Destabilization of activator-DNA complexes by the proteasomal ATPases can inhibit transcription by limiting activator interaction with DNA. Modification of the activator by monoubiquitylation protects the activator from this destabilization activity. In this study, we probe the mechanism of this protective effect of monoubiquitylation. Using novel label transfer and chemical cross-linking techniques, we show that ubiquitin contacts the ATPase complex directly, apparently via Rpn1 and Rpt1. This interaction results in the dissociation of the activation domain-ATPase complex via an allosteric process. A model is proposed in which activator monoubiquitylation serves to limit the lifetime of the activator-ATPase complex interaction and thus the ability of the ATPases to unfold the activator and dissociate the protein-DNA complex.

The ubiquitin-proteasome pathway is responsible for most of the non-lysosomal proteolysis in eukaryotic cells (1). The 26 S proteasome is comprised of a 20 S core particle (CP)3 that contains the three active sites inside of a barrel-like enclosure. The 20 S CP is capped on either end with the 19 S regulatory particle (RP). The 19 S RP is comprised of ~20 proteins including a ring of six ATPases (Rpt1–6) that sit atop the 20 S CP and are thought to unfold substrate proteins and feed them into the interior of the barrel.

Several lines of evidence published over the last few years have revealed that the proteasome and its subcomplexes are intimately involved in RNA polymerase II transcription, as well as other nuclear processes. Recent studies in yeast using chromatin immunoprecipitation (ChIP-on-chip) have shown that a large fraction of active genes are occupied either by the 26 S proteasome or by independent 19 S RP or 20 S CP complexes (2, 3). Genetic links between proteasomal proteins and polymerase II transcription have long been known. For example, specific alleles of SUG1 and SUG2 (sug1-1 and sug2-1), genes that encode two of the proteasomal ATPases (Rpt4 and Rpt6), could rescue the activity of a Saccharomyces cerevisiae Gal4 transactivator derivative lacking about two-thirds of the C-terminal activation domain (Gal4D) (4) by a mechanism that could not be explained by altered proteolysis of Gal4D (5).

What was the biochemical basis of this genetic link between GAL transcription in yeast and the proteasomal ATPases? Investigation into this question demonstrated that the activation domain (AD) of Gal4 binds directly to Sug1/Rpt6 and Sug2/Rpt4 and extracts from the proteasome a complex that includes the six ATPases, Rpn1, Rpn2, and perhaps other associated proteins but not the 20 S core proteasome or the “lid” subunit of the 19 S RP (6). This proteasomal subcomplex, which has been called API5 or the 19 S base (we will use the latter term here), is recruited to GAL promoters by Gal4 and is important for efficient promoter escape and elongation (7, 8). The proteasomal ATPases are also important for efficient promoter escape in other systems (9). Although the precise mechanistic basis of this effect has yet to be determined, a plausible model is that the protein unfolding activity of the ATPases, when uncoupled from proteolysis, acts to somehow remodel the preinitiation complex into an elongation complex, a process that is known to require many alterations in protein-protein and protein-nucleic acid interactions (10). Others later demonstrated that Rpt6, presumably acting in concert with the other proteins in the 19 S base, is also important in mediating histone modifications and in recruiting the SAGA complex to promoters. Neither of these activities require proteolysis (11, 12).

Until recently, one confusing aspect of the published data on the interaction of the ubiquitin-proteasome pathway with GAL gene transcription was the fact that the sug1-1 and sug2-1 mutations were clearly recessive. This is difficult to reconcile with the requirement of Rpt6 protein activity for elongation. Instead, it seems more likely that the sug1-1 and sug2-1 mutations eliminated some repressive activity of the wild-type complex to which Gal4D is hypersensitive. We recently discovered the molecular basis of this putative repressive activity. Specifically, the transactivator-DNA complex is rapidly and reversibly dissociated in an ATP-dependent manner by the proteasomal ATPases in a non-proteolytic fashion (13). The direct interaction of the ATPases and the AD of the transactivator are essential for this reaction to occur. How do wild-type activators resist this potent activity of the proteasomal ATPases and remain resistant to proteolysis by the proteasome?
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associated with the promotor long enough to drive high level transcription? Although it is true that some activators are thought to undergo rapid exchange with their DNA-binding sites in vivo (14), others, including heat shock factor (HSF) (15), do not. We found that the answer lies in the post-translational modification of the Gal4 DNA-binding domain (DBD). When exposed to a HeLa nuclear extract, all of the DNA-bound activator is phosphorylated and monoubiquitylated (Ub) (13), and this form of the protein is insensitive to the destabilization activity of the 19 S base.

