Molecular Cell, Vol. 8, 439–448, August, 2001, Copyright ©2001 by Cell Press

Selective Degradation of Ubiquitinated Sic1 by Purified 26S Proteasome Yields Active S Phase Cyclin-Cdk

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Summary

Selective degradation of single subunits of multimeric complexes by the ubiquitin pathway underlies multiple regulatory switches, including those involving cyclins and Cdk inhibitors. The machinery that segregates ubiquitinated proteins from unmodified partners prior to degradation remains undefined. We report that ubiquitinated Sic1 (Ub-Sic1) embedded within inactive S phase cyclin-Cdk (S-Cdk) complexes was rapidly degraded by purified 26S proteasomes, yielding active S-Cdk. Mutant proteasomes that failed to degrade Ub-Sic1 activated S-Cdk only partially in an ATP-dependent manner. Whereas Ub-Sic1 was degraded within ∼2 min, spontaneous dissociation of Ub-Sic1 from S-Cdk was ∼200-fold slower. We propose that the 26S proteasome has the intrinsic capability to extract, unfold, and degrade ubiquitinated proteins while releasing bound partners untouched. Activation of S-Cdk reported herein represents a complete reconstitution of the regulatory switch underlying the G1/S transition in budding yeast.

Introduction

The eukaryotic 26S proteasome mediates the bulk of cellular ATP-dependent protein turnover (Hershko and Ciechanover, 1998; Voges et al., 1999). It is comprised of two 19S regulatory particles that abut both ends of a 20S proteolytic core. The 20S core consists of a cylindrical stack of four seven-membered rings, with only the two inner rings containing proteolytically active subunits. The entry ports of the cylinder are effectively sealed such that only small peptides and unfolded proteins can be hydrolyzed by the 20S. Docking of the 19S particle on the 20S core confers ATP dependence to the proteolytic process through the proposed ability of the 19S to harness the energy of ATP hydrolysis to unfold and translocate a target substrate into the opened cavity of the 20S core. The 19S particle is comprised of 19 distinct proteins, with six of them being ATPases.

Although the 26S proteasome serves as a garbage disposal where misfolded or short-lived proteins are degraded, it can also be viewed as a dynamic hub where multimeric protein complexes are remodeled via selective degradation of an unstable partner. The ability of the ubiquitin/proteasome pathway to degrade selectively a single subunit of a multisubunit complex is often exploited as a regulatory switch. For example, nuclear factor-κB (NF-κB) is activated in vivo by selective degradation of its inhibitor IκB (Maniatis, 1999). Mitotic Cdk is inactivated at the end of mitosis by selective degradation of tightly bound mitotic cyclin (Peters et al., 1998), and S phase cyclin-Cdk (S-Cdk) is activated in vivo at the G1/S transition upon degradation of its inhibitor, Sic1 (Schwob et al., 1994; Verma et al., 1997a). Because many physiological substrates of the ubiquitin/proteasome pathway, such as IκB, mitotic cyclin, and Sic1, are assembled into multisubunit complexes, subunit-selective degradation is of critical importance to the fundamental role that this system plays in cellular regulation.

Selective degradation by the ubiquitin/26S proteasome system was first demonstrated in vivo with the heterodimeric yeast α2/α1 repressor (Hochstrasser and Varshavsky, 1990) and in crude reticulocyte lysates using engineered tetramers of β-galactosidase (β-gal) assembled from degradable and nondegradable subunits (Johnson et al., 1990). However, since these and more recent in vitro studies on IκB and mitotic cyclin turnover have all been performed in crude or undefined systems, it has not been possible to determine whether the 26S proteasome by itself can carry out subunit-selective degradation, and at which step of the degradation pathway unstable proteins are segregated from their stable partners.

Two competing hypotheses have been advanced to account for subunit-selective degradation. On the one hand, it has been proposed that ubiquitin acts as a "proteinaceous detergent" that destabilizes the folded state of proteins to which it is attached (Johnson et al., 1995). Alternatively, it has been proposed that the ATPases resident in the 26S proteasome act as "unfoldases" that selectively extricate ubiquitinated proteins from multisubunit complexes (Horwich et al., 1999; Larsen and Finley, 1997; Zwickl and Baumeister, 1999). Although the unfoldase hypothesis has great appeal, it was recently reported that purified 26S proteasomes, unlike reticulocyte extracts, are unable to disassemble and degrade purified ubiquitinated β-gal tetramers (Thrower et al., 2000). In contrast, monomeric DHFR was unfolded and degraded by enriched proteasomes. Because ubiquitinated β-gal tetramers bind to the 26S proteasome, the authors suggested that unidentified factors might be required in some cases to help unfold substrates prior to their degradation by the proteasome. Similarly, the activation of NF-κB via destruction of ubiquitinated IκBα was proposed to require heat shock chaperones in addition to the 26S proteasome (Chen et al., 1995; Lee et al., 1996). Finally, it was recently reported that although 26S proteasomes are required for the disassembly of mitotic cyclin-Cdc2 complexes in crude *Xenopus* extracts during the exit from mitosis, purified proteasomes are unable to achieve this feat (Nishiyama et al., 2000).

In the current study, we presented purified ubiquiti-

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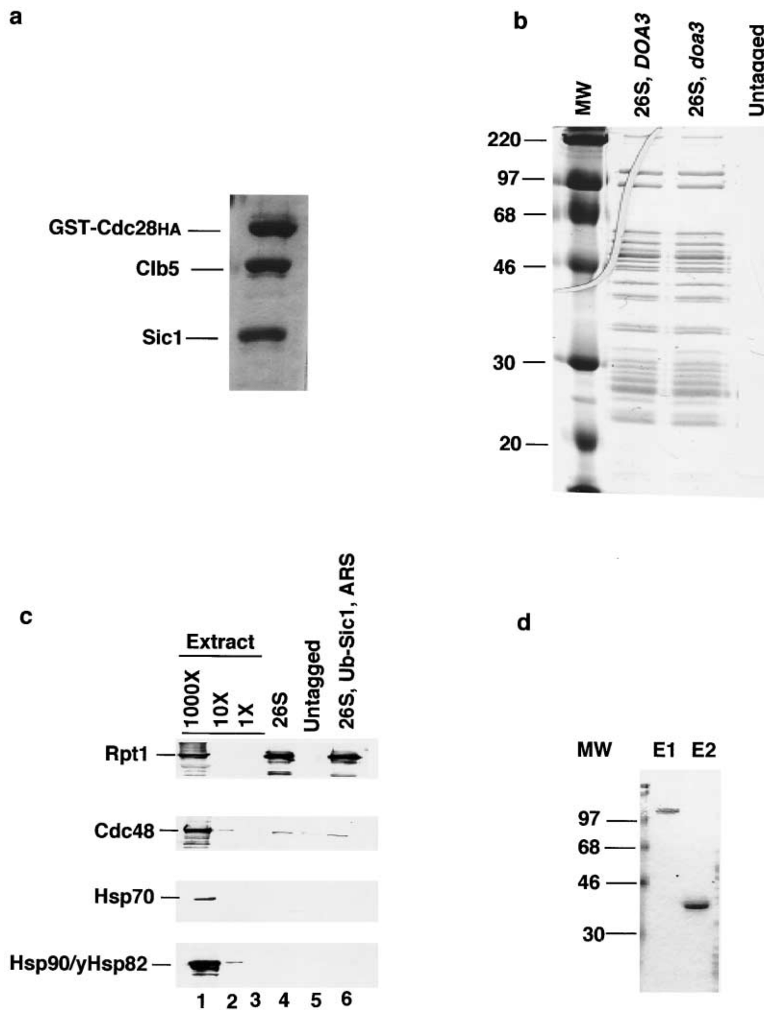

