Isolation and Characterization of SUG2

A NOVEL ATPase FAMILY COMPONENT OF THE YEAST 26 S PROTEASOME*

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Using a genetic strategy designed to find proteins involved in the function of the Saccharomyces cerevisiae transcriptional activator GAL4, we isolated mutants in two genes which rescue a class of gal4 activation domain mutants. One of these genes, SUG1, encodes a member of a large family of putative ATPases, the Conserved ATPase Domain (CAD) proteins (also known as AAA proteins) that are involved in a wide variety of cellular functions. Subsequently, SUG1 was identified as a subunit of the 26 S proteasome. We have now cloned the gene defined by the second complementation group, SUG2 encodes an essential 49-kDa protein that is also a member of the CAD family and is 43% identical to SUG1. The mutation in sug2-1, like that in sug1-1, is found in the CAD near the highly conserved ATPase motif. We present biochemical and genetic evidence that SUG2 is associated in vivo with SUG1 and is a novel CAD protein subunit of the 26 S proteasome. With its highly conserved mammalian homologs, human p42 and ground squirrel CADp44, SUG2 defines a new class of proteasomal CAD proteins.

The GAL4 protein of Saccharomyces cerevisiae is responsible for a very large stimulation (~1000-fold) in the transcription of genes required for galactose metabolism under inducing conditions. The N-terminal 97 amino acids of GAL4 encode the DNA binding domain, while activation function and interaction with the negative regulator GAL80 map to 34 amino acids near the C terminus (1). Partial deletion of this activation domain in the gal4D and gal4-62 alleles leads to a defect in activation by GAL4 and an inability to grow on galactose as the sole carbon source (2, 3). A frameshift mutation of gal4-62 that changes the C-terminal amino acids of the truncated protein from FGITT to FMNV restores the ability to grow on galactose. While the truncated gal4D activation domain subunit of the 26 S proteasome. With its highly conserved mammalian homologs, human p42 and ground squirrel CADp44, SUG2 defines a new class of proteasomal CAD proteins.

This strategy yielded mutants in two complementation groups (2). One of these, sug1-1, had been isolated independently by Matsumoto et al. (3) as a suppressor for galactose (sug) of the gal4-62 allele and was presumed to be an information suppressor. However, sug1-1 suppresses gal4D, which terminates at the same amino acid as the gal4-62 allele with a frameshift and multiple stop codons, arguing that sug1-1 does not act as an information suppressor.

We have previously characterized the sug1-1 allele. It is able to suppress a variety of mutations in the activation domain of GAL4, short of its complete deletion, but is unable to suppress mutations in other regions of the protein. The SUG1 gene was cloned and sequenced and was one of the first identified members of a large family of proteins that contain a highly conserved 220 amino acid motif. We have referred to this as the CAD (Conserved ATPase-containing Domain) (2) because one of its most highly conserved features is a Walker-type nucleotide binding motif. Proteins that share this motif are also collectively known as the AAA family (6). They are involved in a remarkably wide range of cellular processes, including vesicle fusion, proteolysis, peroxisomal, and mitochondrial biogenesis and transcription. The basic function of the CAD is still unknown.

Although sug1 was originally identified as a suppressor of transcriptional defects, subsequent studies suggested that SUG1 is a component of the 26 S proteasome (7–9), a large multiprotein complex that degrades proteins targeted for degradation by the ubiquitin pathway in an ATP-dependent fashion. It is composed of at least two functionally interdependent parts; the 20 S, or core catalytic subunit of the proteasome that can act as a peptidase in isolation, and a 19 S regulatory subunit (also called the PA700 (10)) that is required for degradation of proteins (reviewed in Refs. 11–13). A screen for suppressors of the cdc28N-1 mutation in yeast yielded a sug1 mutation (cim3) along with a mutation in a known component of the 26 S proteasome, CIM5 (14). Both the cim5 mutation and the cim3 mutations affect the half-life of some proteasomal substrates in vivo (14, 15). More convincingly, a protein nearly identical to the human homolog of SUG1 (16) was biochemically isolated as a component of PA700, a complex that stimulates the activity of 20 S proteasome purified from bovine red blood cells (17). Subsequently, it has been physically demonstrated that yeast SUG1 is a component of the 26 S proteasome (7, 9), a localization it shares with four other CAD proteins (listed as homologous pairs, yeast/human): YTA5/S4 (18, 19), CIM5/MISS1 (14, 20), YTA1/TBP1 (18, 21), and YTA2/TBP7 (18, 22). These proteins are postulated to act as “reverse chaperones,” unwinding protein substrates in an ATP-dependent manner for digestion by the 26 S proteasome (13, 23).