Inefficient monoubiquitylation was found to be the defect of Gal4D (16), and it was shown that this results in an inability of the activator to bind stably to promoters in vivo in the face of the “stripping” activity of the proteasomal ATPases. Consistent with this view, Gal4D function can be rescued by massive overexpression, which drives GAL promoter occupancy even in the absence of activator monoubiquitylation (17). This defect can also be partially overcome by genetic fusion of monoubiquitin to Gal4D, indicating that the stereochemical positioning of the monoubiquitin moiety on the protein is not critical. As mentioned above, Gal4D function in vivo is also rescued by the suppressing mutations sug1-1 or sug2-1. We showed that proteasome subcomplexes isolated from these strains have reduced destabilization activity in vitro relative to the wild-type ATPase complex (16).

In summary, these studies have demonstrated a novel aspect of the regulation of Gal4 promoter occupancy in vivo. Gal4 binding to DNA is directly antagonized by the stripping activity of the proteasomal ATPases, but the activator is immunized against this repressive activity by monoubiquitylation of the DNA-binding domain. However, the mechanism by which ubiquitin protects activators from destabilization remains obscure. In this report, we employ a variety of methods, including novel label transfer chemistry, to address this question. First, we confirm that activator monoubiquitylation, and not some other potential modification, is indeed the key to stabilizing the activator-promoter complex. Second, we demonstrate that although mono-Ub does not bind directly to the intact 26 S proteasome, it does engage directly with the 19 S base after it has been extracted from the proteasome by the activator. Evidence is presented that this interaction is the result of direct contact of Ub with Rpt1 and Rpn1. We show that these Ub-19 S base interactions result in the dissociation of the AD-Rpt4/Rpt6 contacts, apparently via an allosteric mechanism. The end result is that the lifetime of the activator-ATPase complex is reduced by Ub, resulting in inhibition of the stripping activity. A model that attempts to integrate these findings with the requirement for the ATPases in promoter escape and elongation is proposed.

EXPERIMENTAL PROCEDURES

Materials—CCPGCC-Ub was constructed by including CCPGCC into the 5′ primer of a pair of PCR primers (FLAG F1asH Ub F, 5′-GCGGAAGCTTGGCGGGTTGCGTG-CGCGGGAATTTTTGTCAAGACAC-3′; FLAG F1asH Ub R,

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time as peptide addition. Cross-linking to detect mono-Ub/proteasome interaction was done as described (19) with the following changes. 110 nM 26 S or 19 S proteasome was mixed with 10 μM CCPGCC-Ub in TR reaction buffer. After 10 min, 10 μM DBF was added and incubated for 10 min in the dark. The reaction was triggered with 5 mM IO₄⁻, and after 2 min, SDS loading buffer containing 100 mM 2,3-dimercapto-1-propanol (DMP or British Anti-Lewisite BAL) was added to quench the reaction and transfer the DBF label to the cross-linked partner. Experiments that included GST-Gal4-VP16 to dissociate 26 S proteasome were done as above, but 100 nM GST-Gal4-VP16 was added to the proteasome for 15 min at room temperature before adding mono-Ub and completing the reaction. The experiment conducted to identify the targets of Ub in the proteasome was scaled up 5-fold relative to that described above (20, 21).