Figure 1. Purification of Sic1/S-Cdk Substrate Complex and the 26S Proteasome and Assessment of Their Purities by SDS-PAGE and Immunological Analysis

(a) Sic1/GST-Cdc28^{HA}/Clb5 complexes were purified from insect cells by glutathione affinity chromatography, fractionated by SDS-PAGE, and stained with Coomassie Blue. Similar results were obtained with complexes that contained ^{HA6}Clb5, but the tagged ^{HA6}Clb5 and GST-Cdc28^{HA} subunits comigrated and could no longer be resolved (Figure 2b).

(b) 26S proteasomes were purified by anti-Flag affinity chromatography (Verma et al., 2000) from *PRE1*$^{Flag\text{-}His6}$ wild-type *DOA3* (lane 2), *PRE1*$^{Flag\text{-}His6}$*doa3* mutant (lane 3), and *PRE1* untagged cells (lane 4), fractionated by SDS-PAGE, and stained with Coomassie Blue.

(c) Aliquots of yeast extracts (lanes 1–3; 1× = 25 ng) or purified 26S proteasomes (3 μg; discussed in the article) containing Ub-Sic1 and 1X ATP regenerating system (lane 6) or not (lane 4), were resolved on SDS-PAGE gels, transferred to nitrocellulose, and immunoblotted for the presence of Rpt1, Cdc48, and the Hsc82 and Hsp 70 families of stress proteins using antisera specific for the proteins.

(d) E1 (Uba1^{His6}) and E2 (Cdc34) enzymes were purified from budding yeast and *E. coli*, respectively, as described previously (Seol et al., 1999). Aliquots were resolved by SDS-PAGE and stained with Coomassie Blue.

nated Sic1 (Ub-Sic1), bound to S-Cdk, to 26S proteasomes purified from budding yeast. We demonstrate that 26S proteasomes selectively degraded Ub-Sic1, but not unmodified Sic1. Degradation of Ub-Sic1 was ~200-fold more rapid than its spontaneous dissociation from S-Cdk, implying that the 26S proteasome actively extracts Ub-Sic1 from S-Cdk as proteolysis proceeds. Remarkably, selective elimination of Sic1 was accompanied by essentially complete activation of its erstwhile partner, S-Cdk. Using protease-dead mutants, we show that S-Cdk activation can be partially, but not entirely, uncoupled from substrate proteolysis.

Results

Evaluation of Purity of Reaction Components

To address the biochemical pathway underlying subunit-selective degradation of Ub-Sic1 and the activation of S-Cdk, we sought to perform a conceptually simple, yet technically challenging, experiment—mixing together pure substrate and pure proteasomes. Recombinant Sic1-Clb5-Cdc28 (S-Cdk) substrate complexes were purified from insect cells (Figures 1a and 4c). Ubiquitinated (Ub-Sic1) and unmodified Sic1/S-Cdk substrate complexes were subsequently generated by sequential treatment with immobilized G1 cyclin-Cdk protein kinase and SCF ubiquitin ligase as described in Figure 2a (see Experimental Procedures). The purity of the E1 and E2 enzymes (which are soluble and thus remain in the substrate preparation) is shown in Figure 1d. 26S proteasomes (Figure 1b) were purified by affinity chromatography from a strain that expresses the Pre1 subunit of the 20S core appended to a bipartite Flag-His6 epitope (Verma et al., 2000). The composition of this preparation was previously evaluated by a mass spectrometry technique (McCormack et al., 1997). Because this analysis employed only a single chromatographic separation (which limits the number of peptides that can be identified), a more thorough investigation of the composition of the 26S proteasomes was undertaken by multidimensional chromatography followed by mass spectrometry (MudPIT; Washburn et al., 2001). The failure of MudPIT to detect peptides from Cdc48, Hsp90 family members, and Ydj1, along with less than 0.3% of the total identified peptides corresponding Hsp70 stress proteins, confirmed that these proteins, if present, were at biochemically inconsequential levels (see supplemental data at http://www.molecule.org/cgi/content/full/8/2/439/DC1). Consistent with these results, direct immunoblotting revealed negligible contamination of 26S proteasome substrate preparations and complete degradation reactions by Hsp70 family

