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Saccharomyces cerevisiae Ub-Conjugating Enzyme Ubc4 Binds the Proteasome in the Presence of Translationally Damaged Proteins

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ABSTRACT

Surveillance mechanisms that monitor protein synthesis can promote rapid elimination of misfolded nascent proteins. We showed that the translation elongation factor eEF1A and the proteasome subunit Rpt1 play a central role in the translocation of nascent-damaged proteins to the proteasome. We show here that multiubiquitinated proteins, and the ubiquitin-conjugating (E2) enzyme Ubc4, are rapidly detected in the proteasome following translational damage. However, Ubc4 levels in the proteasome were reduced significantly in a strain that expressed a mutant Rpt1 subunit. Ubc4 and Ubc5 are functionally redundant E2 enzymes that represent ideal candidates for ubiquitinating damaged nascent proteins because they lack significant substrate specificity, are required for the degradation of bulk, damaged proteins, and contribute to cellular stress-tolerance mechanisms. In agreement with this hypothesis, we determined that *ubc4*Δ *ubc5*Δ is exceedingly sensitive to protein translation inhibitors. Collectively, these studies suggest a specific role for Ubc4 and Ubc5 in the degradation of cotranslationally damaged proteins that are targeted to the proteasome.

PROTEIN synthesis is error prone, and up to 50% of newly synthesized proteins may be degraded cotranslationally (Reits *et al.* 2000; Schubert *et al.* 2000; Turner and Varshavsky 2000). Because nascent proteins that are damaged during synthesis can be ubiquitinated at the ribosome (Sato *et al.* 1988; Benoist and Grand-Perret 1997; Yao *et al.* 1997; Liao *et al.* 1998; Zhou *et al.* 1998; Yewdell 2002), it is likely that a mechanism to recognize and eliminate misfolded proteins is closely coupled to protein synthesis. However, the biochemical events that enable efficient recognition of nascent, misfolded proteins and recruitment of the ubiquitin system have not been determined. Unfolded proteins present a unique hazard that does not arise with native regulatory proteins that are degraded via post-translational mechanisms. Damaged nascent polypeptide chains may be escorted to the proteasome by regulatory factors, such as Rad23, which is the best-characterized member of a family of proteins that contain ubiquitin-like (UbL) and ubiquitin-associated (UBA) domains. These motifs enable Rad23 to bind the proteasome and multiubiquitinated proteins (Chen and Madura 2002). Rad23 and related proteins may promote the trafficking of proteolytic substrates to the proteasome or function as alternate receptors in the proteasome (Chen and Madura 2002; Elsasser *et al.* 2004; Kim *et al.* 2004; Verma *et al.* 2004).

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A yeast strain lacking both Rad23 and Rpn10 (*rad23*Δ *rpn10*Δ) displays pleiotropic defects, including poor growth at low temperature, delay in G2-phase of the cell cycle, accumulation of high-molecular-weight multiubiquitinated (multi-Ub) proteins, and stabilization of substrates of the Ub/proteasome pathway (Lambertson *et al.* 1999). Recent studies showed that *rad23*Δ *rpn10*Δ is also extremely sensitive to inhibitors of protein synthesis (Chuang *et al.* 2005), suggesting that Rad23 and Rpn10 perform an important role in the degradation of nascent, misfolded proteins. A genetic search for dosage (high-copy) suppressors yielded *TEF1* (Chuang *et al.* 2005), a gene encoding the translation elongation factor eEF1A (Anand *et al.* 2001). We proposed that eEF1A provided a link between protein synthesis and protein degradation by promoting the transfer of nascent, damaged proteins to the proteasome (Chuang *et al.* 2005). Specifically, we identified mutants of eEF1A that altered the degradation of test proteins. A mutation in the GTP-coordinating site (eEF1A^{D156N}) that reduced affinity for GTP (Anand *et al.* 2001) caused increased resistance to translational inhibitors and was associated with rapid turnover of nascent, damaged proteins. We detected very low levels of wild-type eEF1A in the proteasome under normal growth conditions. However, eEF1A^{D156N} formed significantly increased interaction with the proteasome, even in the absence of translation inhibitors. One interpretation of these results is that increased eEF1A^{D156N}/proteasome interaction promotes the degradation of nascent, damaged proteins, which could underlie the higher resistance to inhibitors of

protein synthesis. These studies established a potential role for eEF1A in regulating the quality of protein synthesis. Subsequent studies showed that high-molecular-weight ubiquitinated proteins, and the ubiquitin-conjugating enzyme Ubc4, were also present in the proteasome following translational damage.

Protein synthesis inhibitors can rapidly block translation elongation and cause release of truncated polypeptide chains. For instance, disruption of oxidative phosphorylation and reduction of glucose levels depletes intracellular ATP and causes premature translation termination and release of misfolded, nascent polypeptide chains (Giloh and Mager 1975; Moldave 1985; Glickman *et al.* 1998; Ashe *et al.* 2000; Browne and Proud 2002). Nascent, damaged proteins can be conjugated to Ub while associated with the ribosome (Sato *et al.* 1988; Benoist and Grand-Perret 1997; Liao *et al.* 1998; Zhou *et al.* 1998) and rapidly degraded by the proteasome (Reits *et al.* 2000; Schubert *et al.* 2000; Turner and Varshavsky 2000). The cellular response to protein misfolding caused by amino acid analogs, heat shock, and oxidative damage requires Ubc4. The studies described here reveal a prominent role for this ubiquitin-conjugating (E2) enzyme in eliminating nascent, damaged proteins. Since translational errors generate a large fraction of substrates for the proteasome (Reits *et al.* 2000; Schubert *et al.* 2000; Turner and Varshavsky 2000), our findings provide an explanation for the broad substrate specificity of Ubc4, its association with the proteasome, and its key role in cellular stress response mechanisms.