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yet understood.

Only two complementation groups were recovered in our selection for suppressors of gal4Δ. There were 14 independent isolates in each group, suggesting that the selection was saturated. Matsumoto et al. (3) found, and we have confirmed, that a mutation in the second complementation group, sug2-1, is synthetically lethal with sug1-1, suggesting that the two genes are functionally related and may be physically associated with each other. Therefore, we reasoned that characterization of SUG2 could be important to understanding proteasomal function and might provide insight into the mechanism of gal4Δ rescue.

We describe here cloning and characterization of the SUG2 gene and the identification of its protein product as a novel CAD family subunit of the yeast 26 S proteasome. Like SUG1, SUG2 has highly conserved mammalian homologs. With its human homolog p42 (24) and its functionally interchangeable ground squirrel homolog CADp44 (25), SUG2 defines a new, sixth class of proteasomal CAD proteins.

MATERIALS AND METHODS

Strains and Media—Yeast strains used were 21R (GALA4 GAL80 ura3-52 leu2-3,112 ade1-101) (26), Y302 (Δgala4 Δgala80 ura3-52 leu2-3,112 his3 ade2-101 trpl GAL1–lacZ) (27), Sc392 (a sug2-1 gal4Δ ura3 leu2-3,112 ade2 trpl1) (3), Y190 (Δgala4 Δgala80 his3 trp1-901 ade2-1 ura3-52 leu2-3,112 trpl1) (28), Sc342 (a W303 α/α ade2-1 ura3-1 his3-115 trpl-1 leu2-3,112 can1-100) (29), Sc527 (a sug2-1 gal4Δ ura3 leu2 MEL1 gal80) (30), Sc344 (W303 α/α ade2-1 ura3-1 his3-115 trpl-1 leu2-3,112 can1-100) (29), Sc527 (a sug2-1 gal4Δ ura3 leu2 MEL1 gal80) (30), Sc528 (a SUG2 gal4Δ ura3 leu2 MEL1 gal80) (30), Sc344 (W303 α/α ade2-1 ura3-1 his3-115 trpl-1 leu2-3,112 can1-100) (29), Sc527 (a sug2-1 gal4Δ ura3 leu2 MEL1 gal80) (30), Sc342 (pYEP24 (36) allowed moderate expression of SUG2 along with a URA3 marker on integrating vector pRS306 (31)). The genomic SUG2 gene of Sc342 was tagged at the N terminus with the 11-amino acid T7 (S10) epitope tag (Novagen) to produce SUG2:Novel ATPase Family Component of Yeast 26 S Proteasome

The yeast centromeric (single copy) vector YCP50 (35) was used to express physiological amounts of proteins, and the 2-μm (multicopy) pYEP24 (36) allowed moderate overexpression. pMTL1 (a gift of C. Girox) was used to express proteins under the GAL1-10 promoter. Plasmid pRS306 (31) was used as integrating vector for SUG2. Vent polymerase (New England BioLabs) was used for all PCR (2). DNA manipulations were done according to standard protocols (37).

