Crystal Structure of the N-terminal Domain of the Yeast General Corepressor Tup1p and Its Functional Implications

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Background: The yeast Cyc8p-Tup1p protein complex is a general transcriptional corepressor. Cyc8p-Tup1p contains one Cyc8p and four Tup1p subunits and is recruited to gene promoters by a number of different pathway-specific repressor proteins (3, 12–19). Cyc8p-Tup1p appears to repress transcription via two mechanisms. One involves interference with the basal transcription machinery, and the other involves chromatin remodeling (6). Genetic studies have identified additional proteins necessary for repression by Cyc8p-Tup1p, including the Mediator/Srb subunits Med3p, Med21p/Srb7p, and Cdk8p/Srb10p that interact directly with the RNA polymerase II holoenzyme (20–22), and mRNA 5′-triphosphatase Cet1p, which catalyzes the first step in mRNA capping and is associated with RNA polymerase II (23). Cyc8p-Tup1p also directly interacts with class I and II histone deacetylases; the simultaneous mutation of three genes encoding histone deacetylases, RPD3, HOS1, and HOS2, abolishes Cyc8p-Tup1p activity (24, 25). Tup1p preferentially interacts with underacetylated histone H3 and H4 isoforms at their N-terminal regions in vitro (26). Notably, genes repressed by Tup1p are associated with underacetylated histones in vivo, and mutations in H3 and H4 histones synergistically reduce repression levels (26). Recently, Cyc8p-Tup1p has been shown to prevent the recruitment of transcriptional coactivators by DNA-binding proteins (27).

The budding yeast Saccharomyces cerevisiae Cyc8p-Tup1p complex is a functionally well characterized general transcriptional corepressor. Cyc8p-Tup1p is required for the repression of genes that are regulated by cell type (1), glucose (2) and oxygen levels (3), DNA damage (4), osmotic stress (5), and other signaling events (6). Tup1p is highly conserved in most species, e.g. as Drosophila Groucho (7) and human TLE (transducin-like enhancer of split) (8). Two redundant Tup1p homologues, Tup1p and Tup12p, exist in the fission yeast Schizosaccharomyces pombe (9). Cyc8p (also named Snsp6) is conserved in humans as the ubiquitous tetratricopeptide repeat motif Y/X proteins (10, 11). Therefore, Cyc8p-Tup1p-type complexes are also involved in transcriptional repression in other eukaryotes.

The atomic coordinates and structure factors (codes 3VP8 and 3VP9) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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required for normal growth (30), and the *S. pombe ssn6* gene has also been shown to be essential for growth (31).

*In vitro* protein binding experiments and two-hybrid studies have identified three protein-protein binding domains in Tup1p (713 residues, molecular mass of ~78 kDa; see Fig. 1A). The N-terminal 72 residues in Tup1p interact with Cyc8p and are required for self-tetramerization, but this region cannot independently repress transcription (29). Residues 73–386 form the repression domain (29) and interact with the N-terminal regions of histones H3 and H4 (26). The C-terminal region of Tup1p (residues 333–706) directly interacts with DNA-binding repressor proteins, *e.g.* Mata2p (32). This domain contains WD40 motifs (33, 34) that are defined by highly conserved tryptophans and aspartates. Cyc8p contains tetratricopeptide repeats (TPRs)2 in its N-terminal half that interact with the Tup1p N-terminal domain and Mata2p (17, 35, 36). Although many individual WD40 repeat and TPR domain structures have been solved (37, 38), the structures of their complexes have not been solved.

For the study reported herein, we determined the crystal structure of the Tup1p N-terminal domain (NTD, residues 1–92), which is required for Tup1p tetramerization and association with Cyc8p. NTD forms a novel antiparallel four-helix bundle, which is organized as a dimer of dimers with the protomers of each dimer associated via coiled coil interactions at their N- and C-terminal regions. Nonpolar residues in the coiled coil regions are required for self-association and transcriptional repression of genes targeted by Tup1p. A mutant NTD Tup1p-L62R also forms a dimer of dimers, although it contains interactions between protomers that are markedly unlike those of the wild-type NTD and that may be related to the ability of Cyc8p-Tup1p complexes to oligomerize when associated with chromatin targets.

