Activation Domain-dependent Monoubiquitylation of Gal4 Protein Is Essential for Promoter Binding in Vivo

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The Saccharomyces cerevisiae Gal4 protein is a paradigmatic transcriptional activator containing a C-terminal acidic activation domain (AD) of 34 amino acids. A mutation that results in the truncation of about two-thirds of the Gal4AD (gal4D) results in a crippled protein with only 3% of the activity of the wild-type activator. We show here that although the Gal4D protein is not intrinsically deficient in DNA binding, it is nonetheless unable to stably occupy GAL promoters in vivo. This is because of the activity of the proteasomal ATPases, including Sug1/Rpt6, which bind to Gal4D via the remainder of the AD and strip it off of DNA. A mutation that suppressed the Gal4D “no growth on galactose” phenotype repressed the stripping activity of the ATPase complex but not other activities. We further demonstrate that Gal4D is hypersensitive to this stripping activity because of its failure to be monoubiquitylated efficiently in vivo and in vitro. Evidence is presented that the piece of the AD that is deleted in Gal4D protein is likely a recognition element for the E3 ubiquitin-protein ligase that modifies Gal4. These data argue that acidic ADs comprise at least two small peptide subdomains, one of which is responsible for activator monoubiquitylation and another that interacts with the proteasomal ATPases, coactivators and other transcription factors. This study validates the physiologic importance of Gal4 monoubiquitylation and clarifies its major role as that of protecting the activator from being destabilized by the proteasomal ATPases.

The proteasome is a large macromolecular complex that carries out most of the non-lysosomal proteolysis in eukaryotic cells (1). It comprises a 20 S core subunit containing the protease active sites and a 19 S regulatory particle (RP) that caps either end of the barrel-shaped core. Proteins destined for proteasome-mediated destruction are recognized by the 19 S RP, usually, although not always, by virtue of their post-translation modification with a chain of at least four Lys-48-linked ubiquitin (Ub) molecules (2). Once bound, the substrate is unfold-ed/unwound by a heterohexameric ring of six ATPases (Rpt1–6) at the base of the 19 S RP, and the unfolded chain is fed through the narrow opening to the inside of the core complex.

It is now clear that proteins in the Ub-proteasome pathway (UPP) play important roles in a variety of nuclear processes. Recent global chromatin immunoprecipitation (ChIP on chip) studies in yeast have shown that a large fraction of active genes are occupied by either the 26 S proteasome and/or by independent 19 S or 20 S complexes (3, 4). The best studied intersection of the UPP and nucleic acid metabolism is RNA polymerase II transcription. The first hint of such a link was the finding that 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) (5, 6). Subsequent experiments demonstrated that this effect could not be explained by altered proteolysis of Gal4D (7). This stimulated an investigation into potential direct roles of these proteins in transcription that revealed that Rpt6/Sug1 protein activity is important for efficient promoter escape and elongation (8, 9). This is not an activity of the 26 S proteasome. The activation domains (ADs) of Gal4 bind directly to Sug1/Rpt6 and Sug2/Rpt4 and extract from the proteasome a complex that we coined APIS, which includes the six ATPases, Rpn1, and Rpn2 proteins, and perhaps other associated proteins, but not the 20 S core proteasome or the “lid” subunit of the 19 S RP (10, 11). The ability of the proteasomal ATPases to promote elongation has subsequently been demonstrated in other systems (12, 13). Although the 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 (14). Others later demonstrated that Sug1 and presumably other proteasomal ATPases are also important in mediating histone extract; IMAC, immobilized metal affinity chromatography; CTR, C-terminal region; NTR, N-terminal region; qPCR, quantitative PCR; UAS, upstream activation sequence.

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5 The abbreviations used are: RP, regulatory particle; Ub, ubiquitin; UPP, ubiquitin-proteasome pathway; ChIP, chromatin immunoprecipitation; AD, activation domain; GST, glutathione S-transferase; HNE, HeLa nuclear
modifications and in recruiting the SAGA complex to promoters, neither of which require proteolysis (15, 16).

An unsatisfying aspect of these discoveries is that none seemed to explain the ability of sug1-1 and sug2-1 alleles to suppress the “no growth on galactose” phenotype of gal4ΔD. These mutations were clearly recessive (6), which is difficult to reconcile with the requirement of Sug1 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 Gal4ΔD was hypersensitive.

We recently reported a new activity of the proteasomal ATPases that provides a possible rationalization for these genetic data (17). When a transactivator-DNA complex is exposed to the 19 S RP, it is rapidly and reversibly dissociated. This destabilization, or “stripping” activity, does not involve activator proteolysis, is inhibited by the addition of an antibody raised against the Sug1/ Rpt6 ATPase, and requires ATP hydrolysis for activity, demonstrating an important role for the proteasomal ATPases (17). Direct interactions between the ATPases and the AD of the transactivator are essential because 19 S RP does not affect the stability of a protein-DNA complex lacking an AD. This was an unknown activity of the proteasomal ATPases; however, other ATPases are known to have similar activities. For instance, the ATPase Mot1 is known to disrupt TATA-binding protein-DNA complexes in an ATP-dependent manner (18).