For the reactions employing recombinant Rpt proteins, six different *E. coli* strains expressing one ATPase in the pET-28a vector (Novagen) (kindly provided by G. DeMartino, University of Texas Southwestern Medical Center) were grown in individual 1-liter LB cultures to mid-log phase, and expression of the ATPase was induced with isopropyl-1-thio-β-D-galactopyranoside. After 2 h of induction, cells were pelleted at 4000 rpm for 15 min. Cell pellets were resuspended in lysis buffer (50 mM sodium phosphate (pH 7.2), 150 mM NaCl, 6.25 mM MgCl₂, 1 mM dithiothreitol with a protease inhibitor tab (Roche Applied Science)) and lysed by sonication. After centrifugation at 17,000 rpm for 20 min, the protein concentration of the lysate was measured. For cross-linking reactions, 1 mg of total lysate was mixed with 10 μM CCPGCC-Ub and 1 μM of the DBF reagent in a 100-ml reaction. The protein mix was allowed to incubate for 10 min at room temperature, and the reaction was triggered and quenched as above. The reaction was run on SDS-PAGE, and the cross-linked product was detected by blotting with NeutrAvidin-HRP. The total amount of His-tagged ATPase was probed with the pentaHis antibody.

Peptidolysis and ATPase Assays—The peptidolysis activity of the 26 S was tested using the fluorescent substrate Suc-LLVY-AMC (Bachem). 50 nM substrate was mixed with 7 nM 26 S proteasome and 3 mM ATP in peptidolysis buffer (50 mM Tris (pH 8.0) and 20 μM mg β-mercaptoethanol). The change in fluorescence (excitation, 360 nm; emission, 465 nm) was monitored for 30 min at room temperature. The indicated proteins were added at 10 μM, and MG132 was added at 100 nM. The graph shows the average change in fluorescence over 30 min from three measurements with one standard deviation of the mean as error. Experiments done in the presence of NaIO₄ were done as above with the addition of 5 mM NaIO₄.

The measurement of ATP hydrolysis by coupling the production of ADP to the oxidation of NADH has been described (22). The absorbance at 340 nm of 5 nM proteasome in the reaction mix was monitored. GST or mono-Ub was included at 10 μM in the indicated lanes. The change in absorbance was converted to nmol of ATP hydrolyzed per minute for three measurements and graphed with one standard deviation of the mean as error.

**Inhibition of Destabilization Activity of the Proteasome**—The destabilization assay was done as stated in the text. 10 μM DOPA-Gal4 AD peptide or the control DOPA-containing peptide (20) were added to the proteasome prior to the addition of the activator-DNA complex. The graph in Fig. 1A shows the mean and standard error of the mean for three experiments.

**RESULTS**

**Ub Antagonizes Proteasome-mediated Destabilization in Trans**—To begin to probe the mechanistic basis of this phenomenon, we first addressed whether it is indeed monoubiquitin that is responsible for the protective effect. The ability of a monoubiquitin fusion to rescue Gal4D (16) suggests that this is true, but it was difficult to exclude other modifications in the *in vivo* system. If monomeric ubiquitin were solely responsible for the protection, it might be possible to observe the inhibition of destabilization by adding ubiquitin to a reaction containing non-ubiquitylated activator-DNA complex. We previously reported a simple *in vitro* assay to monitor the ability of the proteasomal ATPases to destabilize activator-DNA complexes...
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A

DOPA

FIAsH

Biotin

NaIO₄

B

Boil

DMP

C

19S 26S

110

84

48

NA-HRP

α-Rpt6

α-20S

26S

bFD-CCPGCC Ub

Gal4-VP16

26S

+ + + + +

+ + + + +

- + + + +

No

XL

1 2 3 4 5

α-Rpt6
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As shown in Fig. 2B, when 10 μM CCPGCC-Ub and the DBF reagent were incubated with the 26 S proteasome and sodium periodate, no significant labeling of any of the 26 S proteins was detected by SDS-PAGE analysis followed by Western blotting with a NeutrAvidin-HRP conjugate. However, CCPGCC-Ub inhibited destabilization in trans equally as well as His6-Ub (supplemental Fig. S1D, compare lanes 8 and 9), demonstrating that this Ub derivative is active. Significant cross-linking and label transfer was observed when the same experiment was carried out with the 19 S RP rather than the full 26 S proteasome (Fig. 2B, compare lanes 1 and 2). At least two major biotinylated bands were observable, one of ~55 kDa and a larger species that migrated slightly above the 110-kDa marker. The ability of CCPGCC-Ub to affinity label the 19 S RP but not the 26 S proteasome suggests that the Ub interaction site is obscured in the full 26 S proteasome.