**EXPERIMENTAL PROCEDURES**

**Bacterial and Yeast Strains, and Media—**Escherichia coli strains DH5α and JM109 (39), respectively, served as hosts during plasmid construction and GST-tagged protein expression. *S. cerevisiae* strain YM427 (MATα tup1::HIS3 ura3Δ52 trp1 his3Δ3 pho3Δ pho5Δ leu2::[LEU2, STE6-PHOS]) was used for complementation assays of *TUP1* disruption (9). Cultivation media for *S. cerevisiae* and *E. coli* cells were as described (39, 40).

**Oligonucleotides and Plasmids—**The primer sequences used are listed in supplemental Table S1. pGEX-NTD for expression of GST-tagged NTD was constructed by inserting into pGEX-6P-1 (GE Healthcare) the 0.3-kbp EcoRI-SalI fragment encoding residues 1–92 that had been PCR-amplified using the primers TUP1(1–92)f and TUP1(1–92)r. pCDF-TPRI–3 for the expression of His-tagged TPR1–3 was constructed by inserting into pCDF-1b (Novagen) the 0.3-kbp KpnI-Sacl fragment encoding TPR1–3 that had been PCR-amplified using the primers CYC8TPR1f and CYC8TPR3r. The YCp50-based plasmid pYMCI19, previously referred to as CCC (9), was used for expression of *TUP1* in *S. cerevisiae.*

**Mutagenesis—**Site-directed mutagenesis was performed using QuikChange II site-directed mutagenesis kit reagents (Stratagene) according to the manufacturer’s instructions. The primers used for mutagenesis are listed in supplemental Table S1. pGEX-NTD served as the template. The mutated *TUP1* sequences were sequenced to verify that the desired mutation(s) were present. Mutated *TUP1* sequences, each in a pGEX-6P-1, were PCR-amplified with the primers TUP1(1–72)If and TUP1(1–72)Ir and individually cloned into the HindIII-MluI site in a yeast expression vector pYMCI19 using In-Fusion Advantage PCR cloning kit reagents (Clontech) according to the manufacturer’s instructions. Random mutagenesis by error-prone PCR was performed using GeneMorph II random mutagenesis kit reagents (Stratagene) according to the manufacturer’s instructions. Error-prone PCR was performed with pGEX-NTD as the template and the primers TUP1(1–72)If and TUP1(1–72)Ir. The PCR products were individually cloned into pYMCI19 as described above, and the resulting plasmid library was screened for *TUP1* mutants after insertion into YM427. When the expression product of a mutated gene did not complement Δ*tup1*, the mutated gene was recovered and sequenced with TUP1pf as a sequencing primer to determine the mutation site(s).

**Yeast Genetic and Biochemical Methods—**Preparation and manipulation of DNA and RNA, and transformation with lithium acetate for the complementation assay were as described (40). Acid phosphatase activity in the yeast colonies was detected using the diazo-coupling staining method (41). Quantitative RT-PCR was performed using QuantiTect SYBR Green RT-PCR kit reagents (Qiagen) according to the manufacturer’s instructions.

**Expression and Purification of Tup1p and Cyc8p in E. coli—**Cultures of *E. coli* JM109 cells containing pGEX-NTD were incubated in Luria broth, 100 μg/ml ampicillin at 37 °C until the A_{600} values of the cultures were between 0.6 and 1.0. Expression was induced by addition of 0.1 mM isopropyl-β-d-thiogalactopyranoside (final concentration), and the cultures were then incubated overnight at 20 °C. The harvested cell pellets from 2 liters of culture medium were suspended in 25 ml of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 6.0, 1% (v/v) Triton X-100, protease inhibitor complete mixture (one tablet; Roche Applied Science), and sonicated. The lysate was centrifuged at 323,000 × g for 30 min at 4 °C, and the supernatant was loaded onto a glutathione-Sepharose 4B column (GE Healthcare), which was pre-equilibrated with 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 6.0. The protein was eluted with 20 mM glutathione in the same buffer. Fractions containing GST-tagged NTD were pooled and concentrated. GST was removed by treatment with PreScission Protease (GE Healthcare). After dialysis against 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA-2Na, 1 mM DTT, the protein solution was loaded onto a glutathione-Sepharose 4B column (GE Healthcare) to separate GST from NTD. Recovered NTD was then chromatographed through a Superdex 75 column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl. The eluted fractions were subjected to SDS-PAGE and MALDI mass spectrometry. Fractions containing NTD were pooled and concentrated, and the protein concen-
Dimer of Dimers of Corepressor Tup1p Tetramer

