasome targets ribosome-bound nascent chains that are destined for cotranslational degradation. It has been suggested that ubiquitylation of nascent polypeptides serves as a targeting signal for the proteasome (8, 14–16). This suggestion is supported by the identification of ribosome-bound ubiquitin ligases and the detection of ubiquitylation of nascent polypeptides (14–18). In addition, several Ub-binding proteins, including translation elongation factor 1A (eEF1A) and Cdc48, are associated with the ribosome and involved in cotranslational degradation of aberrant nascent polypeptides (6, 15, 47). Intriguingly, cotranslational ubiquitylation occurs at a rather low level. In budding yeast cells, 1–6% of nascent polypeptides are cotranslationally ubiquitylated (19, 20). This number is apparently lower than the estimated percentage of yeast proteins likely undergoing cotranslational degradation (14). In fact, our quantitative analysis reveals that about one-third of nascent polypeptides are cotranslationally degraded in yeast cells. These results argue...
that a substantial fraction of nascent polypeptides may be degraded cotranslationally by a Ub-independent pathway. In this study, we demonstrate that cotranslational degradation of Rpn4 can occur without prior ubiquitylation. Our attempts to elucidate the underlying mechanism led to the unveiling of a novel function for Srp1 and Sts1 in cotranslational protein degradation.

Previous studies have firmly established Srp1 as a nuclear import factor whose cargoes include the proteasome (36–41, 48, 49). An early report by Tabb et al. (46) implied the involvement of Srp1 and Sts1 in protein degradation. Although the authors suggested that the function of Srp1 in protein degradation may be separate from its role in nuclear import, the mechanism was not explored. In contrast, Chen et al. (45) proposed recently that the defect of protein degradation in srp1–49 and sts1–2 was caused by a reduced nuclear import of proteasomes. The proposed mechanism may be applicable to nuclear proteins, but it cannot explain impaired degradation of cytosolic substrates such as Ub-Pro- and R-β-gal (46, 50). Nevertheless, neither of these two studies revealed the role of Srp1 and Sts1 in cotranslational protein degradation, perhaps because of overlook of the zero time effect in pulse-chase analysis, i.e. the degradation of newly synthesized polypeptides during pulse labeling. In this study, we demonstrate that Srp1 binds nascent chains emerging from the ribosome and that cotranslational protein degradation is severely impaired in srp1–49 (Figs. 2–5). In addition, we show that the association of proteasomes to polysomes is markedly reduced in (Figs. 2–5). In addition, we show that the association of proteasomes to polysomes is markedly reduced in srp1–49 and sts1–2 (Fig. 6). On the basis of these and other data, we propose a model whereby Srp1 and Sts1 cooperate to recruit proteasomes to polysomes to degrade the nascent polypeptides destined for cotranslational degradation. Specifically, Srp1 targets the susceptible nascent polypeptides, whereas Sts1 serves as an adaptor to link the proteasome to Srp1. This model provides a plausible mechanism for cotranslational degradation of Ub-independent substrates, exemplified by the ability of Srp1 to target non-ubiquitylated Rpn4 nascent chains. Of note, Srp1 appears to bind the ribosome as well, although with lower affinity than the nascent chains attached on the ribosome (Fig. 4). This observation suggests that Srp1 may recruit proteasomes to RNCs via interaction with the nascent chains and the ribosome. These two binding activities should not be mutually exclusive but, likely, cooperate with each other to facilitate the delivery of nascent chains to the proteasome. For instance, the dual binding activity may prolong the association of proteasomes with RNCs, allowing enough time for proteasomes to engulf the nascent chains. This may be particularly important for Ub-independent substrates because they probably have a lower affinity for the proteasome than Ub chains.

It is worthy of note that the N-terminal sequence of Rpn4 is critical for recognition by Srp1 (Fig. 4), suggesting that Srp1 has different affinities for different nascent chains. In other words, Srp1 may target specific nascent chains for cotranslational degradation. The specificity is perhaps determined by the structural features of the N-terminal motif of a nascent chain. For instance, a segment of amino acids that cannot fold properly or quickly enough after emerging from the ribosome may be recognized by Srp1. Interestingly, the N-terminal region of Rpn4 indeed harbors a disordered domain (28). It will be of great interest to decipher the determinant or determinants that mark nascent polypeptides for cotranslational degradation. This remains a challenging task because very few protein species have been clearly identified that undergo cotranslational degradation. Given that Srp1 plays a broad role in cotranslational protein degradation, the identification of Srp1-bound nascent chains through global proteomic analysis or ribosome profiling will provide a snapshot of the cellular proteins that are subject to cotranslational degradation.

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