A Complex Composed of Tup1 and Ssn6 Represses Transcription in Vitro*

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The Saccharomyces cerevisiae Tup1 protein is a member of a family of WD repeat containing proteins that are involved in repression of transcription. Tup1, along with the Ssn6 protein, represses a wide variety of genes in yeast including cell type-specific and glucose-repressed genes. Tup1 and Ssn6 are recruited to these specific gene sets by interaction with sequence-specific DNA binding proteins. In this work, a protein complex containing Ssn6 and Tup1 was purified to determine its composition. The size of the complex is estimated to be 440 kDa. Tup1 and Ssn6, which are both phosphoproteins, are the only proteins present in stoichiometric amounts in the complex. We also demonstrate that this purified complex represses transcription in an in vitro assay.

The Tup1 protein of Saccharomyces cerevisiae is one of a family of repressor proteins that contain β-transducin or WD repeats. The majority of the WD repeat containing proteins are homologs of β-transducin and are known to function in signal transduction pathways within the cytoplasm. However, an increasing number of WD repeat proteins have been identified that are nuclear localized and function in the repression of transcription. These include Tup1, Hir1, and Met30 in S. cerevisiae; SCON2 in Neurospora crassa; extra sex combs and groucho in Drosophila; COP1 in Arabidopsis thaliana; and HIRA and the family of TLE proteins in humans (1–10). These WD repeat repressor proteins turn off a wide variety of genes, including those involved in segmentation, sex determination, and neurogenesis (controlled by groucho) and those involved in photomorphogenesis (controlled by COP1) (7, 11). The HIRA protein has been implicated in the human developmental disease DiGeorge syndrome (8, 9).

Of these WD repeat repressor proteins, Tup1 is the best characterized. Tup1 along with another protein, Ssn6, is required for the repression of at least five sets of genes in yeast, including the glucose-repressed genes, genes regulated by the presence of oxygen (hypoxic genes), the α-specific and haploid-specific genes, and a set of genes induced by DNA damage (12–16). A deletion of SSN6 or TUP1 results in the constitutive expression of all of these genes sets. Tup1 and Ssn6 are recruited to these specific gene sets by interaction with sequence-specific DNA binding proteins. In the case of the α-specific and haploid-specific genes in yeast, the homeodomain protein α2 binds to sequences (operators) located upstream of each gene in the set and recruits Ssn6 and Tup1 by direct interaction with each of these proteins (for review, see Ref. 17).

Tup1 and Ssn6 interact directly in vitro and are found associated in a large complex in yeast extracts estimated at 1.2 MDa (18). The size of this complex suggests that it consists of many protein subunits. Genetic experiments have implicated a number of additional proteins in the Ssn6-Tup1 repression pathway including Rox3, Sin4, Srb8, Srb9, Srb10, and Srb11 (19–23). Each of these proteins is required for full repression of transcription by Tup1 and Ssn6 in vivo. To determine the subunit composition of the Ssn6-Tup1 complex, we purified the complex, determined its size, characterized its components, and demonstrated its activity in an in vitro repression assay.

EXPERIMENTAL PROCEDURES

Expression Vectors—The Ssn6HIS construct is comprised of the 3′-1000 base pairs of the SSN6 coding sequence fused to 6 histidine codons followed by a stop codon in the vector pRS304 (24). The final construct was sequenced (U. S. Biochemical Corp.) and integrated into the yeast genome at the native SSN6 locus resulting in the SSN6HIS fusion gene and a duplication of the last 1000 nucleotides of the SSN6 gene. Proper integration was confirmed by PCR. The glutathione S-transferase (GST)-Ssn6* includes the entire Ssn6 coding sequence fused with the GST coding sequence in pBS316-GAL1-GST. 5 Ssn6HIS function was checked by introducing the construct into α and a strains bearing the mfa2::lacZ reporter. Repression was measured by 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside filter β-galactosidase assay (25). Standard mating assays were also performed to assay complementation of the ssn6Δ a-mating defect (26). Gal1-GST-Ssn6 function was checked by introducing the plasmid into an ssn6Δ a cells and assaying mating in media containing 2% galactose.