For mapping the putative SUG2 clone to the sug2-1 locus, a PCR fragment (1.58 kb) from the genome containing the entire SUG2 gene as well as 5′- and 3′-untranslated regions was blunted-end cloned into the Smal site of pUC18. By using Clal sites in the oligonucleotides, the SUG2 fragment containing (from 210 base pairs 5′ of the ATG to 41 base pairs 3′ of the stop codon) was subcloned to integrating vector pRS306 carrying a URA3 marker. The recombinant plasmid was linearized within the gene at the single BgIII site and transformed into Sc392. Genomic DNA was prepared from 2 Ura+ isolates. The locus of integration was confirmed by diagnostic PCR using one oligonucleotide in the genome and another in the URA3 gene.

The SUG2 disruption construct was generated by replacing the 1.05-kb Stu1-Bcl1 fragment (leaving 54 amino acids at the N terminus and 29 amino acids at the C terminus) of SUG2 in pUC118 with a 1.1-kb Sma1-BamH1 fragment containing the URA3 gene by blunt cloning. The SUG2-UARA3 fragment was removed by Clal digestion and transformed into Sc342 carrying pMTL1-SUG2 (SUG2 under GAL1-10 control). Successful integration events were selected on galactose plates lacking uracil and confirmed by diagnostic PCR. Site-directed mutagenesis was performed with the Sculptor kit (Amersham Corp.). pH6-SUG2 was constructed by amplifying the open reading frame of the SUG2 gene by PCR with a 5′ primer that produced an NcoI site at the 3′ end of the product. The product was subcloned into the expression plasmid H6-pQ6E0, a derivative of pQ6E0 (Qiagen), to produce pH6-SUG2 which encodes SUG2 tagged at the N terminus with six histidines (Met-Ala-His6-Ala-SUG2).

The sequence of SUG2 was determined by automated cycle sequencing using fluorescently tagged terminator base analogs (Applied Biosystems Inc.). Comparison of the SUG2 sequence with other CAD proteins in the GenEMBL combined data base was done at the National Center for Biotechnology Information using the BLAST network service. Percent identity values for pairwise comparisons of CAD proteins were calculated using the GAP program, and the dendrogram of yeast CAD proteins was generated using the Pileup program in the Wisconsin Sequence Analysis Package (Genetics Computer Group).

β-Galactosidase Activity—β-Galactosidase assays were performed at 37°C on 300 μl sample buffer.

Generation of Antibodies—SUG2 tagged at the N terminus was produced in bacterial strain BL21 (DE3) carrying pREP4 and pH6-SUG2 and was isolated as inclusion bodies. This protein was approximately 50% pure as estimated after SDS-PAGE and Coomassie Blue staining (data not shown) and was further purified by preparative SDS-PAGE.

The H6-SUG2 band was cut out of the gel, and a suspension of finely macerated acrylamide in phosphate-buffered saline containing purified H6-SUG2 along with an equal mass of Adjuprime (Pierce) was used for all inoculations. After obtaining preimmune serum, antibodies were raised in mice by standard methods (40). Extracts for testing the specificity of the SUG2 antibody were prepared by directly boiling yeast grown in yeast extract peptone glucose medium and harvested at an A600nm of 1.0 in 2 × SDS sample buffer.

Preparation of Yeast Extract and Gel Filtration Chromatography—Two liters of yeast strain SC507 (wild type for GAL4, SUG1, and SUG2) were grown at 30°C to an A600 of 1.0 in yeast medium with 2% glucose as the carbon source. The cells were harvested by centrifugation, washed once with cold water and re-centrifuged, then resuspended in 3 ml/g wet weight SCED (1.2 M sorbitol, 0.1 M sodium chlorate, pH 5.8, 60 mM EDTA, 5 mM dithiothreitol). 50 μl of β-glucuronidase (Sigma) and 1 mg of zymolase 20T (Seikagaku Corp.) was added per g wet weight of cells. The suspension of cells was shaken at 150 rpm in an air incubator at 30°C until completely spheroplasted (30–45 min). The spheroplasts were centrifuged at approximately 700 × g at 4°C for 5 min and then gently resuspended in cold 1.2 M sorbitol and recentrifuged. This washing step was repeated. The pellet spheroplasts were then resuspended in an equal volume of column buffer A (20 mM Tris at pH 8, 2 mM diethiothreitol, 20 mM potassium acetate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 0.0045 mM ATP, and 20% glycerol) made without the glycerol and incubated on ice for 5 min resulting in lysis of the spheroplasts. One spheroplast pellet volume of column buffer A containing 60% glycerol was then added to give a final concentration of glycerol of approximately 20%.