tration was determined using Bio-Rad protein assay kit reagents with bovine serum albumin as the standard. Cultures of E. coli BL21(DE3) cells transformed with pCDF-TPR1–3 were incubated in Luria broth, 20 μg/ml streptomycin at 37 °C until the A600 values of the cultures were between 0.6 and 1.0. Then expression was induced by addition of 0.5 mM isopropyl-β-d-thiogalactopyranoside, and the cultures were incubated overnight at 20 °C. The harvested cell pellets from 2 liters of culture medium were suspended in 23 ml of 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% (v/v) Triton X-100, 20 mM imidazole, protease inhibitor complete EDTA-free mixture (one tablet; Roche Applied Science) and sonicated. The lysate was centrifuged at 81,000 × g for 30 min at 4 °C, and then the supernatant was loaded onto an nickel-nitritotriacetic acid Superflow cartridge (5 ml, Qiagen), which was pre-equilibrated with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% (v/v) Triton X-100, 20 mM imidazole. The protein was eluted with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.1% Triton X-100, 500 mM imidazole. The eluted, pooled fractions were chromatographed through a Hidrof 26/60 Superdex 75 column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 0.01% (w/v) n-dodecyl-β-d-maltoside, 1 mM dithiothreitol. The eluted protein was characterized by SDS-PAGE and MALDI mass spectrometry, and the protein solution was concentrated to 10 mg/ml with centrifugal concentrators (Millipore). Protein concentration was determined using Bio-Rad protein assay kit reagents with bovine serum albumin as the standard.

Crystallization and Data Collection—The purified NTD and SeMet-NTD samples were concentrated to 10 and 12 mg/ml, respectively. The NTD and SeMet-NTD crystals used for x-ray diffraction were each grown in 5 ml solutions that contained 300 μl of MilliQ water, 1 μl of protein solution, and 1 μl of reservoir solution (300 μl) was 13% (v/v) 1, 4-dioxane, 0.1 mM MES monohydrate, pH 6.5, 1.3 M ammonium sulfate and was equilibrated against a drop that contained 1 μl of protein solution and 1 μl of reservoir solution using the sitting drop vapor diffusion method at 20 °C. For SeMet-NTD, the crystal that diffraction to the highest resolution was obtained by stirring the crystallization solution (42). Purified NTD-L62R and SeMet-NTD-L62R samples were each concentrated to 10 mg/ml. For crystallization of NTD-L62R, the reservoir solution (300 μl) was 14% (v/v) 1,4-dioxane, 0.1 mM MES monohydrate, pH 6.5, 1.6 M ammonium sulfate and was equilibrated against a 2-μl drop that contained 1 μl of protein solution and 1 μl of reservoir solution using the hanging drop vapor diffusion method at 20 °C. The SeMet-NTD-L62R crystal was grown using the sitting drop vapor diffusion method. The reservoir solution (300 μl) was 13% (v/v) 1, 4-dioxane, 0.1 mM MES monohydrate, pH 6.7, 1.3 M ammonium sulfate and was equilibrated against a drop that contained 1 μl of protein solution and 1 μl of reservoir solution at 20 °C. Crystals were each mounted into a loop and then flash frozen in a stream of nitrogen at 100 K. The diffraction data were collected at 100 K synchrotron radiation at the SPring8 BL44XU and Photon Factory KEK-PF BL17A beamslines (Japan). The diffraction data sets were processed using HKL2000 (43). The data collection statistics are summarized in Table 1.