These new data, however, provided a conundrum. How do wild-type activators resist this potent stripping activity of the 19 S RP and remain associated with the promoter long enough to drive high level transcription? A detailed study of the effect of the 19 S RP on GST-Gal4-VP16 promoter complexes suggested that the answer lies in the post-translational modification of the Gal4ΔD DNA-binding domain. When exposed to a HeLa nuclear extract, all of the DNA-bound activator is monoubiquitylated (17), and this form of the protein is insensitive to the destabilization activity of the 19 S RP.

In this study, we have addressed the relevance of ATPase-mediated destabilization of activator-DNA complexes and the role of monoubiquitylation in blocking this activity to understand the gal4ΔD phenotype and its suppression by sug1-1 and sug2-1. First, we show that the Gal4ΔD protein, although not intrinsically deficient in DNA binding, is not able to occupy the promoter regions of Gal4-dependent genes in vivo. Moreover, a Gal4ΔD derivative is not effectively ubiquitylated in vitro, and evidence is presented that this is the case in vivo as well. We show the sug1-1 mutation cripples the destabilization activity of the proteasome, but not other functions of the ATPase, and largely restores the ability of the Gal4ΔD protein to occupy DNA in vivo even though it is not monoubiquitylated. The extensive correlation between the genetic and biochemical data argues strongly that the molecular basis of the gal4ΔD phenotype is that truncation of the AD cripples activator monoubiquitylation and that this, in turn, renders the Gal4ΔD-promoter complexes hypersensitive to disruption by the proteasomal ATPases. Finally, we demonstrate that the piece of the Gal4 AD that is lacking in Gal4ΔD can compete activator monoubiquitylation in trans, arguing that it is probably a docking site for the E3 ubiquitin-protein ligase complex that targets Gal4. These data, in addition to further validating the physiological relevance of Gal4 monoubiquitylation, also reveal a previously unsuspected subdomain structure of the Gal4 acidic AD that comprises a short peptide (12 residues) capable of acting as a classical AD and a 22-amino acid piece that is essential for activator monoubiquitylation.

EXPERIMENTAL PROCEDURES

Yeast strains, Plasmids, and Proteins—Pre1-FLAG-tagged 26 S and 19 S have been described (19). A set of congenic strains derived from W303 (MAT a leu2-3 112, his3-115, trp1-1, can1-100, ade2-1, ura3-1) were used in this study. For generation of SUG wild-type and mutant proteasome containing a T7-tagged SUG, the following strains were used: Sc507 (T7-SUG1) and Sc654 (W303 T7-sug1-1, described in Ref. 7). For ChIP experiments, yeast strains Sc726 (SUG1 gal4::HIS3), Sc728 (sug1-1 gal4::HIS3), Sc732 (sug1-20 gal4::HIS3), Sc736 (sug2-1 gal4::HIS3), and Sc738 (sug2-13 gal4::HIS3), as described previously (7), were used. Yeast strains used for ChIP experiments were transformed with single-copy plasmids (derived from pSB32) expressing either wild-type Gal4 (pSJ263) or gal4ΔD (pSJ261). In each case the encoded proteins were tagged at their N termini with three tandem copies of the T7 epitope tag (Novagen). Genetic fusion of ubiquitin to S10-tagged Gal4ΔD in the pSB32 vector was done by removing Ub from GST-Ub-Gap71-VP16 (17) using a NcoI digest and inserting Ub into the Ncol site at the start codon of the T7 tag. For all of these constructs, the Gal4ΔD gene was expressed from its own promoter. Pulldowns to detect ubiquitylated Gal4ΔD were done in Sc726 using a pSB32 plasmid with a His₉ tag. α-Galactosidase assays were done using Sc244 (strain 21; a gal4Δ--2, Gal80, ura3-52, leu2-3 112, ade1, MEL1) transformed with the pSB32 plasmids mentioned above. The transformed strains were grown in complete medium lacking leucine, to select for the plasmids, with raffinose as the carbon source. The use of raffinose ensured that there was no selection for suppressors of gal4ΔD induction. The Gal4ΔD genes was performed by adding galactose directly to the medium (2% final concentration).

26 S and 19 S proteasome were purified using a FLAG affinity tag as described (19) with modifications (8). The sug1-1 26 S proteasome and congenic wild-type 26 S proteasome were purified as for the FLAG affinity tag except using T7-agarose (Novagen) and T7 peptide followed by a Mono-Q (Amersham Biosciences) column using a 20 mM–1 M NaCl gradient over 30 column volumes, which eluted proteasome at ~450 mM NaCl. GST-Gal4 (1–147)-VP16, GST-Gal4 (1–147), GST-Gal4 AD, GST-Gal4ΔD AD, GST-ubiquitin-like domain of Rad23p, His₉-SUMO, His₉-Gal80, and His₉-Ub were purified from Escherichia coli BL21 cells as described (8). The mini-Cla Gal4 and Gal4ΔD constructs have been described previously (20). Antibodies against PentaHis (Qiagen), ubiquitin (Dako Cytomation), GST (Santa Cruz Biotechnology), neutradvin-horseradish peroxidase (Pierce), and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) were used according to instructions. The α-Rpt6, α-Rpt4, α-Rpt1, α-Gal4N terminus, α-Gal6, and α-20 S antibodies were produced in rabbit. α-Rpn1 and α-Rpn2 were produced in mouse.