As mentioned in the Introduction, the Gal4 and VP16 ADs can extract the 19 S base from intact proteasome via contacts with Rpt4 and Rpt6 (6). We wondered whether this activator-19 S base complex would interact with Ub. Adding Gal4-VP16 to the label transfer reaction containing CCPGCC-Ub and the 26 S proteasome indeed resulted in affinity biotinylation of the same bands that were labeled in the 19 S RP-containing experiment (Fig. 2C, compare lane 1 and 2). When CCPGCC-Ub, DBF, and periodate were mixed with GST-Gal4-VP16 in the absence of any proteasomal proteins, no labeled bands were observed (Fig. 2C, lane 3). Thus, the cross-linking/label transfer data indicate an Ub-19 S RP (or 19 S base) interaction but provide no evidence for direct interaction with the activator.

Identification of the Ub-proximal Proteins in the 19 S RP—

We next turned to identification of the proteins that acquired biotin in the label transfer reaction (Fig. 2C, lane 2). The high molecular weight region of a gel run for a longer period of time to achieve maximal separation of the larger products showed an intense band running just above the 110-kDa marker and a less intense band just below the marker (Fig. 3A, left panel). These bands are approximately the size expected for Rpn1 (upper band) and Rpn2 (lower band), respectively. These assignments were confirmed by aligning the NeutrAvidin-HRP blots with Western blots using antibodies raised against the Rpn1 and Rpn2 subunits. The other major band(s) displayed an apparent mass of slightly above 50 kDa (Fig. 2B). Several proteins in the 19 S RP, including all six ATPases, have similar gel mobilities in this region. Therefore, the label transfer products were analyzed by two-dimensional electrophoresis using isoelectric focusing in the first dimension and SDS-PAGE in the second (Fig. 3B). The major biotin-labeled protein has an isoelectric point of 5.3 and a mass of ~55 kDa, suggesting that it is the Rpt1 subunit (26). To confirm this assignment, the membrane was...
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FIGURE 3. Identification of the monoubiquitin-binding proteins. A, the label transfer experiment employing CCPGCC-Ub and the 19 S RP was repeated, and the products were processed as described in the legend for Fig. 2. The panel shows the alignment of blots probed with NeutrAvidin-HRP (NA-HRP) or antibodies raised against Rpn1 or Rpn2, B, identification of the −55-kDa biotinylated protein. The products of the label transfer experiment were subjected to two-dimensional gel electrophoresis. The upper panel shows the NeutrAvidin-HRP-probed blot of this gel. The lower two panels are false-colored overlays of a portion of the membrane probed with the NeutrAvidin-HRP or the indicated antibodies. C, interaction of mono-Ub with recombinant His₆-tagged Rpt proteins. Lysates from E. coli cells expressing the indicated protein were subject to a cross-linking/label transfer reaction in the presence of CCPGCC-Ub. The NeutrAvidin-HRP blot shows the labeled proteins, and the anti-His blot shows the amount of each of the His-tagged ATPases.

stripped and reprobed with antibodies raised against Rpt1 and Rpt4, the latter being the 19 S protein closest in molecular mass and isoelectric point to Rpt1. The false-colored overlays of the NeutrAvidin-HRP blot with the Western blots against the proteasomal subunits are shown in Fig. 3B, in the lower panels. Note that Rpt1, but not Rpt4, showed an overlap with NeutrAvidin-HRP signal.