MATERIALS AND METHODS

Plasmids and strains: Epitope-tagged proteasome subunit Pre1-FLAG was expressed from the copper-inducible P_{CUP1} promoter. Expression was induced by the addition of copper sulfate (0.1 mm) to the growth medium. Sensitivity to translation inhibitors was determined by spotting 10-fold dilutions of exponential phase cultures and incubating at 30° for 4 days. Proteasome mutant strain *rpt1/cim5-1* was grown at 24° and then transferred to semipermissive (30°) or nonpermissive (37°) temperatures. M. Hochstrasser (Yale University, New Haven, CT) provided yeast strains deleted of specific Ubc (E2) encoding genes (Chen *et al.* 1993). Proteasome mutant *rpt4* and *rpt6* strains were provided by E. Friedberg (University of Texas, Dallas) (Gillette *et al.* 2001). Pre1-FLAG was expressed from a 2μ plasmid, bearing a *LEU2* selectable marker (J. Dohmen, University of Cologne).

Protein extracts and immunological methods: Detailed methods were described in Chuang *et al.* (2005). Yeast strains containing plasmids were grown in synthetic medium, pelleted, and frozen at −70°. Yeast cells were suspended in buffer A (50 mm Hepes, pH 7.5, 150 mm NaCl, 5 mm EDTA, and 1% Triton X-100 with the addition of protease inhibitors, including Pefabloc SC, leupeptin, aprotinin, antipain, pepstatin, and chymostatin) and lysed by glass-bead disruption. Extracts were normalized to equal protein concentration and equal amounts were applied to anti-FLAG-M2 affinity agarose (Sigma, St. Louis). Beads were washed three times with buffer A and the bound proteins were released by boiling, separated by electrophoresis in SDS-polyacrylamide gels (SDS-PAGE), transferred to nitrocellulose, and examined by immunoblotting.

ATP depletion: Yeast cells expressing Pre1-FLAG were grown to exponential phase and treated with 0.2 mm 2,4-dinitrophenol (2,4-DNP) and 20 mm 2-deoxy-d-glucose (2-DG) at 30°. However, the *rpt1/cim5-1* mutant was treated at the nonpermissive temperature (37°). Aliquots were removed at various times, as indicated in the figure legends, and equal amounts of protein (~500 μg) were immunoprecipitated with FLAG-agarose. The levels of Rpt1, Ubc4, and ubiquitin were determined by immunoblotting.

Antibodies: FLAG-agarose and antibodies against ubiquitin and FLAG were purchased from Sigma Chemical (St. Louis). Anti-β-gal antibodies were purchased from Promega (Madison, WI). The antibodies were used at dilutions recommended by the manufacturers. Antibodies against Ubc4 and Rpt1 were prepared in rabbits (Pocono Rabbit Farms).

RESULTS

Yeast cells lacking Ubc4 and Ubc5 are highly sensitive to inhibitors of protein synthesis: Ubc4 and Ubc5 are functionally redundant E2 enzymes. However, Ubc4 is expressed at much higher levels than Ubc5 and is primarily detected in actively growing cells. A *ubc4* mutant displays a mild growth defect and shows weak sensitivity to diverse environmental stresses. In contrast, loss of Ubc5 has no effect, due to the presence of high levels of Ubc4. A double mutant displays poor growth and stress sensitivity and is deficient in the degradation of bulk proteolytic substrates (Seufert and Jentsch 1990), as well as certain regulatory and reporter proteins (Chen *et al.* 1993; Johnson *et al.* 1995; Byrd *et al.* 1998). To investigate if Ubc4/Ubc5 affected the quality of protein synthesis, we spotted single- and double-mutant strains on medium containing translation inhibitors. A cell lacking Ubc4 (*ubc4*Δ) showed poor growth in the presence of hygromycin B, which interferes with translation elongation and can cause premature release of nascent polypeptide chains (Figure 1A). In contrast, *ubc4*Δ was not sensitive to paromomycin, which decreases translation fidelity. One interpretation of this result is that truncated polypeptide chains are more cytotoxic than proteins bearing coding errors due to their higher potential to misfold. Because of the functional redundancy of Ubc4 and Ubc5, and the high levels of endogenous Ubc4, the growth of *ubc5*Δ was not impaired in the presence of protein synthesis inhibitors. However, both Ubc4 and Ubc5 may be required for optimal degradation of cotranslationally damaged proteins because *ubc4*Δ *ubc5*Δ displayed 100- to 1000-fold increased sensitivity to all the drugs that were examined.