Destabilization of Activator-DNA Complexes by the Proteasome—The destabilization assay has been described previously (17) but was done with the following changes. An activator-DNA
complex (60 nM final concentration) was added to the reaction mix containing 25 nM 26S proteasome, 3 mM ATP, and 1 μM nonbiotinylated DNA containing Gal4 binding sites in TR reaction buffer (10 mM HEPES, pH 7.8, 50 mM KCL, 6.25 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 2% glucose (v/v)).

**Mono-ubiquitylation of Activators**—The monoubiquitylation of GST-Gal4-VP16 has been described (17). The protocol here was the same, but the stated mini-Cla versions were used in place of GST-Gal4-VP16. The non-ubiquitylated controls were done exactly as stated, but 3 units of hexokinase were used, and the ATP was omitted. At the end of the reaction, 6 m urea was added to denature everything, and TALON (Clontech) affinity resin was used to pull down the proteins covalently linked to His₆-ubiquitin.

For gel-based analysis of ChIPs, 5 ml of the final product was used in a standard PCR reaction with the indicated primer. Reactions were run on a 1% agarose gel, scanned, and analyzed by densitometry to produce the graphs shown in Fig. 1. Quantity PCR of precipitated chromatin was performed using an iCycler thermal cycler and the IQ SYBR Green Supermix (2× mix contained 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 units/ml iTaq DNA polymerase, 6 mM MgCl₂, SYBR Green I, 20 μM fluorescein, stabilizers (Bio-Rad)). Reactions were run on a 1% agarose gel, scanned, and analyzed by densitometry to produce the graphs shown in Fig. 1. Quantitative PCR of precipitated chromatin was performed using an iCycler thermal cycler and the IQ SYBR Green Supermix (2× mix contained 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 units/ml iTaq DNA polymerase, 6 mM MgCl₂, SYBR Green I, 20 μM fluorescein, stabilizers (Bio-Rad)). Relative enrichment of specific DNA was calculated by comparing products derived from primers against the GAL7 promoter from the precipitated samples with the specific antibody and an unspecific control antibody. Data were graphed as a percent of the total DNA from each chromatin sample. Primers used for analysis: GAL1-10 promoter ChIP, UAS F, GTGGAATAATGTA-AAGAGCCCC; UAS R, CTTCATTGGTGGAGACTG; TATA F, CAACCATAGGTGATAAATGGC; and TATA R, CTTCTTGGAATGAGATTGTC; and GAL7 promoter ChIP, GAL7 F, TGCTCTGCATAATATGCC; and GAL7 R, TTGCTTTGCCTCTCTTTTG.

**Measurement of RNA Production**—Total RNA was isolated from 10 ml of cells at an A₆₀₀ of 0.6–0.8 after the addition of galactose. Cells were centrifuged for 5 min at 3000 × g in a Sorvall RT7 centrifuge with a RTH-750 swing bucket rotor. Cells were washed with PBS and centrifuged as described above. Cell pellets were frozen in liquid nitrogen and stored at −80 °C. Cell pellets were resuspended in 400 ml of water, and 400 ml of water-saturated phenol was added and vortexed for 1 min. The mixture was incubated at 65 °C for 45 min. The aqueous layer was removed and extracted with water-saturated phenol followed by chloroform. RNA was treated with RQ1 DNase (Promega Corp., Madison, WI) for 1 h. RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) followed by chloroform. The RNA was precipitated by adding 40 ml of 3 M NaOAc, pH 5.3, and 1 ml of 95% EtOH. RNA quantity was measured by measuring the A₂₆₀. 1 mg of Total RNA was used to make cDNA using the Stratascript first-strand cDNA synthesis kit (Stratagene, La Jolla, CA) and oligo(dT). cDNA was measured by quantitative PCR, as described above, using GAL1 and ACT1 primers. The ratio of GAL1 to ACT1 from three samples was averaged and graphed. Primers used for analysis: RNA analysis, GAL1 F, CTCCTGTTCGGGTAGAAAG; GAL1 R, ACCTTTATTCGTCGTGATCC; ACT1 F, CTCAGATGTTGTGATGAAGCT; ACT1 R, GTCAATCATCTTACCGG.