Because of the nature of the label transfer process, there is always the possibility that labeling of both the Ub-binding protein itself and its nearest neighbors could occur given the length of the chain connecting the DOPA and FlAsH moieties (Fig. 2A). Therefore, to further probe whether Rpt1 is indeed the ATPase targeted by mono-Ub, we repeated the label transfer experiment with crude extracts derived from six different E. coli strains expressing the His₆-tagged version of each ATPase (Fig. 3C). The only ATPase to acquire the biotin tag in these experiments was Rpt1 (Fig. 3C, upper panel), although all six ATPases were present at similar levels (lower panel). This argues that Rpt1 is indeed the direct binding partner of mono-Ub. Note that none of the many bacterial proteins present in the extract are labeled, arguing that the Ub-Rpt1 interaction is quite specific. Unfortunately, we do not have recombinant Rpn1 in hand, so we cannot absolutely confirm that this interaction is also direct but speculate that this is likely to be the case. Importantly, there was no evidence for binding of CCPGCC-Ub to Rpt4 or Rpt6 (Sug2 and Sug1, respectively), which are the direct binding partners of acidic ADs (20). Based on models of the 19 S architecture, these proteins are predicted to be on the opposite side of the ATPase ring (27).

Ubiquitylated Gal4-VP16 Binds the Proteasomal ATPases Weakly—The experiments described above support a model in which the proteasomal ATPase-catalyzed destabilization of activator-DNA complexes is inhibited by Ub in trans through direct Ub-19 S base interactions. This could be the result of a general Ub-mediated down-regulation of 19 S RP activity. However, we found that mono-Ub had little or no effect on either the 19 S RP-dependent peptidolysis activity of the proteasome or, perhaps more importantly, the ATPase activity of the 19 S RP itself (supplemental Fig. S3). Another mechanism by which Ub could confer protection to the activator-DNA complex is by modifying the interaction between the proteasomal ATPases and the AD of the activator. We demonstrated previously that a complex of the Gal4 DBD alone (lacking an AD) with DNA is insensitive to 19 S base-mediated destabilization, arguing that the AD-ATPase interactions are essential for this effect (13). This is consistent with previously reported direct interactions between the Gal4 and VP16 ADs and Rpt4 or Rpt6 (6, 20, 28–30). Thus, if Ub binding to the 19 S weakens or dissociates the AD-ATPase contacts, the strong prediction is that the destabilization process would be attenuated.

A variant of the destabilization assay was used to address the role of the Ub moiety in modulating AD-ATPase interactions in the context of the monoubiquitylated activator. GST-Gal4-VP16 bound to biotinylated DNA was monoubiquitylated, or not, by exposure to HeLa nuclear extract and a 3-fold excess amount of Ub in the presence or absence of ATP. The ability of these two forms of the activator to retain the proteasomal ATPases was then assessed by a pulldown protocol followed by SDS-PAGE and Western blotting with the appropriate antibodies. As shown in Fig. 4, significant ubiquitylation of the activator was observed in the presence of ATP (lane 4, second panel) but not in its absence (lanes 3 and 5, second panel), as expected. The ubiquitylated and non-ubiquitylated forms of the activator-
Mono-Ub Inhibits the Interaction between the AD of Gal4 and the Proteasome—Gal4-VP16 is an artificial activator, and so it is important to determine whether the results described above are applicable to the native Gal4 protein. The VP16 and Gal4 ADs are thought to act in a similar fashion, both belonging to the acidic class of ADs. Nonetheless, it was important to determine whether the Gal4 and VP16 ADs behave similarly with regard to their interaction with the 19 S base during destabilization reaction. First, we asked whether the two ADs bind identical or overlapping surfaces of the 19 S base complex using a cross-linking experiment. We have shown previously that a synthetic biotin-DOPA-Gal4 AD peptide cross-links efficiently to Rpt6 when incubated with 26 S proteasome and NaIO₄ (20). As shown in supplemental Fig. S4, when an increasing amount of Gal4-VP16 was added to this cross-linking reaction, the yield of the Gal4-AD-Rpt6 product was decreased. No such inhibition was observed when the Gal4 DNA-binding domain lacking the VP16 AD was added. Additionally, when the synthetic the Gal4 AD peptide was added in trans to a destabilization reaction containing immobilized Gal4-VP16 and the proteasome, stripping of Gal4-VP16 from the DNA was inhibited strongly. A control peptide had no effect (supplemental Fig. S4).