To examine the specificity of these effects we measured drug sensitivity of strains expressing mutant derivatives of other ubiquitin-conjugating enzymes. The Ubc1 protein is of particular interest because it is related to Ubc4/Ubc5, and overlapping phenotypes were described (Seufert *et al.* 1990). A triple mutant lacking Ubc1, Ubc4, and Ubc5 is inviable, indicating that members of

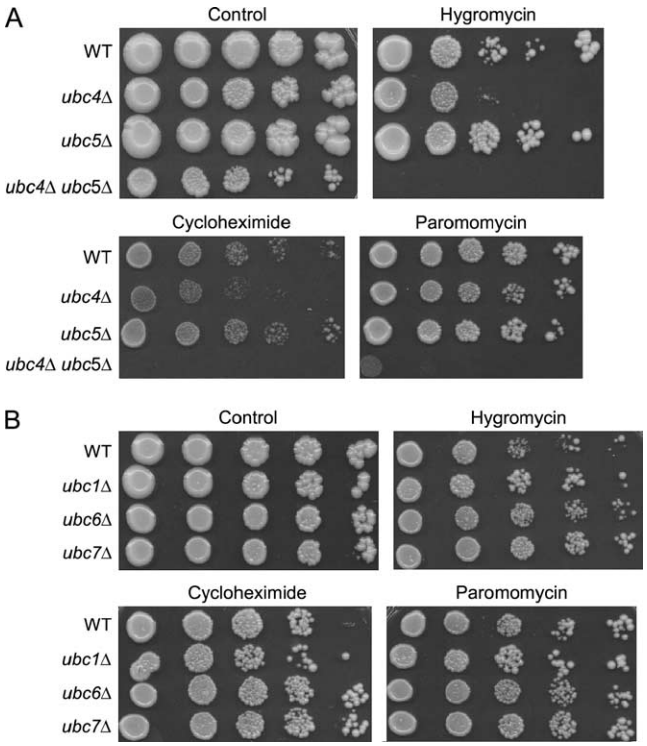

FIGURE 1.—*ubc4*Δ *ubc5*Δ is sensitive to translation inhibitors. (A) Wild-type, *ubc4*Δ, *ubc5*Δ, and *ubc4*Δ *ubc5*Δ cells were grown to exponential phase and diluted to A_{600} ~1.0, and serial 10-fold dilutions were spotted on medium containing translation inhibitors (0.2 mM hygromycin B, 0.001 mM cycloheximide, and 1 mM paromomycin). Plates were incubated at 30° for 4–6 days. (B). Yeast strains with mutations in *ubc1*, *ubc6*, and *ubc7* were grown to exponential phase and adjusted to A_{600} ~1.0, and serial 10-fold dilutions were spotted on medium containing translation inhibitors, as described above.

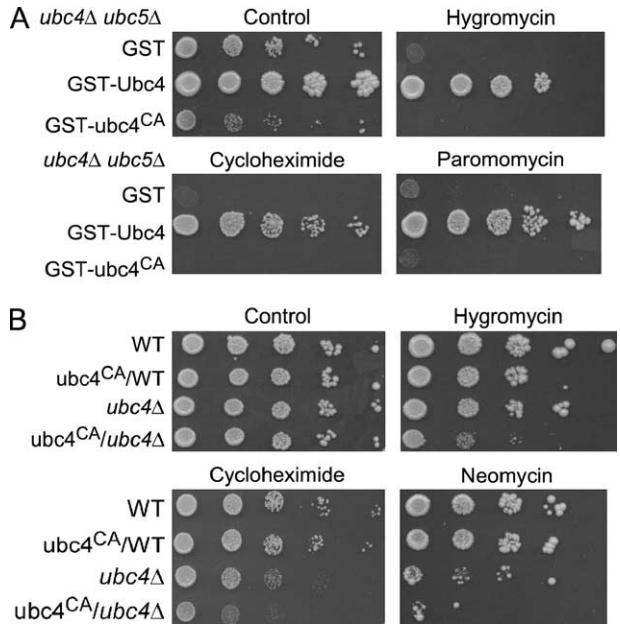

FIGURE 2.—Ubc4 confers resistance to translation inhibitors. (A) GST-Ubc4, but not GST-ubc4^{CA}, suppressed the sensitivity of *ubc4*Δ *ubc5*Δ to translation inhibitors. (B) Expression of high levels of GST-ubc4^{CA} had no effect in a wild-type strain. However, GST-ubc4^{CA} significantly increased the sensitivity of *ubc4*Δ to translation inhibitors. (Neomycin was used at 2 mM.)

this family of E2 enzymes play essential, but broadly redundant functions. However, genetic studies suggested that Ubc1 might not be obligatorily required for cotranslational degradation, due to phenotypic differences between *ubc1*Δ and *ubc4*Δ (JENTSCH *et al.* 1990; SEUFERT *et al.* 1990). For instance, the overlapping function of Ubc1 and Ubc4 plays a significant role in the germination of spores following sporulation. To determine if Ubc1 contributed to translation control, we plated *ubc1*Δ on medium containing translational inhibitors. No obvious growth defect was detected in *ubc1*Δ (Figure 1B), unlike *ubc4*Δ, which showed ~10- to 100-fold reduced growth in medium containing hygromycin B or cycloheximide. However, it is possible that a double mutant lacking both Ubc1 and Ubc4 will reveal a role for Ubc1 in the turnover of nascent, damaged proteins. Similarly, yeast mutants lacking Ubc6 or Ubc7 were not required for providing resistance to inhibitors of protein synthesis (Figure 1B). Ubc6 and Ubc7 are required for the elimination of damaged proteins in the endoplasmic proteins (PLEMPER and WOLF 1999; FRIEDLANDER *et al.* 2000; HILL and COOPER 2000; SWANSON *et al.* 2001) and for the degradation of Matα2 (CHEN *et al.* 1993). We determined that a double mutant (*ubc6*Δ *ubc7*Δ) displayed normal growth on medium containing all the protein synthesis inhibitors that we examined in Figure 1 (data not shown). Previous studies showed that the degradation of Matα2 occurred through two independent pathways involving Ubc4/5 and Ubc6/7 (CHEN *et al.* 1993). Consequently, both pathways may contribute to the degradation of nascent, damaged proteins, although our results suggest that Ubc4/5 represent the primary mechanism since only *ubc4*Δ, but not *ubc6*Δ or *ubc7*Δ, showed a growth defect on medium containing protein synthesis inhibitors.