Yeast Strains and Growth Conditions—The protease-deficient strains BJ5459, MATα, ura3–52 trp1 leu2–801 his3–Δ1 hisΔ200 pep4::HIS3 prb1Δ1.6R can1 (27), and FM135, MATα, leu2–3,112 ura3–52 prb1–112 pep4–3 reg1–501 gal1 (provided by R. Deshaies, Cal-Tech), were used for the purification of Ssn6HIS and GST-Ssn6 proteins, respectively. FM135 cells harboring the GST-SSN6 plasmid were grown in 3 liters of SD minus uracil, minus leucine, plus 2% galactose to an A600 of 0.5. The reg1–501 and gal1 mutations allow growth and induction in glucose and galactose (28). This culture was then used to inoculate 60 liters of YEFP plus 2% galactose in a 60-liter fermentor. Cells were grown until they reached an A600 of 2.4 and then harvested by filtration. Cells containing the Ssn6HIS expression vector were grown in 40 liters of YEFP in the fermentor until they reached an A600 of 2.

Yeast strains SM1196 and SM1179 containing mfa2::lacZ were used to monitor the function of the Ssn6HIS construct and have been described (29). Strains EG123 MATα trp1 leu2 uro3 gal2 and 246–1–1 MATα trp1 leu2 uro3 gal2 transformed with the Ssn6HIS construct were used for mating assays. The strain YCK12 MATα ssn6Δ trp1 leu2 uro3 gal2 containing the GST-SSN6 plasmid was used for mating assays (15).

Antibodies—Ssn6 and Tup1 antibodies were made against GST fusion proteins. The GST-Ssn6 fusion contains most of the tetrasricopep-

1 The abbreviations used are: GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline plus 5% milk and 0.2% Tween; TPR, tetrasricopeptidase repeat.

2 R. Deshaies, unpublished data.

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Polypeptide-encoding portion of Ssn6 fusions were lysed with glass beads (Biosearch Products Inc.) in an equal volume of 2 × lysis buffer (1 M NaCl, 100 mM HEPES, pH 7.9, 20% glycerol, 2% Triton X-100, plus the protease inhibitors 10 mM benzamidine, 2 mM phenylmethylsulfonfyl fluoride, and 2 μg/ml each of bestatin, pepstatin, and leupeptin). Lysed cells were then centrifuged at 33,000 rpm in a type 55 rotor at 1 h at 4 °C. The supernatant was collected and 20% glycerol (Fina) was added. This mixture was rocked for 2.5 h at 4 °C, after which it was poured into a column. The resin was washed with 40 ml of low salt buffer (100 mM NaCl, 20 mM Tris, pH 8.0, 10% glycerol, 0.1% Tween 20) and eluted with the same buffer plus 150 mM imidazole. The Ssn6HIS and Tup1 elution profiles were followed by a dot blot antibody assay. The peak fractions were pooled and loaded over a 5-m1 HiTrap-Q column (Pharmacia). Protein was eluted with a gradient of NaCl from 100 mM to 500 mM. The peak fractions were pooled, concentrated, and loaded over a Superose-6 column (Pharmacia) in 500 mM NaCl, 50 mM HEPES, pH 8.0, 10% glycerol, and 0.1% Tween 20. Fractions were collected, and samples from each were subjected to SDS-PAGE. The fractions bearing Ssn6HIS-Tup1 were identified by Western immunoblotting. The total yield was approximately 50 μg of Ssn6HIS-Tup1. The Stokes radius was determined by comparing the migration of Ssn6HIS-Tup1 through the Superose-6 column with that of molecular size standards (see below for the glycerol gradient) as in Siegel and Monty (32).