These data argue that the mode of interaction of the VP16 and Gal4 ADs with the 19 S base during the stripping reaction is quite similar. Therefore, it seems likely that ubiquitin would also inhibit the interaction between the Gal4 AD and Rpt4/6. To test this, cross-linking and more traditional pulldown experiments were performed with the Gal4 AD (Fig. 5). We used cross-linking with the DOPA-Gal4 AD peptide to determine whether Ub alters the interaction between the Gal4 AD and the proteasomal ATPases (20). The NeutrAvidin-HRP blot in Fig. 5A shows the cross-linked product produced when the DOPA-containing Gal4 AD is mixed with the 26 S proteasome and NaIO₄ (lane 2). The addition of increasing amounts of Ub decreased the amount of AD-Rpt4 cross-linked product with a 77% ± 7.5% reduction at the 10 μM concentration (lanes 3–5). Note that the dose dependence of this effect is similar to that observed for Ub-mediated inhibition of the destabilization process (Fig. 1B). The addition of 10 μM SUMO (lane 6) or 10 μM Lys-48-linked tetra-Ub (lane 7) did little to decrease the amount of cross-linked product. The latter result again highlights the stark differences in the properties of Ub monomers and Lys-48-linked chains in these assays (also see Fig. 1A).

To determine whether Ub would disrupt other Gal4 AD interactions, the binding of Gal4 AD to its repressor Gal80 (31) was examined. The cross-linked product formed between DOPA-Gal4 AD and His₆-Gal80 (Fig. 5B, lane 2) was not significantly decreased by the addition of Ub (lanes 3–5), SUMO (lane 6), or Lys-48-linked tetra-Ub (lane 7). This experiment also demonstrates that Ub does not interfere with the chemistry of the periodate-mediated reaction. We conclude that Ub will inhibit interaction of the Gal4 AD with the proteasomal ATPases but not with other Gal4 AD targets.

A pulldown assay was used to further confirm this result. Immobilized GST-Gal4 AD fusion protein retained the 19 S base, as indicated by probing for Rpt4 protein (Fig. 5C, lane 2) in the absence of Ub, but the addition of mono-Ub to 10 μM almost completely abolished the interaction (Fig. 5C, lane 3).
Poly-Ub chains and SUMO had little effect on the interaction at the same concentration (Fig. 5C, lanes 4 and 5), confirming the results of the cross-linking assay. As a control, we also tested whether Ub would inhibit interactions between the 26 S proteasome and the Ub-like domain (Ubl) of Rad23, a known proteasome-binding protein (32) that contacts Rpn1 directly (33). GST-Ubl pulled down the 26 S proteasome (Fig. 5D, lane 2), and this interaction was unaffected by the addition of Ub, Lys-48-linked tetra-Ub, or SUMO (lanes 3–5). These results show that the Ub effect is specific for the AD-ATPase interaction.

DISCUSSION

Previous studies from our laboratories had demonstrated that activators such as Gal4 must recruit the proteasomal ATPases to drive efficient gene transcription (6–8). ATPase recruitment to the GAL genes requires direct interaction of the Gal4 AD with the Sug1/Rpt6 and Sug2/Rpt4 proteins, two of the six proteasomal ATPases (6, 20). However, we also showed recently that when the ATPases engage activators, they treat them as substrates for their protein unfolding activity (13). In the context of an activator-promoter complex, this results in potent, reversible disruption of the activator-DNA complex, a reaction that inhibits activated transcription in vitro and in vivo. This raised the question of how activators resist this activity to function efficiently. Using various Gal4 derivatives, we correlated the ability to resist proteasomal ATPase-mediated destabilization with ubiquitylation of an as yet uncharacterized lysine residue (13). This ubiquitylation event requires the intact Gal4 AD, which presumably acts as part of the recognition site of the ubiquitin-protein isopeptide ligase (E3) that carries out this process. Inefficient monoubiquitylation of Gal4D, a Gal4 derivative that lacks two-thirds of the native AD, was shown to be the molecular basis for the very low activity of this protein (16).