**GST Fusion Protein Retention Assays**—5 μg of GST, GST-Gal4 AD (GST-AD), or GST-Gal4 AD (GST-G4AD), while on agarose beads, was incubated with the yeast whole cell extracts in MTB (50 mM HEPES, pH 7.5, 100 mM potassium acetate, 5 mM magnesium acetate, 1 mM EGTA, 0.1 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P-40) that also contained 10 mg/ml soluble E. coli lysate as a nonspecific competitor. Glutathione beads and labeled proteins were incubated at 4 °C with gentle agitation for 1 h. Following incubation, the beads were centrifuged (13,000 rpm, 1 min), and washed twice in 1.5 ml of MTB. After the final wash, the entire supernatant was carefully removed, and 20 μl of SDS loading buffer was added to the beads. The beads were then boiled for 5 min and loaded onto a 10% SDS-polyacrylamide gel. The gels were transferred to polyvinylidene difluoride, and membranes were then used for measurement of retained protein by Western blot analysis. Input values represent 10% of total input.

**RESULTS**

The gal4D Mutation Results in a Defect in Promoter Occupancy in Vivo—The recent finding that the proteasomal ATPases could antagonize transcription factor-DNA binding led us to employ ChIP to test the ability of Gal4D to occupy GAL promoters in vivo. Gal4 and Gal4D have been shown to have similar steady-state levels in yeast, so changes seen by ChIP cannot be due to differences in concentration between the two proteins (7). Yeast cells deleted for GAL4 were transformed with single-copy vectors expressing Gal4 or Gal4D from the native GAL4 promoter. In galactose medium, Gal4 was resident on the UAS region of the GAL1/10 promoter, but not on the GAL1 core promoter, as expected (Fig. 1). However, the Gal4D protein was not able to occupy its binding site under the same conditions in the wild-type SLIG1 background. The failure of Gal4D to occupy its binding site is not a defect in the intrinsic DNA binding activity of the protein. In vitro, constructs containing the DNA binding domain and activation domain of Gal4D have been shown previously to exhibit an affinity for GAL promoter-containing DNAs similar to that of the wild-type Gal4 (21).

If the inability of Gal4D to occupy GAL promoters under inducing conditions is indeed the molecular basis of the Gal4D no growth on galactose phenotype, then the strong prediction is that the suppressing sug alleles, but not the non-suppressing alleles, should reconstitute Gal4D-promoter interactions. This was tested by ChIP in a sug1-I or sug1-20 background (Fig. 1B). Compared with the wild-type SLIG1 background, the levels of Gal4D bound to the promoter were much higher in the sug1-I strain (50% of Gal4 levels), whereas the binding of wild-type Gal4 to the GAL1/10 promoter was similar in both strains. In contrast, the non-suppressing mutation, sug1-I-20, did not
sensitive. The effect of the proteasomal ATPase complex on Gal4D-promoter backgrounds (data not shown). We conclude that the molecular monoubiquitylation of Gal4-VP16 being dissociated by the proteasomal ATPases (17). Moreover, modification is essential for Gal4-promoter complexes to resist the extent to which Gal4D is monoubiquitylated, because pre-

FIGURE 1. sug1-1 restores Gal4D promoter occupancy in vivo. A, schematic of the GAL1 promoter region. The Gal4 binding site (UAS), TATA box (TATA), and start site are indicated. The regions amplified by the two different primers sets are indicated in gray below the gene. B, chromatin immunoprecipitation (IP) performed against mid-log cells after a 1-h induction with galactose. An antibody raised against the N terminus was used to precipitate Gal4 or Gal4D in the strain background, indicated at the top of the panel. PCR with the UAS primer shows DNA precipitated from the Gal4 binding site, and the TATA primer shows DNA from the start site. Totals represent a PCR with the UAS primer from chromatin isolated but not precipitated. The graph at right presents the average \pm S.E. of densitometry analysis of three experiments with Gal4 in SUG1 as 100%.

restore the ability of Gal4D to bind to the promoter (Fig. 1B). A similar result was obtained when the experiment was repeated in the sug2-1 (suppressing) and sug2-13 (non-suppressing) backgrounds (data not shown). We conclude that the molecular basis of Gal4D dysfunction must be because of an antagonistic effect of the proteasomal ATPase complex on Gal4D-promoter binding to which wild-type Gal4 is immune or, at least, far less sensitive.

Gal4D Is Not Ubiquitylated Efficiently—We next examined the extent to which Gal4D is monoubiquitylated, because previous in vitro and in vivo experiments had indicated that this modification is essential for Gal4D-promoter complexes to resist being dissociated by the proteasomal ATPases (17). Moreover, monoubiquitylation of Gal4-VP16 in vitro is dependent on the presence of an activation domain. Because Gal4D lacks about two-thirds of the native Gal4 AD (Fig. 2B), inefficient ubiquitylation of Gal4D seemed a reasonable model to explain its promoter-binding properties in vitro.

Because of the difficulty of purifying and conducting biochemical experiments with native, full-length Gal4 we turned to the “mini-Cla” version of Gal4 and Gal4D (20). This protein contains the native DNA-binding domain and the AD of Gal4 but lacks a central segment encoded by DNA flanked by Clal sites. In S. cerevisiae the mini-Cla Gal4 is a potent activator that responds to the same repressive and activating signals as full-length Gal4 and thus serves as an excellent substitute for the native activator in biochemical experiments.