Structure Determination and Refinement—Of the 16 and 8 possible selenium atoms in an asymmetric unit, 12 and 6 were found by SHELXD (44) using the anomalous signals in the SeMet-NTD and SeMet-NTD-L62R peak data sets, respectively. Initial phases for SeMet-NTD were calculated and refined using SHELXE (44) and the graphical interface HKL2MAP (45). Model refinement used CNS (46) and PHENIX (47), with manual inspection and modification in conjunction with the CCP4 program COOT (48). The ϕ-ψ angles of >90% of the residues in the NTD structure are in the most favored regions of the Ramachandran plot as assessed by PROCHECK (49). Phasing and refinement statistics are given in Table 1. The interactions between protomers were analyzed using the PISA server (50). Secondary structure predictions were performed by PSIPRED (51). The probability of coiled coil (CC) formation was estimated by COILS (52). The figures were prepared using Pymol, Molscript (53), and Raster3D (54). The final atomic coordinates and structure-factor amplitudes (Protein Data Bank entries 3VP8 for NTD and 3VP9 for NTD-L62R) have been deposited in the Worldwide Protein Data Bank and the Protein Data Bank Japan at the Institute for Protein Research of Osaka University (Osaka, Japan). The refinement statistics for both coordinate sets are presented in Table 1.

Native Gel Shift Assays—The mixtures of wild-type NTD or an NTD mutant and TPR1–3 were each diluted in 125 mM Tris-HCl, pH 6.8, 20% (w/v) glycerol, and 0.01% (w/v) bromphenol blue and subjected to electrophoresis under native conditions according to the instructions of ATTO. The gels were stained with Coomassie Brilliant Blue.

Surface Plasmon Resonance Analysis—Assays for interactions between mutant and wild-type NTD and TPR1–3 were performed using a Biacore 2000 apparatus with CM5 sensor chips (GE Healthcare). The temperature was maintained at 293 K. NTD (5.9 –51.5 μg/ml) diluted in 10 mM sodium acetate, pH 5.2, was immobilized onto the chip surface through amine chemistry according to standard procedures. Briefly, after washing the chip surface with a 50:50 (v/v) solution of 0.1 M N-hydroxysuccinimide and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride at a rate of 5 μl/min for 7 min to activate it, the NTD solution was washed over the activated surface at a rate of 5 μl/min for 7 min. Subsequently, the remaining activated sites were quenched by washing the chip with 1 M ethanolamine at a rate of 5 μl/min for 7 min and then once with 50 mM NaOH and 1 M NaCl. The relative amount of protein immobilized ranged from 1677.8 to 2166.6 response units. Binding of the TPR1–3 fragments to immobilized NTD was monitored by injecting TPR1–3 (0.03–10 μM) in HBS (10 mM HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20) over the chip surface at a rate of 20 μl/min. After 6 min, the surface was regenerated with 80 μl of HBS, 1 mM NaCl. Kp, kcat, and kcat were calculated using BIAevaluation 3.2 software (GE Healthcare).

RESULTS

Crystal Structure of NTD—The NTD structure was solved using the single-wavelength anomalous diffraction of a SeMet-NTD crystal. The structure was refined to 1.9 Å resolution and has a crystallographic R factor of 24.4% (Rfree = 29.8%; Table 1). The R factor seems to be relatively large, probably because the x-ray diffraction spots and the fiber diffraction pattern were simultaneously recorded on the detector, and certain regions of
the crystal structures are disordered. The asymmetric unit contains four NTD protomers (protomers A–D), each of which is a single, long helix (~105 Å), that interact to form the NTD tetramer (see below and Fig. 1, B and C). The final model contains residues 4–78, 2–81, 4–80, and 2–79 in the A, B, C, and D chains, respectively. Mass spectroscopy of dissolved crystals contains residues 4–78, 2–81, 4–80, and 2–79 in the A, B, C, and D chains, respectively. Mass spectroscopy of dissolved crystals.