In this study, we have determined the biochemical mechanism of protection against the destabilization activity of the proteasomal ATPases. A model incorporating the data presented in this study is shown schematically in Fig. 6. Based on previous biochemical studies (6), the first event that occurs is that the AD extracts the proteasomal ATPases, Rpn1 and Rpn2 (the base of the 19 S RP (34), also known as the APIS complex (6)) from the proteasome, leaving the 20 S core complex and the lid subassembly behind. Note that even if the activator is monoubiquitylated at this point, the Ub moiety will not bind to the 26 S proteasome (Fig. 2). Once engaged with the AD, the 19 S proteasome is dissociated from the activator-promoter complex, allowing the transcriptional machinery to resume its function.
S base will proceed to unfold the activator. If this process is allowed to continue unabated, as would be the case when the Gal4 DBD is not monoubiquitylated, then the activator-promoter complex would be disrupted, and transcription would be inhibited. However, based on the data presented here, the model argues that when the DBD is ubiquitylated, Ub-Rpt1 and Ub-Rpn1 contacts result in disruption of the AD-Rpt4/Rpt6 contacts via an allosteric mechanism. Put another way, the Ub-19 S base contact limits the lifetime of the AD-Rpt4/Rpt6 complex and reduces the degree of activator unfolding that can be achieved by the ATPases before dissociating. Note that Fig. 6 depicts an ordered series of events in which the AD first extracts the 19 S followed by binding of Ub to Rpt1 and Rpn1. This is based on our finding that Ub cannot interact with the intact 26 S proteasome but can bind to 19 S proteins after the AD has extracted the 19 S base complex from the 26 S proteasome (Fig. 2C, lane 2).

In the Fig. 6 model, it is also suggested that once dissociated from the activator, the 19 S base could then move into the nearby transcription complex, where it must eventually arrive to stimulate promoter escape and elongation. However, this aspect of the model is purely speculative and is not addressed by the data presented in this report. Finally, our data provide no conclusive evidence for or against the continued association of the mono-Ub residues with the 19 S base subcomplex after the latter dissociates from the activator, although the poor ability of the ubiquitylated, DNA-bound activator to pull down the ATPases (Fig. 4) is more consistent with rapid dissociation.

The insights provided by the data reported here might also be important in understanding other nuclear processes that involve protein monoubiquitylation and non-proteolytic functions of the proteasomal ATPases. For instance, histone H2B monoubiquitylation functions cooperatively with the FACT (facilitates activation on chromatin templates) complex to help the elongating polymerase move through chromatin templates (35). The proteasomal ATPases are also important for elongation (7, 8) and, furthermore, FACT associates with the APIS complex (36). Another interesting case is the reported linkage of histone H2B monoubiquitylation and histone H3 methylation, a coupling that is somehow dependent on the non-proteolytic activities of the proteasomal ATPases (11). It is possible that monoubiquitin-Rpn1/Rpt1 interactions are important in these events as well, either in attracting the unfoldases to histones (and perhaps thus promoting structural rearrangements that are required for subsequent covalent modifications) and/or in limiting the lifetime of a different chromatin-proximal ATPase-protein complex.

Finally, it is worthwhile reemphasizing the striking differences between the functional and physical interactions of the proteasome with mono-Ub and Lys-48-linked tetra-Ub chains seen in this study. The development of the novel cross-linking/label transfer scheme, shown in Fig. 2A and supplemental Fig. 6.
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S2, has made possible the evaluation of the interaction of mono-Ub with intact proteasome complexes or subcomplexes in a relatively straightforward fashion. The results, buttressed by more classical pulldown studies, argue that mono-Ub is not a 26 S proteasome-binding protein, whereas previous workers have demonstrated that poly-Ub chains bind tightly to the 26 S complex (37, 38). Mono-Ub binding to Rpn1 and Rpt1 is readily detectable by label transfer only in the absence of the 20 S, concentration do not exhibit this activity. These experiments highlight the fact that these different forms of Ub represent completely different modifications of a protein that will cause it to interact with the proteasome in very different ways.

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