We employed a previously reported assay to monitor the monoubiquitylation of mini-Cla Gal4 and mini-Cla Gal4D in vitro (17). In the previous report, we were able to demonstrate a DNA-dependent monoubiquitylation of the activator Gal4-VP16 in a HeLa nuclear extract (HNE) in the presence of ATP. In the current report, the activators were first bound to immobi-

FIGURE 2. Gal4D is not ubiquitylated efficiently in vivo. A—We next examined the extent to which Gal4D is monoubiquitylated, because previous in vitro and in vivo experiments had indicated that this modification is essential for the Gal4D promoter complexes to resist being dissociated by the proteasomal ATPases (17). Moreover, monoubiquitylation of Gal4-VP16 in vitro is dependent on the presence of an activation domain. Because Gal4D lacks about two-thirds of the native Gal4 AD (Fig. 2B), inefficient ubiquitylation of Gal4D seemed a reasonable model to explain its promoter-binding properties in vitro.

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Identification of a Subdomain of the AD Required for Monoubiquitylation—This result argues that the C-terminal two-thirds of the native Gal4 AD is somehow essential for activator monoubiquitylation. One model is that these residues interact with the (as yet unknown) E3 ubiquitin-protein ligase complex that targets Gal4. To test this idea directly, peptides corresponding to the N-terminal 12 residues of the Gal4 AD, which remain intact in Gal4D (residues 841–853, called the AD N-terminal region (NTR) peptide), or the C-terminal piece of the AD missing from Gal4D (residues 855–875, called the AD C-terminal region (CTR) peptide) were synthesized for use in an ubiquitylation assay (Fig. 2B). If the region of the AD missing from gal4D (AD CTR peptide) contacts the ubiquitylation machinery, one would predict that addition of an excess of the peptide would inhibit ubiquitylation of the activator by competing the AD of the protein for the ubiquitylation machinery. The addition of the AD NTR peptide to an ubiquitylation reaction at 10 \muM (an \sim 30-fold excess over DNA bound activator) had no effect on the amount of ubiquitylated mini-Cla Gal4 recovered (Fig. 2C). In contrast, addition of the AD CTR peptide reduced the amount of ubiquitylated mini-Cla Gal4 recovered by 60% (Fig. 2C). The AD CTR peptide, but not the AD NTR peptide, also reduced the amount of ubiquitylated Gal4-VP16 protein recovered (Fig. 2D), which argues that the Gal4 AD and the VP16 AD must have similar properties relative to stimulating activator ubiquitylation.
Ubiquitylation of Gal4 in Vivo

The considerable efforts directed toward the direct biochemical observation of mono-ubiquitylated Gal4 in yeast cells were inconclusive. For example, we attempted to probe the native ubiquitylation state of Gal4 using a Δgal4 strain that had been transformed with the empty pSB32 vector or a vector expressing the His₉-tagged Gal4 under the control of the GAL4 promoter. The His₉ tag allows for lysis of the cells and recovery of the protein by IMAC under denaturing conditions to limit the activity of proteases and deubiquitylases. Cells were grown to mid-exponential phase in either glucose or raffinose and then induced with galactose for 1 h prior to lysis under denaturing conditions in the presence of inhibitors of the proteasome, proteases, and deubiquitylation enzymes. Western blot analysis of the IMAC-retained fraction showed a band at the expected apparent molecular weight that was reactive anti-Gal4 antibodies (supplemental Fig. 1, lane 4). Blots with anti-ubiquitin antibodies suggest that the same band contains ubiquitin. This band was not detected when the cells were grown in glucose (supplemental Fig. 1, lane 3) or in the cells grown in galactose not expressing Gal4 (lane 2), suggesting one major ubiquitylated form of Gal4 under inducing conditions. However, the quality of these blots was not good enough to demonstrate unequivocally that most or all of the Gal4 is monoubiquitylated. We note that, to the best of our knowledge, there are no reported examples of monoubiquitylated transcription factors isolated from a yeast whole cell extract, which perhaps attests to technical difficulties with this experiment.

Therefore, we turned to a different method to assess the physiological relevance of Gal4 monoubiquitylation. Specifically, we employed ChIP assays to address the question of whether promoter-bound Gal4 is ubiquitylated in vivo. Cells were grown to mid-log phase in raffinose and then induced with galactose for 1 h. ChIP assays were performed with antibodies directed against Gal4 or against ubiquitin, and the amount of DNA recovered from the GAL7 UAS₉₅ was quantified by quantitative PCR (qPCR). Similar to the results shown in Fig. 1B, Gal4 was able to