**NTD Coiled Coil Structures**—The NTD protomer contains α-helical CC sequences that are important for dimer and tetramer formation. The CC sequences are characterized by heptad sequence repeats (abcdefg), with a, d, e, and g often nonpolar or hydrophobic “knob” residues that mediate interchain interactions through knobs into holes packing to form coiled coils (55, 56). The structure-based algorithm SOCKET (57) (packing cutoff = 7.0 Å) identified two coiled coil segments in the NTD protomer (CC-1 and CC-2), in which residues assigned to d and g positions are knob residues (Fig. 2A). CC-1 is located in the N-terminal region, with Thr8, Leu13, Leu15, and Ile19 as knob residues; the C-terminal CC-2 contains Gln54, Thr58, Leu62, Thr65, and Met69 as knob residues (Fig. 2B and supplemental Fig. S1B). Although CC-1 and CC-2 tightly associate via nonpolar coiled coil interactions to form a dimer (see below), intradimeric interactions for the internal regions (residues 20–54) are formed by hydrophilic residues. Glu22, Lys44, and Gln57 in chain A form hydrogen bonds with Thr58, Gln36, and Tyr33 of chain B, respectively. The interstitial space between residues 20–54 in the two chains is occupied by more than 130 water molecules (65% of total water molecules; supplemental Fig. S1C); therefore, the orientations of the chains are also stabilized by water-mediated hydrogen bonds. CC-1 and CC-2 of chain A (or C) associate with CC-2 and CC-1 of chain B (or D), respectively, and account for most of the buried surface area described above. Two residues (Phe23 and Arg24) are responsible for the association of the neighboring AB and
Tup1p Residues Important for Transcriptional Repression—The surface of tetrameric NTD is negatively charged (supplemental Fig. S2), and the negatively charged surface residues are highly conserved among Tup1p homologues (Fig. 2A). We therefore hypothesized that these residues may electrostatically interact with Cyc8p. To test this hypothesis, we constructed genes for the single-point Tup1p mutants E14A, D17A, E22A, E29A, E51A, E63A, E14K, and E29K, the double-point mutants E14A/D17A and E29A/E51A, and the quadruple-point mutant, E14A/D17A/E29A/E51A. Samples of the S. cerevisiae strain YMH427, which harbors a deletion in TUP1, were separately transformed with a plasmid containing a mutated gene. Transcriptional repression of genes targeted by Cyc8p-Tup1p, i.e. the hypoxic ANBI, the a-mating type-specific STE2, and glucose-repressed SUC2, was measured by quantitative RT-PCR (Fig. 3A). All of the mutants repressed transcription of the Tup1p-regulated genes in the same manner, as did the wild-type strain, indicating that these surface acidic residues are not important for in vivo repression by Tup1p and also possibly not for Cyc8p-Tup1p association.

To identify residues in Tup1p that are important for transcriptional repression, we screened for mutants that could not repress genes targeted by Tup1p. To do so, TUP1 in a centromeric YCp50 plasmid was randomly PCR mutagenized, and the resulting plasmid library was introduced into YMH427, which also contained STE6-PHOS, a reporter gene under the control of Tup1p. Approximately 1000 yeast transformants were screened for acid phosphatase activity (red stain), which resulted from the derepression of STE6-PHOS; 82 positive clones were isolated. Sequencing of the mutated TUP1s identified four clones, Tup1p-L12R, -L24P, -L49P, and -L62V, that contained a single mutation. The other clones contained multiple mutations, nonsense mutations, or frameshift mutations. Leu<sup>12</sup> and Leu<sup>62</sup> are knob residues (Fig. 2B). Replacement of a leucine in an α-helix by a proline is expected to disrupt a helical structure. Non-alanine replacements might disrupt or have unanticipated effects on the helical structure of Tup1p and consequently its functions. To exclude this possibility, we generated a set of single-point Tup1p mutants, each of which had an alanine in place of the hydrophobic amino acids, Leu<sup>12</sup>, Leu<sup>15</sup>, Leu<sup>16</sup>, Phe<sup>23</sup>, or Leu<sup>62</sup>. Additionally, Tup1p-L62R (58), -L12R, -L24P, -L49P, -L62V, and -H66A were used in the study. Derepression of ANBI, STE2, and SUC2 by the aforementioned mutants were assessed by quantitative RT-PCR (Fig. 3B). As expected, Tup1p-L12R, -L24P, -L49P, -L62R, and -H66A did not repress expression of the reporter genes. Tup1p-L12A, -F23A, -L62A, and -L62V moderately inhibited repression. Tup1p-L15A and -L16A repressed expression similar to that of wild-type Tup1p. Therefore, residues that were predicted to stabilize the tetrameric structure were important for the native structure.