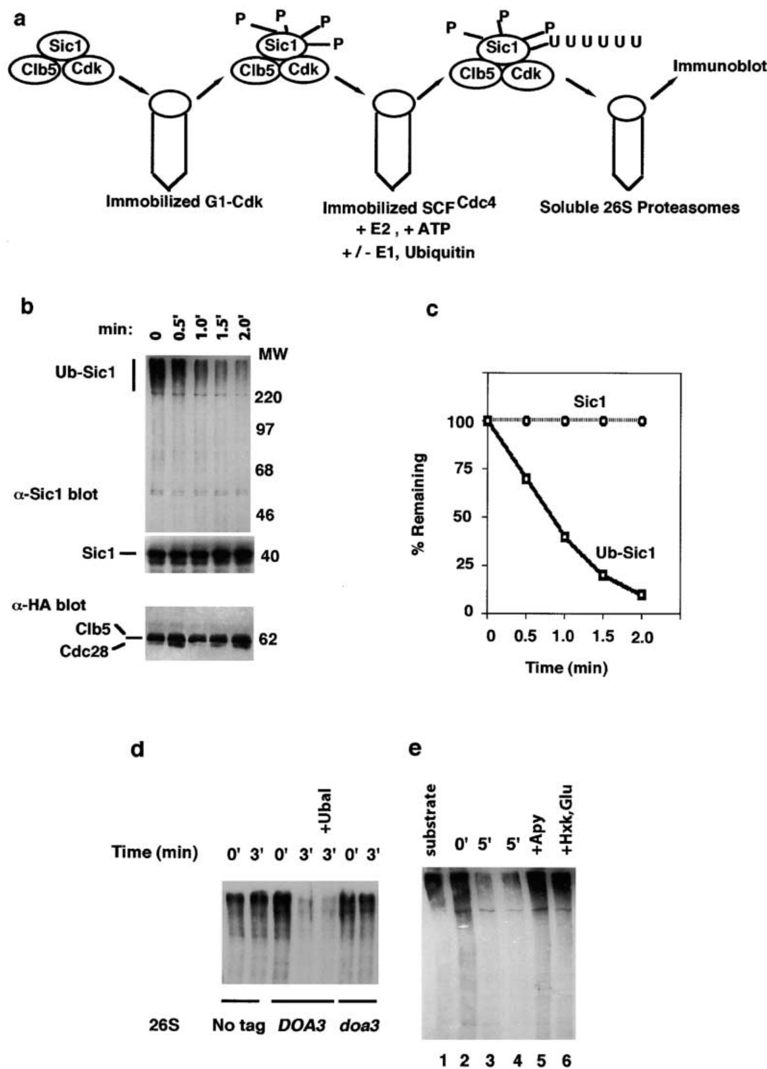

Figure 2. Subunit-Selective Degradation of Ubiquitinated Sic1 by Purified 26S Proteasomes Is ATP and *DOA3* Dependent

(a) Sic1, complexed with GST-Cdc28^{HA} and ^{HA6}Clb5, was phosphorylated with immobilized recombinant G1-Cdk. Phosphorylated substrate was recovered in the supernatant fraction and subsequently incubated with Cdc34 (E2) and immobilized recombinant SCF^{Cdc4} in the absence or presence of ubiquitin and E1 to yield unmodified or Ub-Sic1 substrate complexes, respectively (Seol et al., 1999). Under the conditions used, essentially 100% of the input Sic1 was converted to ubiquitinated species (Figure 4a, lane 5).

(b) To provide an internal control for the degradation reactions, Ub-Sic1 and unmodified Sic1 were mixed in equimolar amounts (approximately 300 nM each) and supplemented with purified 26S proteasome (100 nM) and 3 mM ATP, or the Sic1 substrate complexes (modified and unmodified) were assayed separately (Figure 4a). Degradation reactions were stopped by the addition of 5× SDS buffer, fractionated by SDS-PAGE, and evaluated by immunoblotting with anti-Sic1 polyclonal antibodies (b, top two panels) or anti-HA monoclonal antibody 12CA5 (b, bottom panel). Ub-Sic1 and unmodified Sic1 were evaluated on 8% and 10% polyacrylamide gels, respectively. Immunoblot analysis of individual proteins revealed that GST-Cdc28^{HA} and ^{HA6}Clb5 comigrated. A lack of Cdc28 degradation was confirmed using anti-GST serum (data not shown). A lack of Clb5 degradation was independently confirmed by the generation of Cdc28/Clb5 kinase activity (Figure 4).

(c) Quantitation of the degradation reactions shown in (b). Similar results were obtained by either PhosphorImager (Molecular Dynamics STORM system; Amersham Pharmacia Biotech) or by analysis of scanned autoradiograms with NIH Image.

(d) 26S proteasomes were prepared from either untagged (lanes 1 and 2), *PRE1*$^{Flag\text{-}His6}$ wild-type *DOA3* (lanes 3–5), or *PRE1*$^{Flag\text{-}His6}$ *doa3* mutant (lanes 6 and 7) cells, as indicated. Degradation reactions were performed and visualized as described in (b). The reaction shown in lane 5 was supplemented with 2 μM ubiquitin aldehyde (Ubal).

(e) Affinity-purified 26S proteasomes were incubated with Ub-Sic1/S-Cdk substrate in the presence or absence of ATP. Lane 1 is a zero time point sampled prior to addition of 26S, whereas all other lanes contained 26S proteasomes. ATP depletion was achieved by preincubating 26S proteasomes at 30°C for 5 min in the absence (lane 4) or presence of 15 U/ml apyrase (+Apy; lane 5) or 5 U/ml hexokinase plus 30 mM glucose (+Hxk,Glu; lane 6) before the addition of Ub-Sic1/S-Cdk. No preincubation was done in lane 3. Degradation was allowed to proceed for 5 min at 30°C after addition of Ub-Sic1/S-Cdk, and reactions were stopped and visualized as described in (b).

members, the AAA ATPase Cdc48 (both of which have been suggested to serve as chaperonins or unfoldases in proteasome-dependent proteolysis; see Dai et al., 1998; Ghislain et al., 1996; Lee et al., 1996) and Hsp90 (Figure 1c; see Experimental Procedures). Together, these data indicate that substrate and proteasomes were present in great stoichiometric excess over any potential contaminants other than E1, E2, ubiquitin, and variable amounts of Cdc4^{PyHA} that leaches off the polyoma beads.

Sic1 Is Degraded in an ATP- and Ubiquitin-Dependent Reaction by Purified 26S Proteasomes

To evaluate whether Ub-Sic1, tightly assembled into heterotrimeric complexes, was competent to serve as a substrate for degradation, Ub-Sic1/S-Cdk or unmodified Sic1/S-Cdk substrate complexes were incubated with affinity-purified 26S proteasomes in the presence of ATP. As shown in Figures 2b and 2c, 90% of Ub-Sic1 conjugates were degraded within 2 min at 30°C, whereas unmodified Sic1 was spared. The disappearance of Ub-Sic1 was due to degradation and not deubiquitination because (1) little or no unmodified Sic1 was regenerated during the course of a reaction (Figure 4a, lanes 5–8), (2) ubiquitin aldehyde, a potent inhibitor of deubiquitinating enzymes (Wilkinson and Hochstrasser, 1998), did not prevent consumption of Ub-Sic1 by 26S proteasomes (Figure 2d, lane 5), and (3) the disappearance was dependent on ATP (Figure 2e, lanes 4–6). The extremely rapid and selective degradation of Ub-Sic1 attests to the functional and structural integrity of the purified 26S complex.

To confirm the specificity of the reactions shown in Figure 2b, we performed three control experiments. First, no degradation was observed when purified 26S proteasomes and ubiquitination reactions were depleted of ATP by treatment with either apyrase or glucose plus hexokinase (Figure 2e). Second, Ub-Sic1 was not degraded by preparations derived from strains lacking tagged Pre1 (Figure 2d, lanes 1 and 2). Finally, 26S proteasomes prepared from a *doa3* mutant strain (Figure 1b), which is defective in the chymotrypsin-like activity of the 20S core (Chen and Hochstrasser, 1996), were unable to degrade Ub-Sic1 (Figure 2d, lanes 6 and 7). The 20S core possesses at least three types of proteolytic activities: chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing (PGPH) activity. Of these, genetic studies (Arendt and Hochstrasser, 1997) indicate that the chymotrypsin-like activity is the most important for viability, and thus, it is not surprising that the *doa3* proteasomes are so dramatically impaired. Likewise, proteasomes prepared from a strain with a mutant 19S ATPase subunit, *rpt2RF* (Rubin et al., 1998), were unable to degrade Ub-Sic1 (data not shown).