The viscous extract was sonicated for seven bursts of 1 s using the microprobe of a Branson Sonifier 450 and then centrifuged at 35,000 × g for 45 min at 4°C. The supernatant was transferred to a fresh tube and resupplemented. The cleared extract was then diluted to 10 mg/ml protein with column buffer A. 10 ml (100 mg) was fractionated on a 2.5 × 100 cm Sphacyl S400 HR (Pharmacia Biotech Inc.) gel filtration column running at 1 ml/min at 4°C, and 15 ml fractions were taken. The remaining of the extract and all fractions were snap-frozen in liquid nitrogen in aliquots.

10 ml from 26 S peak fraction 8 of the crude extract fractionation was depleted of ATP by treatment with 4 units of apyrase type 5 (Sigma) for 1 h on ice. An appropriate amount of 2 mM KC1 was added to bring the fraction to 300 mM KC1, and it was then refractionated on the S400 HR column in column buffer B (20 mM Tris, pH 8, 2 mM diethiothreitol, 20 μM EDTA). The fractions containing SUG2 were pooled, dialyzed against buffer A, and then fractionated on a 20 × 100 cm Sphacyl S400 HR gel filtration column running at 1 ml/min at 4°C, and 15 ml fractions were taken. The remaining of the extract and all fractions were snap-frozen in liquid nitrogen in aliquots.

The abbreviations used are: PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; AMC, 7-amino-4-methylcoumarin; kb, kilobase pairs; 2-DG, 2-deoxyglyceraldehyde; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
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mm potassium acetate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 300 μM βME.

The Sephacryl S400 HR column was calibrated with the following standards (Sigma) in column buffer A: dextran blue, thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylose (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

Proteasome peptidase activity was assayed, in the absence or presence of 0.05% SDS, against Suc-Leu-Leu-Val-Tyr-AMC (Bachem) as described (41). Protein concentrations were determined with the Bio-Rad protein assay using bovine serum albumin as the standard.

Fractions were separated by SDS-PAGE and blotted to polyvinylidene difluoride. Tagged SUG1 was detected with a monoclonal antibody to the T7 S10 epitope (Novagen). SUG2 was detected with antiserum against the yeast enzyme generously provided by K. Tanaka. ImmunobLOTS were visualized using horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs (Pierce), the Renaissance chemiluminescent detection system (Amersham Corp.), and x-ray film (Amersham Corp.).

Immunoprecipitations—Fixed protein A+ S. aureus cells were purchased from Boehringer Mannheim and prepared as suggested by Mark Biggin. A 10% suspension of the cells was washed three times with 1 volume of TES (50 mM Tris, pH 8, 2 mM EDTA, 0.2% Sarcosyl). They were then resuspended in 2 volumes of phosphate-buffered saline supplemented with 3% SDS and 10% β-mercaptoethanol and incubated in boiling water for 30 min. After washing two times in TES, cells were frozen aliquoted at −80°C as a 20% (w/v) suspension in TES until use.

To load cells with antibody, 100 μl of cell suspension was washed three times with 1 ml of NET buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris, pH 8) and then incubated with 40 μl of anti-yeast 20S proteasome or preimmune serum in a total volume of 1 ml of NET for 2 h at 4°C with inversion. Cells were washed three times with 1 ml of NET buffer, taken up to a final concentration of 10% w/v in immunoprecipitation buffer, and stored at −80°C.