**FIGURE 2.** Gal4D cannot be monoubiquitylated. A, mini-Cla versions of Gal4 (lanes 1–3) and Gal4D (lanes 4–6) were tested in an ubiquitylation reaction in a solution supplemented with His₉-tagged Ub. Ubiquitylated proteins were pulled down by IMAC under denaturing conditions. 5% of the mini-Cla Gal4-DNA complex input into the reaction is shown in the lower panel B, schematic of the Gal4AD. A close-up view of the region that contains the AD (amino acids 841–875) is shown. Gal4D is truncated at residue 853. The sequences of the peptides made are indicated in the lower part of the panel C, ubiquitylation experiment of mini-Cla Gal4 same as in A but with the addition of the indicated peptide at 10 μM. The graph shows the average ± S.E. with the amount of ubiquitylated product recovered with no peptide added set at 100%. D, same as in C, but GST-Gal4-VP16 is used in place of mini-Cla Gal4.
occupy the promoter in the sug1-1, sug1-20, and sug1-20 strains (Fig. 3A). In the sug1-1 or non-suppressing (sug1-20) strains, Gal4D did not occupy the promoter. In the sug1-1 background, Gal4D occupancy was increased to ~75% of the level observed for Gal4 in the same background. The partial, but not complete, recovery of occupancy of Gal4D promoter binding in the sug1-1 strain correlates with the partial recovery of activity of GAL gene expression in this background seen previously (6).

The presence of ubiquitin on the GAL7 UAS region was probed using the same chromatin samples but substituting an anti-ubiquitin antibody for the anti-Gal4 antibody, again quantifying the results by qPCR (Fig. 3B). An ubiquitin-dependent signal was seen when Gal4 was bound to the promoter in all cases. Of course, no Gal4- or ubiquitin-dependent signal was observed in the sug1-1 or sug1-20 strains, which express Gal4D protein, as expected. These data are consistent with the idea that Gal4 is monoubiquitylated, but we cannot exclude the possibility that one or more different proteins bound to the GAL7 UAS region are ubiquitylated in a Gal4-dependent fashion. However, the ChIP data obtained in the sug1-1 strain expressing Gal4D shed considerable light on this issue. Whereas Gal4D binding to the UAS is reconstituted in the sug1-1 background, no ubiquitin-dependent signal was observed from the same chromatin sample. Taken together, these experiments argue strongly that although Gal4D is resident on the promoter in the sug1-1 strain, it is not ubiquitylated. This, in turn, strongly suggests that the ChIP signal observed in cells containing wild-type Gal4 is due to ubiquitylated Gal4 itself. If the ubiquitin-dependent ChIP signal were due to an ubiquitylated histone or some other transcription factor, then one would expect to also observe this in the gal4D/sug1-1 strain, because GAL transcription is quite active.

Gal4D Function Can Be Rescued by Genetic Fusion of Ubiquitin—The biochemical experiments shown in Fig. 2A and the in vivo results in Fig. 3 argue that the reason Gal4D is deficient in promoter occupancy is that the activator is not monoubiquitylated efficiently. To test this model further, we asked whether genetic fusion of a monoubiquitin to the N terminus of Gal4D would rescue its activity. Genetic fusion of monoubiquitin has been shown to restore partial activity of other proteins in yeast that are not efficiently monoubiquitylated due to either the absence of the cognate E3 ubiquitin-protein ligase or mutations in the activator (17, 22). As seen above, Gal4D cannot occupy the UASs in a sug1-1 strain (Figs. 1B and 3A). In contrast, ChIP analysis revealed that expression of a fusion protein in which ubiquitin was fused to the N terminus of Gal4D resulted in occupancy of the promoter at 50% of the wild-type Gal4 level, as determined by qPCR (Fig. 4A). Consistent with the recovery of promoter occupancy, the Ub-Gal4D fusion protein drove GAL1 transcription to ~40% of the level observed in cells containing wild-type Gal4 (Fig. 4B). Thus, as anticipated by the biochemical model, the defect in Gal4D can be partially overcome by fusion of monoubiquitin to Gal4D, arguing that natural monoubiquitylation is important for Gal4 function.

Decreased "Stripping" Activity of Sug1-1-containing Proteasomes—The previous experiments demonstrate the importance of monoubiquitin to Gal4 function and support the model that Gal4D cannot occupy promoters because of a defect in ubiquitylation that makes it hypersensitive to destabilization. We hypothesized that the sug1-1 mutation suppresses this defect, because this mutation decreases the destabilization activity of the proteasomal ATPases. In this model, the reduced destabilization activity allows Gal4D to occupy promoters even without the protective effect of monoubiquitylation.

To test this model directly, 26 S proteasome was purified from either wild-type cells or cells that contained the sug1-1 mutation, and the destabilization activity was measured. We previously reported a simple in vitro assay to monitor the ability of the proteasomal ATPases to destabilize activator-DNA complexes (17), shown schematically in supplemental Fig. S2. An excess of mini-Cla Gal4D was bound to 60 nmol of biotinylated DNA containing five Gal4 binding sites (300 nM binding sites) immobilized on a streptavidin-agarose bead. The mini-Cla Gal4D-DNA complex was exposed to 25 nmol of highly purified 26 S yeast proteasome in the presence of 1 mM ATP and a 15-fold excess of soluble DNA containing Gal4 binding sites. The bead-bound DNA and associated proteins were isolated after the incubation, washed, and probed by SDS-PAGE and Western blotting to monitor the amount of mini-Cla Gal4D still associated with the immobilized DNA.
The wild-type 26 S proteasome removed the majority of the mini-Cla Gal4D from the DNA (Fig. 5A) in the destabilization assay. In stark contrast, when Sug1-1-containing 26 S proteasome was used, most of the mini-Cla Gal4D was retained on the bead-bound DNA. We conclude that the sug1-1 mutation indeed attenuates the stripping activity of the proteasomal ATPases.