Cells harboring the GST-Ssn6 and Tup1 expression vectors were lysed with glass beads following the addition of an equal volume of 2 × lysis buffer (1 M NaCl, 100 mM HEPES, pH 7.9, 10 mM EDTA, 10 mM DTT, 20% glycerol, with protease inhibitors as above). The lysate was then spun at 7000 rpm in a SW50.1 rotor for 1 h to remove cellular debris. The supernatant was collected. (NH4)2SO4 was then added to 70%. The mixture was stirred on ice for 30 min. Protein was then loaded by a stepwise addition of 35%, 30%, 25%, 20%, and 15% glycerol, and 20 mM HEPES, pH 7.6, 10 mM EDTA, and 10 mM DTT. 15% glycerol. The column was eluted in the same plus 10% glutathione. The proteins were concentrated approximately 10-fold by Centricon (Amicon). The total yield of GST-Ssn6-Tup1 was approximately 200 μg.

**Immunoblotting**—Polymeraseattached to peroxidase obtained by electrophoresis to polyvinylidene difluoride membrane. The membrane was blocked in Tris-buffered saline plus 5% milk (5% Tween (TBST)) for 1 h, then incubated with Ssn6 or Tup1 antibodies at 1:1000 dilution for 1 h. Membranes were washed 3 × in TBST for 5 min. Anti-rabbit antibodies conjugated to horseradish peroxidase (Amersham Life Sciences, Inc.) were then incubated with the filters in TBST at a dilution of 1:10,000 for 1 h. Blots were washed 3 × in TBST for 10 min. The blots were then developed with ECL development reagents (Amersham Corp.) and exposed to Kodak XAR film. Dot blot assays were performed by dotting and column fraction on nitrocellulose followed by the above immunoblotting protocol.

**RESULTS**

**Purification of Ssn6-Tup1**—The large size of the complex in which Ssn6 and Tup1 are found suggests that it has many subunits. To determine whether it contains components other than Ssn6 and Tup1, we purified the complex to near homogeneity. To facilitate purification, the Ssn6Tup1 fusion was affinity tagged at its C terminus with a nickel binding six histidine sequence of the tetratricopeptide coding portion of Ssn6 (31). Ssn6 and Tup1 maltose binding fusion proteins contain the SSN6 coding region as well as the TPR coding region of Tup1. They were co-eluted at approximately 170 mM NaCl. The peak fractions were pooled and loaded over a HiTrap-Q column. The Ssn6HIS and Tup1 co-fractionated, as expected for proteins that are tightly associated. The peak fractions were pooled and loaded over a HiTrap-Q anion exchange column eluted with a salt gradient (100–500 mM NaCl). Ssn6HIS and Tup1 bound to the resin and co-eluted at approximately 170 mM NaCl. The peak fractions were loaded and further fractionated by gel filtration. As determined by Western immunoblotting, Ssn6HIS and Tup1 were among the first proteins to flow through the filtration column, consistent with the results of Williams et al. (18) that these proteins are present together in a large complex (Fig. 1A, fraction 9 and 10). The protein elution profile was visualized by silver staining an SDS-PAGE gel containing samples of the column fractions. Purified Ssn6HIS and Tup1 migrated as doublets on polyacrylamide gels as was previously noted for Ssn6 and Tup1 in crude yeast extracts (18) (Fig. 1A, lanes 9 and 10). No other protein fractionated stoichiometrically with Ssn6HIS and Tup1 in this or in any other preparation, suggesting that
this large complex is primarily if not exclusively composed of Ssn6 and Tup1. We estimate that the Ssn6HIS-Tup1 complex was purified approximately 1800-fold and that approximately 1% of the Ssn6HIS-Tup1 complex was recovered from the original lysate. The total yield of the Ssn6HIS-Tup1 complex was approximately 50 µg from 150 g of cells.