A catalytically inactive derivative of Ubc4 can increase sensitivity to translation inhibitors: We expressed a catalytically inactive Ubc4 mutant (GST-ubc4^{CA}; TONGAONKAR *et al.* 2000) in *ubc4*Δ *ubc5*Δ, to further characterize its role in translation control. GST-ubc4^{CA} can bind the proteasome, but is unable to form a thioester intermediate with Ub (TONGAONKAR *et al.* 2000). Therefore, this allele has the potential to function as a dominant interfering mutant. We determined that GST-Ubc4 suppressed the sensitivity of *ubc4*Δ *ubc5*Δ to translation inhibitors (Figure 2A), while GST-ubc4^{CA} failed to suppress any of the pleiotropic defects of *ubc4*Δ *ubc5*Δ. The drug sensitivity of *ubc4*Δ *ubc5*Δ cells expressing GST-ubc4^{CA} was indistinguishable from the GST control. On the basis of this result, we investigated if high-level expression of GST-ubc4^{CA} would increase sensitivity to translational inhibitors by competing with native Ubc4 or Ubc5 (Figure 2B). The expression of GST-ubc4^{CA} did not affect the growth of

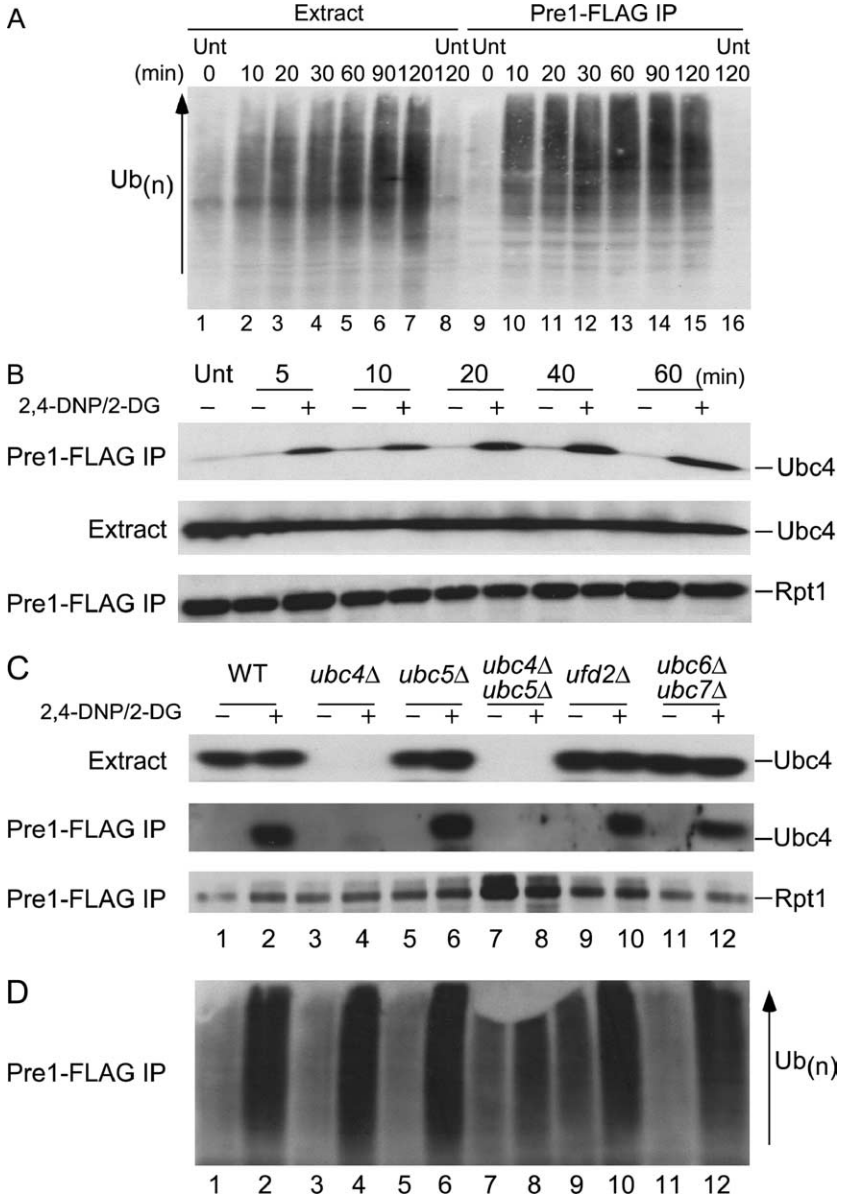

FIGURE 3.—Proteasome interaction with multi-Ub proteins and Ubc4 increases following ATP depletion. Wild-type yeast expressing epitope-tagged 20S proteasome subunit Pre1-FLAG were suspended in medium containing 2-DG + 2,4-DNP for 120 min. (A) Aliquots were withdrawn from the culture (time indicated in minutes on the top), and equal amounts of protein extract were resolved in SDS/PAGE and examined by immunoblotting with antibodies against Ub (Extract; lanes 1–8). Lanes 1 and 8 contain untreated samples (Unt). The sample in lane 1 was prepared before addition of 2-DG + 2,4-DNP, while the extract examined in lane 8 was obtained from the same culture after incubation for 120 min without ATP depletion. In lanes 9–16, Pre1-FLAG was precipitated from the extracts examined in lanes 1–8 to investigate the interaction of ubiquitinated proteins with the proteasome (FLAG-IP). (B) Pre1-FLAG was precipitated from wild-type cell extracts prepared after ATP depletion to examine the interaction of Ubc4 with the proteasome (Ubc4). Ubc4 levels were unchanged during the course of the experiment (Extract), and the efficiency of proteasome purification, as gauged by the copurification of Rpt1 with Pre1-FLAG, was similar in all samples. Rapid and sustained increase in Ubc4/proteasome interaction is observed in all treated lanes (+), while very low levels are detected in the untreated extracts (−). (C) The rapid association of Ubc4 with the proteasome, following ATP depletion, was confirmed in an independent set of strains (see lane 2). Ubc4 was not detected in *ubc4*Δ and *ubc4*Δ *ubc5*Δ (lanes 4 and 8), confirming the specificity of the antibody reaction. Ubc4/proteasome interaction was also examined in *ufd2*Δ (lanes 9 and 10) and in *ubc6*Δ *ubc7*Δ (lanes 11 and 12). (D) Pre1-FLAG was immunoprecipitated from the same extracts described in C, and proteasomes were resolved by SDS-PAGE. An immunoblot was incubated with antibodies against ubiquitin, and the rapid increase in the levels of multiubiquitinated proteins, following ATP depletion, was specifically reduced in *ubc4*Δ *ubc5*Δ.

wild-type and *ubc5*Δ strains (data not shown), suggesting that it is unable to compete with the high levels of endogenous Ubc4. In contrast, expression of GST-ubc4^{CA} in *ubc4*Δ increased sensitivity to protein synthesis inhibitors ~10- to 100-fold, indicating that the low levels of Ubc5 are insufficient to compete effectively with the elevated levels of ubc4^{CA} mutant protein. These results are in agreement with previous studies, which showed that Ubc4 is the primary E2 enzyme required for the elimination of bulk cellular proteins (SEUFERT and JENTSCH 1990).