To ensure that this was not simply a result of poor activity of the Sug1-1 proteasome preparation in general, two other functions of the ATPase were checked. First, the peptidolysis activity of either wild-type proteasome or proteasome containing Sug1-1 was compared. Cleavage of the profluorescent peptide by the chymotrypsin-like activity of the proteasome results in a fluorescent signal that can be monitored by a spectrophotometrically. For the peptide to enter into the cavity of the proteasome efficiently, the ATPases must hold open a “flap” that otherwise blocks substrate access, thus providing an independent assay for ATPase function (23). Both wild-type and Sug1-1-containing proteasome peptidolysis activity was stimulated by the addition of ATP to similar levels (Fig. 5B). Second, Sug1 interacts directly with the Gal4 AD, as does Sug2 (10, 11). To
determine whether the Sug1-1-containing ATPase complex can bind to the Gal4 AD, the ADs of wild-type Gal4 and Gal4D (Fig. 2B) fused to GST were used as the bait in pulldown assays with yeast lysates made from strains carrying different sug mutations. The results (Fig. 5C) show that the Gal4 AD binds equally well to ATPase complexes containing Sug1, Sug1-1, or Sug1-20 proteins, validating that the Sug1-1 protein is active. Interestingly, we also found that all three ATPase complexes bind equally well to the truncated AD present in Gal4D, demonstrating directly that this region (NTR; see Fig. 2B) is fully capable of binding the ATPase complex. We conclude that the ATPase complex derived from Sug1-1-containing proteasomes is specifically deficient in destabilizing activator-promoter complexes but is not generally inactive.

DISCUSSION

Previous studies from our laboratories demonstrated that activators such as Gal4 must recruit the proteasomal ATPases in order to achieve efficient gene transcription (8–10). In vitro experiments have suggested that the ATPases are important for efficient promoter escape and elongation by RNA polymerase II (8), and others have presented evidence that these proteins are also essential for SAGA recruitment (16). 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 (10, 11). However, we also showed recently that when the ATPases engage activators, the ATPases treat these activators as substrates for their protein unfolding activity (17). 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 in order to function efficiently. Using various Gal4 derivatives, we correlated the ability to resist proteasomal ATPase-mediated destabilization with modification in the DNA-binding domain, specifically monoubiquitylation of an as yet uncharacterized lysine residue (17). Further experiments demonstrated this monoubiquitylation of the Gal4 DNA-binding domain to be dependent on binding of the activator to DNA and the presence of an AD (17).

The discovery of destabilization of activator-DNA complexes by the proteasomal ATPases caused us to revisit the truncation mutant of Gal4, Gal4D, and suppression of the no growth on galactose phenotype by the sug1-1 or sug2-1 mutations. As mentioned above, the mechanistic underpinnings of these genetic observations have never been explained adequately. In this work we set out to test whether Gal4D was hypersensitive to destabilization and whether the suppressing sug alleles reduced the destabilization activity. We found that although Gal4D was able to bind to the UASG in vitro (Fig. 2A), it was unable to occupy promoters in the activated state in vivo (galactose-containing media) unless the yeast strain carried a suppressing sug1-1 mutation (Figs. 1B and 3A). Furthermore, it had been reported previously that the gal4D phenotype could also be partially suppressed by forcing occupancy by massive overexpression of the protein (5). Finally, biochemical analysis of the stripping activity of proteasomes isolated from sug1-1 yeast showed that this mutation indeed down-regulates this activity (Fig. 5A). Taken together, these results argue that the molecular basis of the gal4D phenotype is almost certainly hypersensitivity to stripping by the proteasomal ATPase complex.

Why is Gal4D hypersensitive to this stripping activity of the ATPase complex? We showed that a derivative of Gal4D, called mini-Cla Gal4D, is not monoubiquitylated efficiently in vitro (Fig. 2A). Moreover, ChIP analysis of Gal4 and Gal4D promoter binding in vivo strongly suggested that the wild-type activator is ubiquitylated but that Gal4D is not. This is based on the finding that even when Gal4D is resident on the GAL promoter (in a sug1-1 strain), immunoprecipitation with an anti-ubiquitin antibody fails to enrich the GAL promoters. In contrast, when wild-type Gal4 is resident on the promoter, strong co-immunoprecipitation of GAL promoters with ubiquitin was observed (Fig. 3). Taking into account these results and our previously reported work (17), we believe that monoubiquitylation acts to protect the activator from the stripping activity of the ATPase complex and that the inability of Gal4D to be monoubiquitylated is the root cause of its poor activity in vivo. This is further supported by the fact that genetic fusion of monoubiquitin to Gal4D significantly rescues its activity even in a SUG1 strain. These ideas are incorporated into the model shown in Fig. 6.