Samples were pre-cleared before use in immunoprecipitation reactions. Extract or column fractions were diluted to a final volume of 400 μl with IP buffer (the appropriate column buffer supplemented with 0.05% Nonidet P-40, 5 mM MgCl₂, 2 mM ATP, and 0.5 mM EDTA). 20 μl of 20% unladen protein A+ S. aureus cells were added, and the reaction was incubated at 4°C for 1 h and then spun at 12,000 rpm in a microcentrifuge. The supernatant was used as the input for immunoprecipitations.

For immunoprecipitations, 20 μl of loaded S. aureus cells were added to the pre-cleared extract and incubated at 4°C for 1 h. After gently pelleting the cells (7,500 rpm in a microcentrifuge), the supernatant was removed and concentrated by precipitation with 10% trichloroacetic acid using 150 μg/ml deoxycholate as carrier. The pellet was then subjected to three 5-min washes with 1 ml of IP buffer. Both the concentrated supernatant proteins and the pellet were resuspended in equal volumes of 1× SDS-PAGE loading buffer supplemented with 6 M urea and boiled for 5 min. Equal aliquots of the samples were separated on a 10% Tricine SDS-polyacrylamide gel (42) and transferred to a polyvinylidene difluoride membrane, and detected by Western blotting. The 20S proteasome was detected in immunoprecipitations using the anti-20S antibody that had been biotinylated using Biotin (Long Arm) NHS i.e.

RESULTS

Cloning of SUG2 by Complementation—A strain relying on gal4-69 (which, like gal4-62, encodes a protein truncated at amino acid 853) for activation of genes in the GAL regulon is unable to grow on galactose as the sole carbon source (Gal−). In order to isolate the dominant, wild type SUG2 gene, we transformed Sc392 (a sug2-1 gal4-69 strain) with multi-copy and single-copy genomic libraries.

For each library approximately 10,000 transformants were subjected to three 5-min washes with 1 ml of IP buffer. Both the concentrated supernatant proteins and the pellet were resuspended in equal volumes of 1× SDS-PAGE loading buffer supplemented with 6 M urea and boiled for 5 min. Equal aliquots of the samples were separated on a 10% Tricine SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and detected by Western blotting. The 20S proteasome was detected in immunoprecipitations using the anti-20S antibody that had been biotinylated using Biotin (Long Arm) NHS i.e.

Identity: 43%

CAD Identity: 54%

Fig. 1. Comparison of SUG2 and SUG1 protein sequences showing 43% identity overall and 54% identity within the CAD. The CAD region is underlined. The core Walker motif is in bold. Vertical lines indicate identity. Colon and periods indicate stronger and weaker similarity of amino acids, respectively. The EMBL accession number for SUG2 is U43720.
was integrated into a sug2-1 strain. A clone of the novel CAD gene on the URA3 integration vector pRS306 was cut within the CAD open reading frame and transformed into Sc392 (a sug2-1 gal4-69). All 12 integrants selected for uracil prototrophy were Gal- Integration of this fragment diminished gal4-69 activity to a level equal to that found in a SUG2 wild type background (Table I), consistent with integration at the sug2-1 locus and generation of one wild type copy of SUG2 by recombination. To further determine that the novel CAD gene maps to the sug2-1 locus, we performed crosses and random spore analysis. A strain in which the URA3 gene was targeted to the novel CAD protein locus by integration was crossed with a mating type switched derivative of the parental strain, Sc527 (a sug2-1 gal4-69) carrying a LEU2 marker plasmid (to allow selection of diploids). Diploids selected on glucose medium lacking uracil and leucine were used for random spore analysis. A total of 49 spores were assayed for Ura, Gal, and 2-DG phenotypes. 21 of the 49 spores were Ura+. All of the Ura+ spores were Gal'/2-DG' (no complementation of gal4-69), and all Ura- spores were Gal'/2-DG-, verifying that the URA3 marker integrated at or very close to the sug2-1 locus. We conclude that sug2-1 is an allele of the CAD gene isolated, hereafter called SUG2.