Increased levels of ubiquitinated proteins in the proteasome following ATP depletion: We showed previously that ATP depletion mirrored the effects of protein synthesis inhibitors (including paromomycin and cycloheximide; CHUANG *et al.* 2005). We determined that after 30 min of ATP depletion, the degradation of a well-characterized substrate of the Ub/proteasome system (Ub-Pro-βgal) was not reduced (data not shown), indicating that proteasome activity is not significantly reduced. To examine the effect of translational damage we monitored the levels of ubiquitinated proteins in cell extracts and in association with the proteasome following ATP depletion. Yeast cells were grown in medium containing 2-deoxyglucose + 2,4-dinitrophenol (2-DG + 2,4-DNP), and protein extracts were examined with antibodies against Ub. The levels of high-molecular-weight Ub cross-reacting material increased significantly in the extracts within 10 min of treatment and accumulated progressively over the next ~2 hr (Figure 3A). The accumulation of multi-Ub proteins during this period suggested that substrate-capture and ubiquitination was not significantly reduced under these conditions. Pre1-FLAG was immunoprecipitated (IP) to purify the proteasome and examine the levels of multi-Ub proteins. We determined that the levels of multi-Ub

proteins bound to the proteasome increased ~10-fold (Figure 3A; compare Unt and 10 min, lanes 9 and 10), indicating the capacity of the proteasome for interacting with proteolytic substrates is typically not limiting. Intriguingly, the multi-Ub proteins associated with the proteasome displayed a distinctly larger size distribution. One interpretation of this result is that free multi-Ub proteins (in extracts) are vulnerable to the action of deubiquitinating enzymes, while proteasome-associated multi-Ub chains are shielded from this activity. However, these results are also consistent with the possibility that multi-Ub chains are expanded, following their interaction with the proteasome. This conjecture is in agreement with our previous observation that purified proteasomes have the capacity to add ^{32}P-labeled Ub to a test protein (Tongaonkar *et al.* 2000).

Ubc4 levels in the proteasome increase dramatically following translation damage: Ubc4 and Ubc5 play an important role in cellular stress-response mechanisms (Seufert and Jentsch 1990), and we showed previously that heat stress increased Ubc4/proteasome binding (Tongaonkar *et al.* 2000). We surmised that since heat stress causes protein misfolding, it was conceivable the Ubc4 might also participate in the degradation of nascent, misfolded proteins. We therefore depleted intracellular ATP to generate damaged nascent polypeptide chains and examined Ubc4/proteasome interaction. Pre1-FLAG was immunoprecipitated from control and ATP-depleted cell extracts, and an immunoblot was incubated with antibodies against Ubc4. High levels of Ubc4 were detected in the proteasome within 5 min following ATP depletion (Figure 3B), and maximal levels (>10-fold) were observed ~20 min after ATP-depleting conditions were initiated. However, Ubc4 abundance in extracts from untreated and treated cells remained unchanged during the 60-min time course of the experiment. This result suggested that only a small fraction of cellular Ubc4 was bound to the proteasome. The copurification of Rpt1 (a subunit in the 19S regulatory particle) with Pre1-FLAG (a 20S subunit) decreased slightly following ATP depletion, indicating that 19S + 20S subunit interactions were only modestly reduced. We note that the timescale of Ubc4/proteasome binding mirrored the association between ubiquitinated proteins and the proteasome (Figure 3A). Taken together, the extreme sensitivity of *ubc4*Δ *ubc5*Δ to translational inhibitors and the ability of Ubc4 to rapidly bind the proteasome in the presence of nascent, damaged proteins provide compelling evidence of a role for this highly conserved E2 enzyme in protein synthesis quality control.