Purified Proteasomes Degrade Ub-Sic1 but Not Tightly Bound Clb5 and Cdc28

Because we were able to establish that purified Ub-Sic1 was specifically degraded by purified 26S proteasomes, we tested whether the 26S proteasome has the intrinsic ability to destroy Ub-Sic1 without harming the associated Clb5 and Cdc28 subunits of S-Cdk. To address this point, we followed the fate of both ^{HA6}Clb5 and GST-Cdc28^{HA} in our reactions. Immunoblotting with anti-HA revealed that these proteins were not detectably conjugated with ubiquitin in the same reaction in which the entire pool of Sic1 was extensively ubiquitinated (data not shown). Consistent with the lack of ubiquitination, the comigrating GST-Cdc28^{HA} and ^{HA6}Clb5 antigens were not degraded upon incubation of Ub-Sic1/S-Cdk complexes with purified 26S proteasomes (Figure 2b). That two normally unstable substrates of the ubiquitin-proteasome system (Sic1 and Clb5) were not degraded when present in an unmodified form underscores the selectivity of the in vitro Ub-Sic1 degradation reaction.

Stability of the Ub-Sic1/S-Cdk Complex

Sic1 has been reported to be very stably bound to the cyclin/Cdk complex (Mendenhall, 1993). To determine whether Ub-Sic1 was also bound tightly to S-Cdk, we assessed the rate of spontaneous disassembly of the Ub-Sic1/S-Cdk complex by a subunit-exchange protocol. A 30-fold molar excess of MbpSic1^{mycHis6} was incubated with Ub-Sic1/S-Cdk for various lengths of time, and the rate at which Ub-Sic1 was replaced by MbpSic1^{mycHis6} was evaluated by immunoprecipitation of S-Cdk followed by immunoblotting with anti-Mbp (Figures 3a and 3b). Ub-Sic1 was displaced by Mbp-Sic1^{mycHis6} with a half-life of ~3.2 hr at 30°C. A control experiment confirmed that unfused Sic1 and Mbp-Sic1^{mycHis6} bound naive Clb5/GST-Cdc28 complexes with equivalent efficiency (data not shown). As demonstrated in Figure 2, the half-life of the Ub-Sic1 complex was less than 1 min in the presence of 26S proteasomes. Thus, Ub-Sic1 was degraded by the 26S proteasome approximately 200 times faster than the spontaneous rate of Ub-Sic1 dissociation from S-Cdk. This observation suggests that the 26S proteasome actively disassembled Ub-Sic1 from S-Cdk complexes, as opposed to trapping Ub-Sic1 that spontaneously dissociated from S-Cdk.

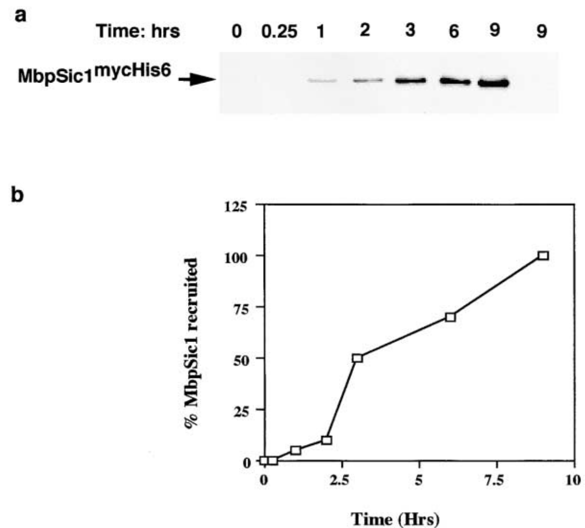

Figure 3. Stability of the Ub-Sic1/S-Cdk Complex

(a) Ub-Sic1/S-Cdk (300 nM), prepared as described in Figure 2, was incubated at 30°C for varying lengths of time with 9 μM Mbp-Sic1^{mycHis6} purified from *E. coli* as described (Verma et al., 1997b). At the end of the incubation, S-Cdk was precipitated by glutathione beads, and the amount of Mbp-Sic1^{mycHis6} recruited into the complex in exchange for Ub-Sic1 was determined by immunoblotting with affinity-purified anti-Mbp polyclonal antibody.

(b) Quantitation was performed as described in Figure 2c. Maximal recruitment was achieved at 9 hr, with no further increases at 16 and 24 hr (not shown), and the amount of Mbp-Sic1^{mycHis6} recovered was stoichiometric with the amount of Clb5 present in the original Ub-Sic1/S-Cdk complex. For the sample shown in lane 8, 9 μM Mbp-Sic1^{mycHis6} was incubated alone for 9 hr before being incubated with glutathione beads.

Degradation of Ub-Sic1 by Purified 26S Proteasomes Efficiently Regenerates Active S-Cdk

Our observation that Ub-Sic1, but not its ^{HA6}Clb5 and GST-Cdc28^{HA} partners, was degraded by purified 26S proteasomes prompted us to ask whether the 26S proteasome was sufficient to switch on S-Cdk by selectively degrading Ub-Sic1. If so, this would recapitulate, using purified proteins, a key aspect of the regulatory switch that underlies the G1/S transition in budding yeast. To address this question, separate degradation reactions were set up with ubiquitinated or unmodified Sic1/S-Cdk complexes. Following a brief incubation with purified 26S proteasomes, degradation reactions were terminated. S-Cdk was recovered with glutathione resin, and protein kinase activity was measured (see Experimental Procedures). Concomitant with degradation of Ub-Sic1, histone H1 kinase activity increased 12-fold within 3 min (Figure 4a, lanes 5–8). In contrast, 26S proteasomes neither degraded unmodified Sic1 nor activated the associated S-Cdk (Figure 4a, lanes 1–4). Note that extensive ubiquitination was not sufficient to dislodge Sic1 from its partners and activate S-Cdk (Figure