SUG2 is an Essential Gene—SUG1 (2) and other tested yeast proteosomal CAD proteins (YTA1, YTA2 (18), and CIM5 (14)) are encoded by essential genes. To test whether SUG2 is also an essential gene, we generated a disruption construct in which all of the predicted SUG2 open reading frame except the 54 N-terminal and 29 C-terminal amino acids was replaced with the URA3 gene. This construct was used in a one-step disruption protocol by digesting at restriction sites flanking N-terminal and 29 C-terminal amino acids was replaced with all of the predicted an essential gene, we generated a disruption construct in which as expected if sug2-1 were to supply the yeast homolog of the 26S proteasomal subunit TBPF (22).

Characterization of the sug2-1 Mutation—The mutation responsible for the rescue of gal4D by sug1-1 is the change of conserved glycine 214 in the CAD to aspartate. This finding suggests that an alteration in ATPase function may be responsible for the sug1-1 phenotype (2). A similar mutation in sug2-1 would suggest that sug1-1 and sug2-1 might share a common mechanism for suppression of truncated GAL4.

In order to identify the sug2-1 mutation, we amplified the mutant (from Sc392) and wild type (from Sc311) SUG2 alleles in two pieces from genomic DNA by PCR and entirely sequenced them. Two differences between the wild type clone and sug2-1 were found. One coded for an alanine to valine substitution at amino acid 56, the other for a glutamic acid to lysine substitution at amino acid 300. Glutamate 300 is near the Walker nucleotide binding motif of the CAD and is conserved in most members of this family of proteins, whereas alanine 56 is outside of the CAD region.

Site-directed mutagenesis was used to insert the E300K mutation into the wild type sequence. The synthetic E300K allele was used in a one-step gene replacement of SUG2 in YJ02 (AG4AL4) carrying gal4D on a plasmid. Transformants were screened for the ability to grow on galactose. Sequencing of the SUG2 allele from one of these GAL alleles isolated revealed only the E300K mutation, demonstrating that this mutation is sufficient for suppression of the gal4D phenotype. We conclude that the SUG2 mutation responsible for the rescue of gal4-69 is a glutamate to lysine alteration within the CAD region (Fig. 1).

Two-hybrid Interaction between SUG2 and SUG1—The sug1-1 and sug2-1 mutations have similar phenotypes, and the combination of these two alleles is lethal (3). This synthetic lethality suggests that these two proteins may physically interact with each other in a protein complex that is essential for viability. Two-hybrid analysis (28) provided additional evidence of SUG1-SUG2 interaction. SUG1 fused to the DNA binding domain of GAL4 was used as bait in a screen for cDNAs encoding SUG1 interacting proteins. Approximately 100,000 transformants were screened yielding 8 clones that displayed significant reporter gene activity. Plasmids were isolated from these isolates and colony-purified in E. coli. Upon re-transformation, 5 of these remained positive. Each of these was further tested and eliminated from consideration if they could activate transcription in collaboration with nonspecific GAL4 DNA binding domain fusions. Three bona fide interacting clones were isolated. Upon sequencing, one of these was found to be identical to SUG2, reinforcing the conclusion that SUG1 and SUG2 associate with each other in vivo (Table I). The other two clones isolated in this screen encoded YTA2 (18) (data not shown), the yeast homolog of the 26S proteasomal subunit TBPF (22).
cally localized SUG2. Antibodies were generated to recombinant SUG2 produced in E. coli. These antibodies detected a single protein with an apparent molecular mass of approximately 60 kDa in Western blots of crude yeast extract. A larger immunoreactive band was seen in a strain in which SUG2 is increased in size by the addition of a peptide tag, demonstrating the specificity of the antibody which was used for detection of SUG2 in the following experiments (Fig. 3).