To confirm the interaction of Ubc4 with the proteasome, we examined the binding in several strains that are defective in the Ub/proteasome pathway (Figure 3C). Consistent with the results described above, Ubc4/proteasome interaction increased rapidly following ATP depletion in wild-type cells (Pre1-FLAG IP; lanes 1 and 2). ATP depletion did not affect the levels of Ubc4 in cell extracts, and the total amount of intact proteasome that was recovered with Pre1-FLAG was constant (Rpt1). We observed similar results in wild-type, *ubc5*Δ, and *ubc6*Δ *ubc7*Δ cells. As expected, Ubc4 was not detected in extracts prepared from *ubc4*Δ and *ubc4*Δ *ubc5*Δ cells. Additionally, Ubc4/proteasome binding was unaffected in *ufd2*Δ cells. Ufd2 encodes an E4 multi-Ub chain processivity factor that cooperates with Ubc4 to conjugate ubiquitin to substrates of the Ub-fusion degradation (UFD) pathway (Koegl *et al.* 1999).

Because Ubc4/Ubc5 facilitate the elimination of bulk cellular proteins (Seufert and Jentsch 1990) and bind the proteasome following heat stress (Tongaonkar *et al.* 2000), we examined the level of ubiquitinated proteins associated with the proteasome in *ubc4*Δ *ubc5*Δ cells. Pre1-FLAG was immunoprecipitated from untreated and ATP-depleted extracts, and an immunoblot was incubated with antibodies against ubiquitin (Figure 3D). We detected significantly decreased levels of proteasome-associated ubiquitinated proteins in *ubc4*Δ *ubc5*Δ following ATP depletion (compare lanes 1–2 and 7–8).

The Rpt1 proteasome subunit performs a specific role in mediating Ubc4/proteasome interaction: Mutations in the conserved ATP-binding domains of the six ATPase subunits in the 19S proteasome particle reveal multiple roles (Rubin *et al.* 1998). These findings suggest that the ATPase subunits may confer unique substrate selectivity or exert distinct effects on the degradation of proteolytic substrates. We examined Ubc4/proteasome interaction in yeast mutant strains expressing defective ATPase subunits and detected a profound defect in *rpt1*, which encodes a temperature-sensitive allele of Rpt1/Cim5 (Ghislain *et al.* 1993) (Figure 4A). Wild-type and congenic *rpt1* strains were treated with 2-DG + 2,4-DNP and the proteasome was immunoprecipitated (Pre1-FLAG IP). Although the interaction between Ubc4 and the proteasome increased significantly in the wild-type strain, we detected markedly reduced binding in *rpt1*. In contrast, the level of Rpt1 that was copurified with Pre1-FLAG was similar, and overall levels of Ubc4 were unaffected. Consistent with these results, the previously reported heat-induced interaction between Ubc4 and the proteasome (Tongaonkar *et al.* 2000) was not observed in *rpt1* (Figure 4B). Extracts were prepared from yeast cells (*RPT1* and *rpt1*) that were propagated at 24°, 30°, and 37° and applied to FLAG-agarose to purify proteasomes. The image shown in Figure 4B was overexposed ~10-fold (compared to Figure 4A) to demonstrate the complete absence of Ubc4/proteasome interaction in *rpt1* mutant cells following heat stress. This effect was specific, because Ubc4/proteasome interaction was not reduced in *rpt4* or *rpt6* (Figure 4C). However, since this was not a comprehensive survey, we recognize that other proteasome subunits might also affect Ubc4/proteasome binding. The copurification of both Rpt1 and rpt1 (mutant) proteins with Pre1-FLAG were similar following ATP depletion