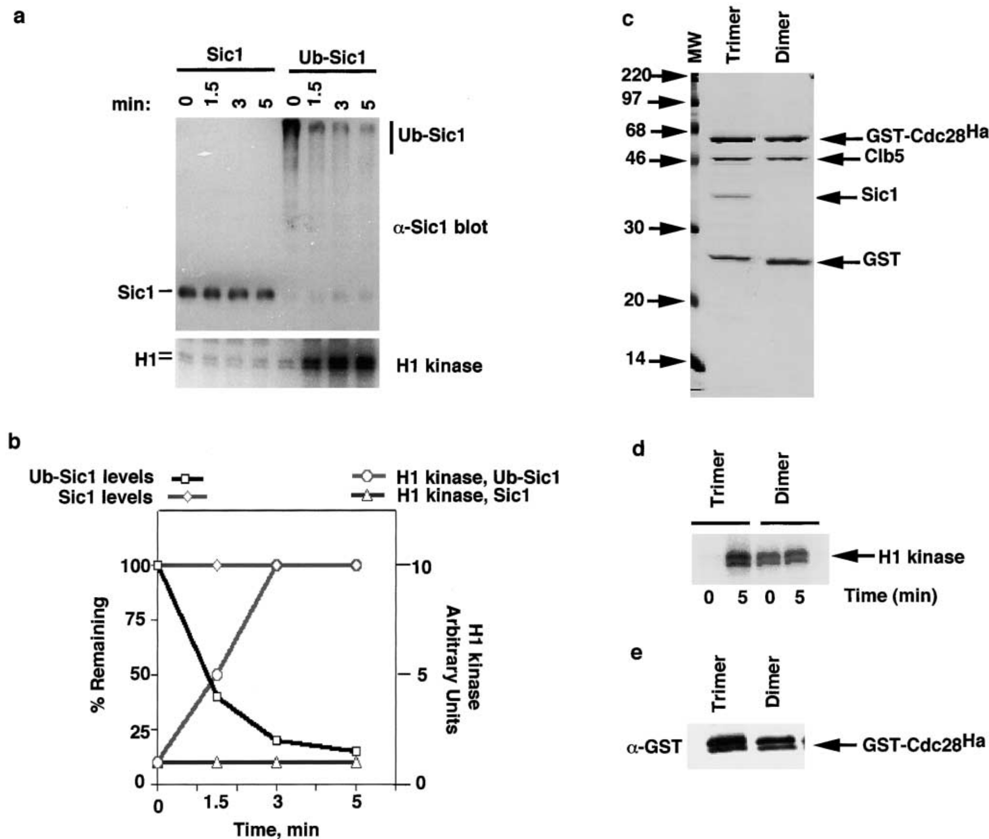

Figure 4. S-Cdk Is Activated Efficiently upon Degradation of Ubiquitinated Sic1

(a) Ubiquitinated or unmodified Sic1 (300 nM), complexed with GST-Cdc28^{HA} and Clb5 (see Figure 1a), was incubated with purified 26S proteasomes (100 nM) at 30°C for the indicated length of time. Each reaction was stopped by the addition of 1ml ice-cold STOP solution (see Experimental Procedures), and the diluted mix was bound to glutathione beads. Glutathione agarose-bound protein kinase was assayed as described in Experimental Procedures, and reactions were fractionated by SDS-PAGE followed by autoradiography to reveal phosphorylated histone H1 (bottom panel) and by immunoblotting with Sic1 antiserum to visualize Sic1 levels (top panel).

(b) Reactions were quantitated as described in Figure 2c.

(c–e) Efficiency of S-Cdk activation. S-Cdk complexes were expressed in insect cells in the absence (dimer) or presence (trimer) of Sic1 and purified by glutathione affinity chromatography as detailed in Experimental Procedures. (c) Aliquots were analyzed by SDS-PAGE and Coomassie Blue. (d) Equivalent amounts were then incubated with the ubiquitination machinery as in Figure 2a. Two microliters of the supernatants from the SCF ubiquitination reaction were incubated with 26S proteasomes, and H1 kinase activity was determined as described in the main body of the text. (e) The amount of GST-Cdc28 recovered was determined by immunoblotting with affinity purified anti-GST antibody.

4a, lane 1 versus lane 5), even though the Ub-Sic1/S-Cdk complexes were washed extensively with buffers containing high salt and detergent prior to the protein kinase assay. Similar to Ub-Sic1, degradation-competent ubiquitinated IκB has been demonstrated to remain bound to NF-κB (Chen et al., 1995). Interestingly, the kinetics of S-Cdk activation mirrored the kinetics of Ub-Sic1 degradation, suggesting that Cdk activation is tightly coupled to Sic1 degradation with no discernible lag time.

To address the efficiency with which 26S proteasome activates S-Cdk via selective destruction of Ub-Sic1, we prepared S-Cdk complexes that either contained (trimer) or lacked (dimer) Sic1. Equivalent amounts of the two complexes (Figure 4c) were subjected to the ubiquitination regimen outlined in Figure 2a. When presented to 26S proteasomes, S-Cdk was activated following degradation of Ub-Sic1. Remarkably, the specific activity, normalized to GST-Cdc28 (Figure 4e), recovered for the trimeric complex was comparable to that observed for the dimeric S-Cdk complex (Figure 4d).

Limited Activation of S-Cdk in the Absence of Proteolysis

An appealing idea is that the proteasome first extracts Ub-Sic1 from Clb5-Cdc28 to yield active S-Cdk and subsequently degrades the Ub-Sic1. To determine whether the activation of S-Cdk could be uncoupled from Ub-Sic1 degradation, we purified 26S proteasomes from *doa3* mutants (Figure 1). This yielded a preparation that was unable to degrade both chymotryptic substrate peptides (data not shown) and Ub-Sic1 (Figures 2d and 5a). Whereas incubation of Ub-Sic1 substrate complexes with wild-type 26S proteasomes resulted in >12-fold activation of S-Cdk, incubation with mutant 26S proteasomes resulted in only 3-fold activation of the kinase (Figure 5b). The kinase activation observed in this experiment was ATP dependent (Figure 5a), consistent

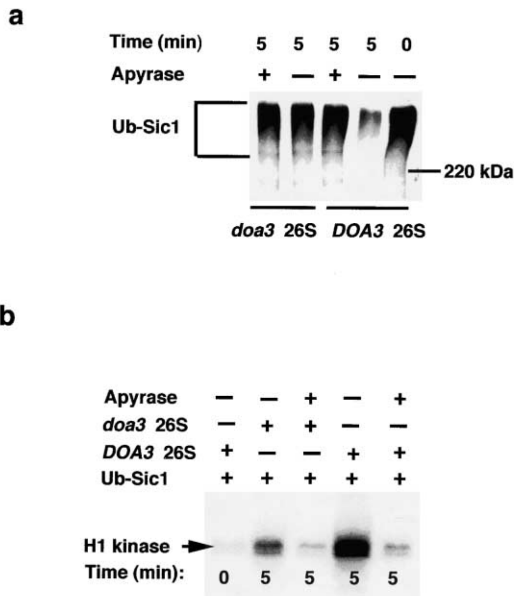

Figure 5. Limited Activation of S-Cdk Kinase by *doa3* Mutant Proteasomes

(a) 26S proteasomes were prepared from *DOA3* wild-type or *doa3* mutant cells as described in Figure 1 and treated with (+) or without (−) Apyrase as described in Figure 2. Ub Sic1/S-Cdk (300 nM) was incubated with the different 26S preparations (100 nM) for 5 min at 30°C at the end of which aliquots were withdrawn for monitoring degradation by immunoblotting with anti-Sic1 polyclonal antibody. (b) The remainder of the reaction mix was diluted with ice-cold STOP mix and was processed for H1 kinase activity as described in Figure 4.

with the notion that the ATPases of the 19S cap were required for disassembly of Ub-Sic1/S-Cdk substrate complexes that occurs in the absence of proteolysis. These results suggest that, although it is not strictly necessary, ongoing proteolysis is required to achieve maximal activation of S-Cdk, perhaps because it renders the activation process irreversible.