Immunoprecipitations using antibodies raised against the purified 20S proteasome were performed on crude yeast extract. Both SUG2 and SUG1 co-immunoprecipitated with subunits of the 20S proteasome but were not precipitated by pre-immune serum (Fig. 4A). To determine the size of the complex containing SUG2, SUG1, and 20S subunits, an aliquot of the extract was separated on a Sephacryl S400 HR gel filtration column. SUG2 co-fractionated with SUG1, subunits of the catalytic 20S core of the 26S proteasome and 20S peptidase activity, with the peak at a molecular mass of approximately 2000 kDa, consistent with the size of the 26S proteasome (8, 9, 13) (Fig. 4B). To confirm that SUG2 was associated with SUG1 and 20S subunits in these fractions, we performed immunoprecipitations. SUG1 and SUG2 co-immunoprecipitated with antibodies against the 20S proteasome in both peak fractions (Fig. 4C). These results imply that, like SUG1 (7, 9, 17), SUG2 is a component of the 26S proteasome.

Increased dissociation of SUG1 and SUG2 from 20S proteins during immunoprecipitations in fraction 8 versus fraction 7 may reflect heterogeneity in the 26S peak.

The 19S/PA700 regulator of the proteasome can be dissociated from the 20S core of the 26S proteasome by depletion of ATP and/or high salt treatment (44). 26S peak fraction 8 was treated with apyrase to deplete it of ATP and brought to 300 mM KCl. The treated fraction was then re-fractionated on the S400 HR gel filtration column equilibrated with buffer B which contains no ATP, no glycerol, and 300 mM KCl. SUG1, SUG2, and 20S subunits still co-fractionated but at a much smaller apparent molecular mass. Since both the 20S proteasome and the 19S/PA700 are estimated to have very similar molecular masses of approximately 700 kDa (13, 45), this is consistent with the splitting of

\[ \text{G. DeMartino, personal communication.} \]
the 26 S proteasome into 20 S and 19S/PA700 components under these conditions (Fig. 5A). Addition of 0.05% SDS to peptide assays of column fractions stimulated the activity of the ~500-kDa peak fraction approximately 14-fold. Stimulation by SDS is characteristic of 20 S proteasome not associated with activating proteins (44). We believe that an estimated molecular mass of approximately 500 kDa for proteins in this fraction by comparison to molecular weight standards is consistent with this interpretation within experimental error. Immunoprecipitation experiments show that in these fractions SUG2 and SUG1 no longer co-immunoprecipitate with 20 S proteasome subunits (Fig. 5B) consistent with co-migration of dissociated 19S/PA700 and 20 S proteasome because of their similar molecular masses. Considering the two hybrid and biochemical data together, we conclude that SUG2 is physically associated with SUG1 as a component of the 19S/PA700 regulatory complex of the yeast 26 S proteasome.

**DISCUSSION**

We have cloned and characterized SUG2, an essential gene of the CAD family in yeast. A mutation in SUG2 that rescues a partial deletion of the activation domain of the GAL4 maps to the CAD of SUG2, as does a mutation in SUG1 which has the same phenotype. Like the closely related SUG1, our data show that SUG2 is a component of the 19S/PA700 regulatory cap of the 26 S proteasome.

Comparison of the SUG2 sequence with all other CAD proteins reveals that, as expected, it is most related to the other yeast family members localized to the 19S/PA700 subunit of the 26 S proteasome (Fig. 6). However, generation of dendrogram for this subset of CAD proteins from all species (see Fig. 4 in Ref. 25) reveals that SUG2 can be classified as a member of a novel subgroup. In the same class are its recently cloned human and ground squirrel homologs p42 and CADp44, respectively. Although the human p42 (24) and ground squirrel CADp44 (25) are 48 and 49 amino acids shorter at the N terminus than SUG2, they are 65 and 67% identical, respectively, over the region they share. Despite its N-terminal truncation compared with yeast SUG2, CADp44 is able to complement the deletion of SUG2 in yeast (25). This is another example of the remarkable functional conservation of these proteins and an indication that the N terminus of SUG2 may not be required for its essential function(s). The identities between SUG2 and its mammalian homologs are similar to those found between yeast SUG1 and its human homolog, TRIP1 (76%), which can functionally replace SUG1 in yeast (16).