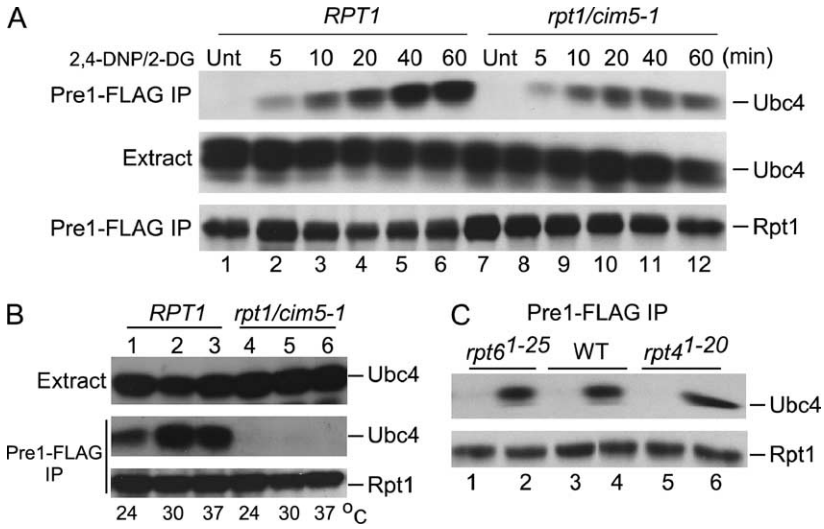

FIGURE 4.—Functional Rpt1 is required for efficient Ubc4/proteasome binding. (A) Pre1-FLAG was precipitated from wild-type and *rpt1* strains following ATP depletion and Ubc4 levels associated with the proteasome, and in extracts, were determined. We measured the recovery of Rpt1 with Pre1-FLAG to confirm that intact proteasomes were purified. ATP depletion resulted in reduced Ubc4/proteasome association in *rpt1* (lanes 7–12), although the levels in total extracts were essentially unchanged. (B) Wild-type and *rpt1* cells were grown for 18 hr at 24° and then transferred to 30° and 37° for 6 hr. Pre1-FLAG was purified and an immunoblot was incubated with antibodies against Ubc4 to test the heat-inducible interaction of Ubc4 with the proteasome. To underscore the failure of *rpt1* proteasomes to bind Ubc4 following heat shock we overexposed this autoradiogram ~10-fold longer (compared to A). Despite the overexposure, it is evident that heat stress resulted in increased Ubc4/proteasome interaction in the wild-type cell but not in *rpt1* mutant. (C) Ubc4/proteasome interaction was determined in $rpt6^{1\text{-}25}$ and $rpt4^{1\text{-}20}$ proteasome mutants and their corresponding wild-type counterparts, following ATP depletion. Ubc4/proteasome interaction following ATP depletion was similar in all three strains. The copurification of Rpt1 with Pre1-FLAG in these mutant proteasomes was constant in the presence or absence of ATP-depleting conditions (bottom). Even numbered lanes correspond to ATP-depleted extracts.

and heat stress, suggesting that the compositional integrity of the proteasome was not altered. On the basis of these studies, we speculate that Ubc4/proteasome interaction is mediated by Rpt1 and is required for the efficient degradation of nascent, damaged proteins.

DISCUSSION

Rad23 and Rpn10 can interact with ubiquitinated proteins and promote their degradation by the proteasome (CHEN and MADURA 2002; ELSASSER *et al.* 2004; KIM *et al.* 2004; VERMA *et al.* 2004). We reported that the translational elongation factor eEF1A plays a key role in binding nascent, damaged proteins and promotes their translocation to the proteasome (CHUANG *et al.* 2005). Because eEF1A was isolated as a high-copy suppressor of *rad23*Δ *rpn10*Δ, we speculate that Rad23 and Rpn10 may mediate the degradation of nascent, damaged proteins. Although it is uncertain how the proteasome recognizes cotranslationally damaged proteins, we determined that the proteasome subunit Rpt1 was required for efficient eEF1A/proteasome binding. A mutation in Rpt1 [*cim5-1*; (GHISLAIN *et al.* 1993)] was associated with reduced eEF1A/proteasome binding and stabilization of translationally damaged proteins. It is significant in this regard that Rpt1 is also required for efficient interaction with Ubc4 (Figure 4).

Protein synthesis is acutely sensitive to ATP levels because a major fraction of cellular energy is consumed during translation elongation (GILOH and MAGER 1975; MOLDAVE 1985; GLICKMAN *et al.* 1998; ASHE *et al.* 2000; BROWNE and PROUD 2002). The translation elongation factor eEF1A interacted with ubiquitinated proteins and was rapidly detected in the proteasome following ATP depletion. Protein synthesis inhibitors (such as paromomycin and hygromycin B) also caused increased interaction between eEF1A and the proteasome, although this effect occurred after a longer incubation period (4 hr *vs.* 10 min of ATP depletion). We therefore focused on the effect of ATP depletion because of the rapidity and reproducibility of the cellular response. Our findings are consistent with previous studies, which showed that nascent, damaged proteins can be ubiquitinated (SATO *et al.* 1988; BENOIST and GRAND-PERRET 1997; YAO *et al.* 1997; LIAO *et al.* 1998; ZHOU *et al.* 1998; YEWDELL 2002) and rapidly degraded by the proteasome (TURNER and VARSHAVSKY 2000). However, the mechanism that ensures their prompt translocation to the proteasome is unknown. E2 and E3 proteins, as well as other regulatory factors, form regulated interactions with the proteasome (TONGAONKAR *et al.* 2000; VERMA *et al.* 2000; XIE and VARSHAVSKY 2000, 2002). The finding that Ub-cross-reacting material in the proteasome displayed a larger size distribution than that present in extracts raises the possibility that proteasome-associated E2/E3 enzymes might expand substrate-linked multi-Ub chains. This idea is consistent with previous studies that showed that proteasome-associated E2 enzymes can conjugate Ub to a test substrate (TONGAONKAR *et al.* 2000).

ATP depletion affects myriad cellular functions, although its effect on protein synthesis is immediate since translation elongation consumes a major fraction of cellular energy. Partially formed polypeptide chains are likely to encounter folding difficulties and could be highly deleterious to the cell. To examine the effect of translation damage on the Ub/proteasome pathway, we subjected yeast cells to ATP-depleting conditions and monitored the levels of multi-Ub proteins in total cellular extracts and in association with the proteasome. Translational stress resulted in higher levels of multi-Ub

proteins, but not Ubc4. However, increased levels of both multi-Ub proteins and Ubc4 were detected in the proteasomes following ATP depletion. These results suggested that Ubc4/proteasome interaction might facilitate the recognition and further ubiquitination of cotranslationally damaged substrates. In agreement with this hypothesis, we determined that yeast cells lacking the Ubc4/Ubc5 family of E2 proteins are extremely sensitive to translation inhibitors. Furthermore, overexpression of a catalytically inactive derivative of Ubc4 (ubc4^{CA}) interfered with cotranslational protein degradation and increased sensitivity to protein synthesis inhibitors. ubc4^{CA} is unable to form a thioester bond with Ub, although it is fully capable of binding the proteasome (Tongaonkar *et al.* 2000). Therefore, it is possible that Ubc4 can ligate Ub to substrates, after they have bound the proteasome.