Discussion

Protein Unfolding/Disassembly by Prokaryotic ATP-Dependent Proteases and Archael and Eukaryotic Proteasomes

Recent studies using green fluorescent protein fused to an 11-residue degradation signal from ssrA at its carboxy terminus (GFP11) have shown that prokaryotic ClpA and ClpX ATPases, which assemble with the ClpP protease and are members of the Clp/Hsp100 family of ATP-dependent molecular chaperones, can unfold GFP11 as a prelude to its degradation. A similar observation was also reported for the archaebacterial PAN ATPase, which is more closely related to the ATPases of the 26S proteasome (Benaroudj and Goldberg, 2000; Hoskins et al., 2000; Kim et al., 2000; Singh et al., 2000; Weber-Ban et al., 1999).

In contrast, detailed analyses of how the 26S proteasome extracts ubiquitinated subunits from multimeric complexes and unfolds them has proven to be more difficult, perhaps because purified eukaryotic proteasomes and ubiquitinated substrates are more difficult to obtain and are more heterogeneous. Nonetheless, in the most rigorous and systematic study on this topic conducted to date, it was demonstrated that a monomeric ubiquitin-dihydrofolate reductase chimera engineered to contain a tetraubiquitin chain attached to its N-terminal ubiquitin (Ub_5DHFR) is degraded by enriched proteasomes in a methotrexate-inhibitable manner (Thrower et al., 2000). A similar observation was reported earlier (Johnston et al., 1995) using reticulocyte lysate as a source of 26S proteolytic activity. From these experiments, it was inferred that substrate unfolding by the 26S proteasome, which can be impeded by methotrexate, is a prerequisite for degradation. Notably, the rate of proteolysis reported for Ub_5DHFR was considerably slower than what we observe for Ub-Sic1.

In contrast to monomeric Ub_5DHFR, no degradation occurred when tetrameric $Ub_5\beta$-gal was used as a substrate for enriched 26S proteasomes. To address this discrepancy, the authors speculated that perhaps "additional factors sometimes assist proteasomes in vivo" (Thrower et al., 2000). Because DHFR is monomeric and β-gal is tetrameric, one possibility is that 26S proteasome might require assistance to process multimeric substrates. Purified 26S proteasomes were reported to be unable to disengage ubiquitinated cyclin B from Cdc2, even though 26S proteasomes are required for the segregation of these two proteins in crude *Xenopus* extract (Nishiyama et al., 2000). However, the results of the latter experiments must be interpreted cautiously. The 26S proteasome may irreversibly bias reaction equilibria by sequestering disassembled substrates within the cavity of the 20S complex (Murakami et al., 1999), even if the 26S complex does not catalyze disassembly per se. By analogy, the addition of the GroEL mutant D87K is required to observe the ClpA-dependent unfolding of GFP11, even though GroEL-D87K is considered to serve only as a passive "trap" in this process (Weber-Ban et al., 1999). Taken together, the balance of evidence to date suggested that, although the 26S proteasome can slowly unfold and degrade a monomeric substrate, it is unable, by itself, to extract ubiquitinated subunits from multisubunit complexes.

Subunit-Selective Degradation of Ub-Sic1 and Activation of S-Cdk Does Not Appear to Require Extra Proteasomal Chaperones

The considerations raised previously in this article provoke an obvious and important question: why did we observe selective and efficient degradation of Ub-Sic1 by yeast 26S proteasomes in a purified system, even though the Ub-Sic1 was tightly engaged with S-Cdk? The most likely explanation lies in the characteristics of either the proteasomes or substrate employed in our assays. We first considered the possibility that either the substrate or proteasome preparation was contaminated with unfoldases. We believe this possibility to be unlikely for three reasons. First, both multidimensional mass spectrometry (Washburn et al., 2001) and immunoblotting revealed that the major chaperones Hsp70 and Hsp90, as well as the AAA ATPase Cdc48, which has been speculated to be involved in unfolding of proteasome substrates (Dai et al., 1998; Nishiyama et al., 2000),

were present at either extremely low levels or were essentially absent from the degradation reactions. Second, none of the most prominent contaminants of the 26S preparation detected by MudPIT were plausible candidates for a Ub-Sic1 unfoldase (see supplemental data at http://www.molecule.org/cgi/content/full/8/2/439/DC1). Third, for Ub-Sic1 to be extracted from S-Cdk and unfolded by an inabundant contaminant in the preparations, the chaperone would be required to operate at a rate that is unprecedented among well-characterized unfoldase reactions. Nevertheless, one can never formally exclude the possibility of a trace contaminant with a prodigious capacity for disassembling Ub-Sic1 from S-Cdk.

As noted previously in this article, Ub-Sic1 remained productively engaged with S-Cdk. This rules out the notion that ubiquitination brought about global unfolding of Sic1 but leaves open the possibility that ubiquitination causes local perturbations in Sic1 structure that may enable substrate degradation (Lee et al., 2001). Consistent with our observation that Ub-Sic1 remained tightly bound to S-Cdk until it was extricated by the 26S proteasome, conditional *rpn12* (19S subunit) mutants that accumulate ubiquitin conjugates and are defective in Sic1 turnover arrest with low Cdc28-associated protein kinase activity (Bailly and Reed, 1999; Kominami et al., 1995).

How is active S-Cdk generated upon elimination of Ub-Sic1? It remains unclear whether functional S-Cdk was released passively as Ub-Sic1 was degraded, or whether it was actively refolded upon removal of Ub-Sic1. The ATPases of the 19S regulatory cap have previously been demonstrated to refold denatured citrase synthase (Braun et al., 1999), suggesting the possibility of an additional role for the 19S cap in the generation of active S-Cdk. Regardless, the efficiency of S-Cdk reactivation argues that this process does not require extraproteasomal chaperones.

In the only other case in which subunit-selective degradation of a ubiquitinated protein was reproduced outside of crude extracts, Chen et al. (1995) demonstrated that purified 26S proteasomes can degrade immunoprecipitated Ub-IκB conjugates bound to NF-κB. However, neither the composition of the immunoprecipitated substrate nor the competence of the liberated NF-κB were characterized by those authors. Significantly, it was subsequently shown that IκB can coprecipitate from extracts with the putative unfoldase Cdc48 (Dai et al., 1998), and Chen et al. (1995) proposed that extraproteasomal chaperones contribute to the disengagement of Ub-IκB from NF-κB.