Besides the 200-amino acid region of homology that all proteins of this family share, all of the previously identified proteasomal CAD proteins except for YTA5/S4 are also notable for the presence in their N-terminal region of a strongly predicted coiled-coil region of approximately 42 amino acids, or 6 repeats of the 7 amino acid motif (Fig. 7) (13). SUG2 also has a strongly predicted coiled-coil motif of 43 amino acids. The significance of this motif is not clear, but it has been proposed to play a structural role in the assembly of the PA700 regulatory complex. It could also potentially play a role in the attachment of the PA700 to the α subunits of the 20 S proteasome. Notably,
both human and ground squirrel homologs of SUG2 also have predicted coiled coil domains despite their N-terminal truncation relative to SUG2 (not shown).

DeMartino et al. (24) have shown that the apparent bovine homolog of SUG2, p42, is present in both the PA700/19S and a separate complex, the modulator, which is able to further stimulate the 20 S proteasome's proteolytic activity in a PA700-dependent fashion. The modulator is a 300-kDa complex that contains two additional proteins, TBP1, present in the PA700 as well, and a novel 27-kDa protein of unknown function that is associated with modulator. Likewise, a 300-kDa complex would be expected to migrate very near the 19S/PA700 on our column, so that the tail of the SUG2 peak in Fig. 5A could correspond to modulator-associated SUG2. In order to determine if there is a yeast modulator, it will be important to follow a modulator component that is not a subunit of the PA700/19S.

SUG2 is the second CAD protein in the regulatory subunit of the 26 S proteasome to be identified by mutations that rescue the function of weak transcriptional activators. The discovery that SUG2, like SUG1, encodes a protein of the proteasomal regulatory subunit helps to explain the synthetic lethality of sug1-1 and sug2-1 but emphasizes the paradox of proteasomal proteins having strong effects on transcription. It has been proposed that proteasomal CAD proteins are responsible for unwinding substrates for transport into the interior of the 20 S proteasome for degradation (13, 23). An obvious hypothesis to explain the rescue of gal4D by sug1-1 and sug2-1 is that altered proteolysis as a result of these mutations leads to an accumulation of the activators, compensating for their decreased potency with increased concentration. It is also possible that accumulation of another protein or proteins due to defective proteolysis may be responsible for amplifying the residual activity of gal4D. If this is true, the genetics of gal4D suppression may provide clues as to the substrate specificity of proteasomal CAD proteins. Multiple alleles of SUG1 and SUG2, and no mutants in other 19S/PA700 CAD proteins, were isolated by the selection for suppressors of gal4D, implying that proteasomal CAD proteins may have overlapping but non-identical protein substrate specificities. Therefore, the presence of six CAD proteins in the PA700 may reflect a requirement for recognizing a wide variety of cellular proteins with varying primary and secondary structure to be unfolded for degradation.

An alternative to mechanisms involving alterations in the proteolytic activity of the 26 S is that the rescue of gal4D may be due to changes in the proposed "reverse chaperone" activity of SUG1 and SUG2 on transcription factors or complexes. In this regard we note that while under our conditions almost all of the 19S/PA700 proteins can be found in association with the 20 S proteasome, an earlier report (46) found that unusual growth conditions were required to stabilize the 19S-20 S interaction. Given the dependence of the stability of 26 S to extract and growth conditions, and the possibility that other activators of the 20 S proteasome may compete with 19S/PA700 for binding to the 20 S, we feel that the extent to which yeast 19S/PA700 is associated with the 20 S proteasome in vivo is still not clear. Thus it is possible that there is a significant population of free PA700 which might perform "reverse chaperone" activity independent of the 20 S proteasome.

Regardless of mechanism, the phenotypes of SUG2 and SUG1 mutants point to an interesting relationship between transcriptional regulation and protein degradation.

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