The rapid cotranslational degradation of bulk cellular proteins (Reits *et al.* 2000; Schubert *et al.* 2000; Turner and Varshavsky 2000; Chuang *et al.* 2005), as well as specific proteins (Sato *et al.* 1988; Benoist and Grand-Perret 1997; Yao *et al.* 1997; Zhou *et al.* 1998; Yewdell 2002), has been described. The Ubc4/Ubc5 E2 enzymes are required for the degradation of canavanyl proteins and proteins damaged by heat stress (Seufert and Jentsch 1990), which are known to cause protein unfolding. Therefore, we investigated the requirement for Ubc4 in the cotranslational degradation of nascent, misfolded proteins.

Ubc4 and Ubc5 are required for the turnover of overall cellular proteins, and a double mutant (*ubc4*Δ *ubc5*Δ) is sensitive to a broad range of agents that cause nonspecific protein damage (Seufert and Jentsch 1990) and translation damage (Grant *et al.* 1989). Most E2 enzymes contain amino- and carboxy-terminal extensions that facilitate interactions with regulatory molecules and substrates. However, Ubc4 and Ubc5 consist primarily of a catalytic domain (Seufert and Jentsch 1990), suggesting that they might operate with other substrate-recognition factors, possibly the proteasome, to conjugate Ub to substrates. We showed previously that Ubc4 could bind the proteasome following heat stress, suggesting that its functions may be coupled to this interaction. *ubc4*Δ mutant displayed significant sensitivity to translation inhibitors (Figure 1A) and might represent a key E2 enzyme for the elimination of nascent, damaged proteins. The more severe defects of the *ubc4*Δ *ubc5*Δ double mutant indicate that Ubc5 can augment the function of Ubc4. A previous study reported that *ubc1*Δ is sensitive to the presence of L-canavanine (Seufert *et al.* 1990), suggesting that it might also control the quality of protein synthesis. However, in contrast to *ubc4*Δ, we observed no growth defect of *ubc1*Δ on medium containing hygromycin B. Further study will be required to determine how the degradation of canavanyl proteins differs from the removal of prematurely truncated proteins. It is also noteworthy that Ubc6 and Ubc7, which play a key role in the degradation of misfolded proteins in the endoplasmic reticulum, do not appear to play a significant role in protein synthesis quality control, as the *ubc6*Δ and *ubc7*Δ single mutants were not sensitive to translation inhibitors. Furthermore, ATP depletion did not prevent the accumulation of multi-Ub proteins in proteasomes isolated from *ubc6*Δ *ubc7*Δ double mutant. In contrast, ATP depletion reduced the levels of ubiquitinated proteins in proteasomes isolated from *ubc4*Δ *ubc5*Δ (Figure 3D). One interpretation of this result is that proteasomes in *ubc4*Δ may bind substrates, but fail to ubiquitinate them adequately, resulting in inefficient degradation. If proteolytic substrate has a longer "resident time" in proteasomes in the *ubc4*Δ mutant, it could adversely affect the degradation of other substrates. The failure to degrade these proteins could underlie the extreme sensitivity of *ubc4*Δ *ubc5*Δ to protein synthesis inhibitors and heat stress. Further study will be required to determine if nascent, damaged proteins are multiubiquitinated prior to their interaction with the proteasome or if Ubc4 ubiquitinates substrates following their interaction with the proteasome. Ubc4 is not expected to be inadvertently translocated to the proteasome through an association with substrate-linked multi-Ub chains, because a catalytically inactive mutant (ubc4^{C86A}) also bound the proteasome (Tongaonkar *et al.* 2000).

The six ATPase subunits in the 19S particle may have distinct effects on proteolysis, because mutations in their ATP-binding motifs have multiple effects (Rubin *et al.* 1998). Our studies suggest that Rpt1 might perform a specific role in the degradation of nascent polypeptide chains. The failure of Ubc4 to bind the *rpt1* mutant proteasome (Figure 4) provides compelling genetic confirmation of our biochemical results. Proteasomes containing the rpt1 mutant protein are also deficient in binding eEF1A and is associated with inefficient degradation of nascent, misfolded proteins (Chuang *et al.* 2005). We propose that Ubc4/proteasome interaction is required for the degradation of translationally damaged proteins, because both *ubc4*Δ *ubc5*Δ and *rpt1* are sensitive to translation inhibitors (Figure 1 and our unpublished results).

Depletion of intracellular Ub also causes sensitivity to translation inhibitors (Hanna *et al.* 2003), and a yeast mutant that is unable to recycle Ub efficiently (*ubp6*) is sensitive to anisomycin and cycloheximide (Hanna *et al.* 2003). However, *ubp6* is moderately resistant to hygromycin B, while *ubc4*Δ *ubc5*Δ is highly sensitive to this drug (Figure 1A), suggesting that multiple mechanisms regulate the quality of protein synthesis. These and earlier studies show that Ubc4, Rpt1, and eEF1A (as well as Rad23 and Rpn10) are required for efficient response to translation damage and degradation of nascent, damaged proteins by the Ub/proteasome pathway. On the basis of these findings, we speculate that Ubc4/proteasome interaction could play a central role in cellular stress response mechanisms.

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