Potential Effects of Ubiquitin Chain Structure on Degradation

Given that Ub-Sic1 degradation does not appear to require extraproteasomal chaperones, we hypothesize that the extremely rapid and efficient proteolysis we observe is related to properties of the substrate itself. With this in mind, there are significant caveats to the interpretation of prior attempts to reconstitute degradation of folded or multimeric substrates with enriched 26S proteasome preparations. First, both Ub_5DHFR and $Ub_5\beta$-gal were engineered to contain a single tetra-ubiquitin chain, which binds to the 26S proteasome with an equilibrium dissociation constant of 60 nM (Thrower et al., 2000). If the association constant of $Ub_5\beta$-gal for the 26S proteasome is near the diffusion-controlled limit (10^6–10^7 $M^{-1}s^{-1}$), then $t_{1/2}$ for dissociation of proteasome-bound $Ub_5\beta$-gal is predicted to be ~1–10 s. This may simply not be enough time for the proteasome to disassemble a stable protein complex. In a more recent example, it was reported that purified proteasomes are unable to extract and degrade ubiquitinated cyclin bound to Cdc2 (Nishiyama et al., 2000). An inspection of the substrate utilized in this study reveals that the Ub-cyclin conjugates generated in vitro primarily contained only four to five ubiquitins distributed over an unknown number of lysines, suggesting that the Ub-cyclin was probably not a competent substrate for the 26S proteasome.

In contrast to Ub_5-DHFR and cyclin B, Sic1 was extensively multiubiquitinated by SCF in vitro on multiple acceptor lysines in the N-terminal domain (R. Feldman and R.V., unpublished data). If the 26S proteasome contains multiple tetraubiquitin binding sites or if natural substrates, such as Sic1, contain secondary peptide signals that bind 26S proteasome, multidentate interaction would help ensure that Sic1 remains bound to 26S proteasome for a sufficiently long time to be completely unfolded and degraded (Verma and Deshaies, 2000). The difference in number and length of multiubiquitin chains between Ub_5DHFR and Ub-Sic1 might also explain why Ub-Sic1 is efficiently degraded by metazoan 26S proteasomes in the absence of Ub-aldehyde (H. Holzl and R.V., unpublished data), whereas Ub_5DHFR is not (Thrower et al., 2000). In the absence of ubiquitin aldehyde, Ub_5-DHFR is deubiquitinated by proteasome-bound deubiquitinating enzymes (Holzl et al., 2000) faster than it is degraded, whereas the converse is true for Ub-Sic1.

Possible Effects of Substrate Structure on Degradation

A second class of explanation for the distinct behavior of Ub_5-β-gal and Ub-Sic1 relates to the folded structure of these proteins. Recently, Lee et al. (2001) made the fascinating observation that the rates of substrate unfolding and degradation depend on the relative position of the degradation signal within the folded structure of the substrate. Hence, the Ub_5 moiety in Ub_5-DHFR and Ub_5-β-gal might not be optimally positioned to sustain processive unfolding initiated from the Ub_5 signal. In contrast to Sic1, neither DHFR nor β-gal is an unstable protein, and, thus, neither one has evolved to be unraveled from an N-terminal degradation signal. This consideration underscores the importance of employing natural substrates to dissect the mechanism of protein complex disassembly and substrate unfolding by the 26S proteasome.

Concluding Remarks

Defining the minimum components of the ubiquitin/proteasome system required for subunit-selective degradation of Ub-Sic1 sets the stage for future studies on how the 26S proteasome carries out this remarkable feat. Recent data from prokaryotes suggest that, even though they do not contain ubiquitin, the bacterial ATP-depen-

dent protease ClpXP can selectively degrade the UmuD′ subunit of a UmuD/UmuD′ heterodimer (Gonzalez et al., 2000), albeit at a far slower rate ($t_{1/2}$ of 30 min) than we observed for the subunit-selective degradation of Ub-Sic1. Nevertheless, this observation demonstrates that the capacity to selectively degrade single subunits of multisubunit complexes has been conserved throughout evolution, suggesting that this activity is of vital importance to cellular regulation.

Experimental Procedures

Purification of Sic1/S-Cdk Complex

GST-Cdc28^{HA}, ^{HA6}Clb5 or untagged Clb5, and Sic1, with or without Cdk-activating kinase (CAK), were coexpressed in Hi5 insect cells infected with the respective recombinant baculovirus vectors in a 1:2:2:1 ratio. CAK was included in some experiments to boost Cdc28 protein kinase activity and had no effect on the degradation of ubiquitinated or unmodified Sic1. Substrate complexes derived from triple (−CAK) or quadruple (+CAK) infections were bound to glutathione sepharose beads for 90 min at 4°C. Bound complexes were washed with 25 mM Tris (pH 7.5), 250 mM NaCl, and 0.2% Triton and were eluted with 50 mM Tris (pH 8.8), 40 mM glutathione, 2 mM dithiothreitol, 2 mM ethylenediaminetetraacetic acid (EDTA), and 150 mM NaCl for 3 hr at 4°C (the presence of glutathione reduced the pH to neutral). The eluate was dialyzed to remove glutathione and EDTA for 4 hr against a buffer containing 25 mM Tris (pH 7.5), 150 mM NaCl, and 15% glycerol. All baculovirus vectors have been described previously (Seol et al., 1999; Skowyra et al., 1997), except for the vector that expresses ^{HA6}Clb5. To construct this vector, a 2.2 kb ^{HA6}CLB5 fragment was excised from pADH-^{HA6}Clb5 (gift from Etienne Schwob) with HindIII and inserted into a HindIII-linearized baculovirus-transfer vector (pFastBac1). Expression was confirmed by immunoblotting insect cell lysates with 12CA5 anti-HA monoclonal antibody.

Ubiquitination of Sic1/S-Cdk Complex

Eluted Sic1/S-Cdk substrate complexes, derived from +CAK and −CAK infections, were phosphorylated to completion with Cln2/GST-Cdc28HA/CAK/Cks1 kinase complex immobilized on GSH beads. The supernatant containing phosphorylated Sic1 was ubiquitinated as described (Seol et al., 1999) in the presence of E1, E2 (Cdc34), ATP, ubiquitin, and SCFCdc4^{PYHA} ubiquitin ligase immobilized on Py beads. The SCF complex comprised of Cdc4^{PYHA}, Cdc53, Skp1^{HA}, and Hrt1 was coexpressed in insect cells as a tetrameric complex. Following ubiquitination, the supernatant containing the heteromeric complex of Ub-Sic1, GST-Cdc28^{HA}, and Clb5 (tagged or untagged) was presented to purified proteasomes in degradation assays.