Migration through gel filtration is a function of the Stokes radius of a protein complex. To determine the Stokes radius of the Ssn6HIS-Tup1 complex, size standards of known Stokes radii were also fractionated over the same gel filtration column. Using the methods of Siegal and Monty (32), the Stokes radius of the Ssn6HIS-Tup1 complex was determined to be approximately 14.7 nm.

To obtain a greater yield of the Ssn6-Tup1 complex, the GST-Ssn6 fusion protein was purified from a strain that overexpressed both GST-Ssn6 and Tup1. Purification of the overexpressed complex was carried out in a single step using a glutathione-agarose column. Again, Tup1 co-purified with GST-Ssn6. Fig. 1C shows the purified proteins on a silver-stained SDS-polyacrylamide gel. The higher mobility doublet at approximately 75 kDa associates with GST alone (data not shown). The overall yield of the GST-Ssn6-Tup1 complex was approximately 200 µg from 150 g of yeast. Only a small percentage (approximately 1%) of the GST-Ssn6 within the extract bound to the glutathione-agarose column.

Determination of the Size of the Ssn6-Tup1 Complex—It has been suggested that the previous estimate of the molecular mass of the Ssn6-Tup1 complex of 1.2 MDa may be an overestimate if the complex is elongated or extended (18). Glycerol gradient sedimentation in conjunction with gel filtration can be used to estimate the molecular mass of a molecule with greater accuracy than can either method alone (32). To this end, the purified Ssn6HIS-Tup1 complex was subjected to glycerol gradient sedimentation in the presence of molecular standards of known S value (Fig. 1B). The peak of the Ssn6HIS-Tup1 complex (fraction 7) was in close proximity to that of the lactate dehydrogenase standard and corresponded to an S value of 7.3 (Fig. 1B, fraction 7). Taking into account both the Stokes radius and the S value, the molecular mass of the Ssn6-Tup1 complex was estimated at 440,000 Da. The behavior of Ssn6HIS-Tup1 on gel filtration and gradient sedimentation suggests that this complex is indeed asymmetrical shaped. Since the molecular mass of Ssn6 is approximately 107,000 Da and Tup1 is 78,000 Da, the complex must be composed of multiple Ssn6 and Tup1 molecules.

Densitometry of a Coomassie Blue-stained gel can be used to estimate stoichiometry of subunits in a complex. Coomassie Blue binding is approximately proportional to the number of positively charged groups in a protein (34). Since the Ssn6HIS and Tup1 proteins contain similar densities (9.2% and 10.6%, respectively) of arginine, lysine, and histidine, densitometry of a Coomassie-stained SDS gel was performed to obtain a crude estimate of the stoichiometry of these protein subunits in the complex. This analysis yielded a stoichiometry of one Ssn6 to three Tup1 molecules.

Tup1 Is a Phosphoprotein—While Ssn6 is a known phosphoprotein (35), it has not been established whether Tup1 is also modified in this way. The appearance of both Ssn6 and Tup1 as multiple bands after SDS-PAGE (see above) is consistent with both proteins being phosphorylated. If phosphorylation is responsible for the observed multiple-banding pattern, then removal of the phosphates should result in each protein migrating as a single band on an SDS-polyacrylamide gel. This is indeed the case as is shown in Fig. 2. Purified GST-Ssn6-Tup1 complex was subjected to λ phosphatase treatment followed by SDS-PAGE and Western blotting with antibodies against both Tup1 and Ssn6. As seen in Fig. 2, phosphatase treatment of GST-Ssn6-Tup1 results in the loss of the lower mobility forms of Tup1. Since Ssn6 is known to be phosphorylated, the phosphatase-dependent disappearance of the lower mobility Ssn6 species can be viewed as a control for λ phosphatase activity.