The data reported here and previous studies of Gal4 truncation mutants also reveal important and interesting new insights into the subdomain structure of acidic activation domains. Gal4-(1–841), which lacks the 34-amino acid AD entirely (see supplemental Fig. S3), is completely inactive in any strain background even when overexpressed (6). Gal4D (Gal4-(1–853) contains an additional 12 amino acids but lacks 22 residues of the classical 34-residue Gal4 AD (residues 854–875; note that the residues in native Gal4 that are C-terminal to the AD (876–881) are not important for activity (24–26)). Gal4D is a potent activator when its promoter occupancy is rescued by suppressing mutations in SUG1 or SUG2 or by massive overexpression, displaying about 60% of the activity of wild-type Gal4 (i.e. ~600-fold activation of GAL1) (5, 6). This shows that the 12-amino acid peptide present in Gal4D, but absent in Gal4-(1–841), is the real core activation domain and that it must be competent to interact with coactivators Sug1/Rpt6 and Sug2/Rpt4 (this study) and with whatever other factors are critical targets for activated transcription. The fact that Gal4D is not monoubiquitylated efficiently, whereas Gal4 is, shows that the 22-amino acid C-terminal region of the AD is largely if not completely involved in mediating ubiquitylation of the activator. There have been previous reports of the partial overlap of ADs and degrons in activators such as Myc (27), but this finding is somewhat different in that the C-terminal region of the Gal4 AD signals monoubiquitylation rather than Lys-48-linked polyubiquitylation and thus is not a degron.

Moreover, we found that a synthetic peptide containing the 22 residues of the Gal4 AD CTR (Fig. 2) significantly inhibited mini-Cla Gal4 and Gal4-VP16 ubiquitylation in HNE when added in excess (Fig. 2). In contrast, a synthetic peptide containing the 12 N-terminal residues of the AD present in Gal4D did not affect the efficiency of ubiquitylation. We propose that this peptide is likely to be a recognition site for the E3 ubiquitin-protein ligase(s) that target monoubiquitin to Gal4. More work
Gal4 Ubiquitylation in Vivo

FIGURE 6. A model for the activator monoubiquitylation-promoting function. The subdomains of the Gal4 activation domain determine the ability of Gal4 to occupy DNA and promote transcription. The AD CTR (red line, top panel) is responsible for recruiting the ubiquitylation machinery, which monoubiquitylates the activator in the DNA binding domain. The AD NTR (black line, top panel) recruits the transcriptional machinery and binds to the proteasomal ATPases Rpt4 and Rpt6. Gal4 is able to recruit the ubiquitylation machinery (top panel), which leads to the monoubiquitylation that protects it from destabilization due to the interaction with the proteasomal ATPases (middle panel). This allows Gal4 to promote transcription (bottom panel, right side). Gal4D lacks the AD CTR, and the lack of monoubiquitylation allows it to be destabilized from DNA as a consequence of interaction with the proteasomal ATPases and prevents transcription (bottom panel, left side). However, mutations that reduce destabilization activity (such as sug1-1) allow Gal4D to occupy DNA and promote transcription even in the absence of monoubiquitylation.

will be required to determine the residues in the CTR peptide that are required for this activity, but once this is accomplished the Gal4 CTR AD peptide and a suitable inactive mutant may be valuable tools for the affinity purification of the ligase, which has thus far eluded identification.

Another important insight provided by these data is that the dramatic difference between the activity of Gal4 and Gal4D in the sug1-1 background (>30-fold) and the modest difference in activity in the sug1-1 strain (<2-fold) argue that the major effect, by far, of Gal4 monoubiquitylation is to protect the activator from ATPase-mediated stripping. Any effect of this modification downstream of that event must not contribute more than a small degree to the overall activity of the activator. However, this conclusion may or may not be generalized to other activators, a point that will obviously require more investigation to address. For example, there is a report that monoubiquitylation of the artificial activator LexA-VP16 results in more efficient recruitment of the elongation factor P-TEFb (28).

The lack of detectable ubiquitylation of Gal4D provides a reasonable explanation for its lack of activity in the context of a model that incorporates destabilization by the proteasomal ATPases. This claim would be strengthened if one could force the ubiquitylation of Gal4D and demonstrate that modification would rescue activity. The identification of the ligase responsible is still under investigation, so we turned to other methods. Genetic fusion of ubiquitin has been used for other activators to argue for the importance of the modification (17, 22). A genetic fusion of monoubiquitin to Gal4D will partially rescue DNA occupancy and transcriptional activity of Gal4D in vivo (Fig. 4). This is the final piece of data that strongly supports the model that monoubiquitylation of Gal4 is required for DNA occupancy and activity.

In summary, we have deduced the molecular basis of the defect in the truncation mutant Gal4D as well as its rescue by the sug1-1 mutation. These experiments have revealed a novel subdomain structure of the Gal4 AD and shed important new light on the process of activator monoubiquitylation.

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