Affinity Purification of 26S Proteasomes

26S proteasomes were purified as described (Verma et al., 2000) from the following strains: RJD 1144 (*MATa*, *his3*, *leu2-3, 112*, *lys2-801*, *trp*, *PRE1-Flag-His6::YIplac211 URA3*), RJD 1380 (*MATa*, *his3*, *leu2-3*, *112*, *lys2-801*, *trp1-1*, *PRE1-Flag-His6::YIplac211* [*URA3*], *doa3::HIS3* [*YEplac181-Doa3LS LEU2*] [*YCplac22-doa3LS-T76A-His6 TRP1*]), and RJD 487 (*MATa*, *leu2*, *ura3*, *trp1*). Briefly, epitope-tagged 26S proteasomes were recovered from cell extract by adsorption to an anti-Flag monoclonal antibody resin, followed by elution with Flag peptide.

Evaluation of 26S Proteasome Purity

Several chaperones have been implicated in protein degradation (as part of the generic ubiquitin/proteasome system) but are not bona fide subunits of the 26S particle, including Cdc48 and Ssa (Hsp70), Hsc82 (Hsp90), and Ydj1 (dnaJ) family members. Of these, Cdc48 belongs to the same AAA ATPase family to which the Rpt subunits of the 26S belong. Yeast Cdc48 is an essential ATPase that catalyzes homotypic fusion between endoplasmic reticulum membranes (Acharya et al., 1995; Latterich et al., 1995). Cdc48 is also required for the degradation of substrates of the ubiquitin fusion degradation (UFD) pathway (Ghislain et al., 1996). In mammalian cells, the Cdc48 homolog (VCP) has been shown to be complexed to ubiquitinated IκB and postulated to function at a postubiquitination step in IκB turnover (Dai et al., 1998). Hsp70 family members have been shown to promote degradation of mammalian proteins in vitro (Bercovich et al., 1997). They have also been indirectly implicated in protein turnover in vivo because conditional inactivation of budding yeast Ydj1 resulted in the stabilization of some short-lived proteins (Ydj1 functions in cooperation with Hsp70 [Lee et al., 1996]).

To assess the potential contamination of 26S proteasomes by known chaperones, immunoblotting experiments were performed with anti-Ssa1 (to detect 70 kDa stress proteins), anti-Hsc82 (to detect 90 kDa stress proteins), and anti-Cdc48 antisera (Figure 1c). To evaluate their purity further, 60 μg of purified 26S proteasomes were subjected to multidimensional mass spectometry as described (Washburn et al., 2001). Every 26S proteasome subunit was identified by a minimum of 13 spectra (see supplemental data at http://www.molecule.org/cgi/content/full/8/2/439/DC1). In contrast, only 10 spectra (out of 3448 total) were observed for members of the 70 kDa family of stress proteins, and not a single spectrum corresponded to either Cdc48 or a member of the DnaJ or Hsp90 families of proteins.

Evaluation of Purity of Ub-Sic1

The eluted Sic1/S-Cdk complex from glutathione beads was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Blue staining. A typical preparation is shown in Figures 1A and 4C. The eluted complex was ubiquitinated on immunopurified and immobilized (anti-polyoma beads) SCF ubiquitin ligase using E1 (Uba1^{His6}) purified from budding yeast and E2 (Cdc34) from *E. coli* as described (Seol et al., 1999). The purity of these two enzymes was assessed by Coomassie Blue staining of SDS-PAGE resolved aliquots (Figure 1D). The polyoma supernatant containing Ub-Sic1/S-Cdk was presented to purified 26S proteasomes whose purity was evaluated as described previously in this article. Typically, 2 μl of Ub-Sic1 was used in a 50 μl degradation reaction. 26S proteasomes, characterized by MudPIT, were evaluated for the presence of the abundant budding yeast chaperones by immunoblotting before and after the addition of substrates. As shown in Figure 1C, no further increase in antigen signal was observed by the addition of substrate to 26S proteasomes.

Degradation Assays

Degradation reactions contained, in addition to substrate and 26S proteasome, 1× ATP regenerating system (Verma et al., 1997b), 2 mM ATP, 5 mM $MgCl_2$, 4 mM MgOAc, 25 mM Tris (pH 7.5), 10 nM E1, 50 nM Cdc34 (E2), 150 mm NaCl, and 200 μM ubiquitin. Reactions were incubated at 30°C for the indicated length of time and were stopped by the addition of 5× SDS buffer. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with the appropriate antibody to monitor degradation.

H1 Kinase Assays

Ubiquitinated or unmodified heteromeric Sic1 complexes were incubated with purified 26S proteasomes in 50 μl total volume as described in Figure 2. An aliquot (10 μl) was withdrawn to assess the amount of Sic1 degraded. One milliliter of ice-cold Stop solution, containing 25 mM Tris (pH 7.5), 200 mM NaCl, 0.2% Triton, 50 μM polyvinyl sulfone, 2 mM EDTA, and 100 μg/ml bovine serum albumin, was added to the sample remaining in each tube. The entire diluted mix was then transferred to 20 μl settled glutathione beads and incubated for 30 min at 4°C. Bound complexes were washed three times with buffer containing 25 mM Tris (pH 7.5), 250 mM NaCl, and 0.2% Triton and twice with kinase buffer containing 25 mM Tris (pH 7.5) and 10 mM $MgCl_2$. The washed beads were resuspended in 20 μl kinase buffer to which 1 μg histone H1 and 10 μCi γ-ATP (3000 Ci/mmol) had been added. After incubation for 1 hr at 25°C, the reaction was stopped by the addition of an equal volume of 2× SDS Laemmli buffer. Samples were resolved by SDS-PAGE and subjected to autoradiography.

Acknowledgments

This work was supported in part by Searle/Chicago Community Trust and Lucille P. Markey Charitable Trust Scholar awards, How-

ard Hughes Medical Institute, and a grant from the National Institutes of Health (RO1 GM52466) to R.J.D. J.R.Y. and W.H.M. were supported by Merck (MGRI-241) and National Institutes of Health (RR11823-05). We thank Elizabeth Craig for antibody to Hsp 70, Dan Finley for the *rpt2RF* mutant, Mark Hochstrasser for the *doa3* mutant, Martin Latterich for the Cdc48 antibody, Susan Lindquist for antibodies to Hsp70 and Hsc82, Etienne Schwob for pADH-HA6Clb5, Wade Harper for Sic1 baculovirus, S. and A. Dasgupta for support, and D. Chan, M. Budd, W. Shou, J. Campbell, B. Dunphy, and A. Varshavsky for critically reading the manuscript.

Received January 5, 2001; revised May 31, 2001.

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