Purified GST-Ssn6-Tup1 Complex Has Repression Activity in Vitro—As discussed in the Introduction, the DNA binding pro-
Protein α2 recruits Tup1 and Ssn6 to DNA and thereby directs repression of many target genes. Transcriptional repression directed by the α2 protein in vivo is thus completely dependent on both Ssn6 and Tup1. α2-directed repression has also been observed in an in vitro transcription system utilizing whole cell yeast extracts (33). In vitro, α2 repression is dependent on the overexpression of Ssn6 and Tup1 in the yeast from which the transcription extracts are prepared, suggesting that the amount of Ssn6 and Tup1 is a limiting factor for repression in this in vitro system. To test this idea and to determine whether the purified Ssn6-Tup1 complex analyzed above can supply repressor activity, the following experiments were carried out. Transcription from a reporter containing two a-specific gene operators (α2/Mcm1 sites) upstream of a UAS-less CYC1 promoter is measured in parallel reactions, one lacking α2 and one containing 86 nM α2. Mcm1, which binds cooperatively with α2, is present in the yeast extract. Each reaction also contains a control reporter that lacks α-specific gene operators. Transcription extracts were prepared from yeast that do not overexpress Ssn6 and Tup1 and thus show no significant repression upon addition of purified α2 (Fig. 3, lanes 1 and 2). The addition of purified GST-Ssn6-Tup1 gives approximately 5-fold repression, which is also dependent on added α2 (Fig. 3, lanes 3 and 4).

**DISCUSSION**

In this report, we describe the purification of the Ssn6-Tup1 complex from yeast. We determined that the complex contains only Ssn6 and Tup1 proteins in stoichiometric quantities, and its size (approximately 440,000 Da) is smaller than the original estimate by Williams et al. (18), probably due to the asymmetric shape of the complex. The molecular mass estimate of 440 kDa is consistent with the complex being composed of multiple Ssn6 (107 kDa) and Tup1 (78 kDa) proteins, estimated at one Ssn6HIS to three Tup1 molecules. The purified Ssn6-Tup1 complex is active in an in vitro repression assay. Finally, we show that, like Ssn6, Tup1 is a phosphoprotein.

Ssn6 contains a repeated motif termed the tetratricopeptide repeats, TPRs (35). This 34 amino acid repeat is found in proteins involved in a variety of cellular functions from cell cycle progression to mitochondrial protein import (for review, see Ref. 36). The only known function of TPRs is to mediate protein-protein interactions. The TPRs of Ssn6 are essential for repression in vivo and mediate in vitro binding to both Tup1 and α2 (35, 37, 38). A complex composed of several Tup1 proteins and Ssn6 would, therefore, contain numerous protein-protein interaction domains. There are a number of proteins that have been predicted to interact with the Ssn6-Tup1 complex, i.e. Mig1 and Rox1, the DNA binding proteins required for glucose repression and hypoxia gene regulation (15). After recruitment to a particular gene set by interaction with a DNA binding protein, the Ssn6-Tup1 repression complex may interact with downstream elements, possibly Rox3, histones, Sin4, or Srb8, Srb9, Srb10, and Srb11 (19–23, 39).

The phosphorylation of Tup1 may have a regulatory significance. Groucho, another member of the WD repeat repressor proteins, is also phosphorylated, and phosphorylation has been implicated in increasing the affinity of groucho for the nucleus (40). Ste4, a WD repeat protein involved in signal transduction in yeast, is also known to be phosphorylated. Ste4 is the β-subunit of a heterotrimeric G protein complex and is multiply phosphorylated in response to mating pheromones. This phosphorylation is required for the adaptation or down-regulation of Ste4 activity in the continued presence of mating factors (41). Phosphorylation of WD proteins may be a general way of regulating protein-protein interactions. In the case of Tup1, phosphorylation could regulate interaction with one or more of the DNA binding proteins with which it interacts, Ssn6, Tup1 itself, or with its downstream targets.

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**Addendum**—After the submission of this manuscript, Varnasssi et al. (1996) reported that the Ssn6-Tup1 complex is composed of one Ssn6 to four Tup1 subunits (42).

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