Interaction of the NTD Mutants with Cyc8p—We assessed whether the mutants could bind Cyc8p using a native gel shift assay and an N-terminal domain construct of S. cerevisiae Cyc8p that contained the first three tetratricopeptide repeats (TPR1–3). TPR1–3 binds Tuplp (36). The NTD mutants in which Glu<sup>22</sup> or Glu<sup>51</sup> were substituted with an alanine (NTD-E22A and NTD-E51A, respectively) were expressed in E. coli, and their ability to bind TPR1–3 was assessed (Fig. 4A). These mutants interacted with TPR1–3. We also quantified the strengths of the interactions between NTD, NTD-E22A, and NTD-E51A and TPR1–3 by surface plasmon resonance (Fig. 4B and Table 2). The kinetic parameters for NTD-E22A and NTD-E51A are similar to those of wild-type NTD.

We then tested NTD-L62R and NTD-L62A. In the crystal structure, Leu<sup>62</sup> is a CC-2 knob residue (Fig. 2A). The native gel shift assay demonstrated that NTD-L62R almost lost its ability...
to bind TPR1–3 (Fig. 4A). This result is consistent with those of previous reports, suggesting that Tup1p-L62R lost its ability to repress transcription because it could not bind Cyc8p (58). We also assayed the interaction between TPR1–3 and NTD-L62R/A by surface plasmon resonance and again found that the mutations in NTD-L62R/A largely decrease the affinity of binding to TPR1–3 (Fig. 4B and Table 2), suggesting that Leu62 is necessary for NTD-TPR1–3 binding.

Crystal Structure of NTD-L62R—To understand how the arginine substitution at position 62 affects the structure of NTD-L62R, we solved the NTD-L62R crystal structure using the single-wavelength anomalous diffraction of a SeMet-NTD-L62R crystal. The structure was refined to 1.8-Å resolution and had an R factor of 22.0% (Rfree = 25.8%; Table 1). The asymmetric unit contains two NTD-L62R molecules (chains A and B). A tetrameric arrangement of two pairs of NTD-L62R molecules (Fig. 5A) can be generated by a crystallographic 2-fold symmetry rotation, which agrees with an analytical ultracentrifugation study of NTD-L62R that the mutant exists as a tetramer (59). The crystallized tetramer has dimensions of 170 × 20 Å, and each protomer is an α-helical structure. Residues 1–13 in chain A and residues 1–16 in chain B are largely disordered, unlike the wild-type NTD structure. However, the C-terminal α-helices extend farther in L62R than they do in wild-type NTD (Figs. 1B and 5A). Only residues 91–92 in chain A and 87–92 of in chain B of the mutant are disordered.

The quaternary structure of NTD-L62R is markedly different from that of wild-type NTD. Although the NTD-L62R tetramer is also organized as a dimer of dimers, the dimers (~100 Å in length) consist of two intertwined α-helices aligned in parallel,
whereas the wild-type dimer (~120 Å in length) is a pair of intertwined helices arranged in an antiparallel fashion. SOCKET (57) identified a distinctive packing pattern in the NTD-L62R coiled coil dimeric interface (Figs. 2A and 5B). The C-terminal region of chain B interacts with the C-terminal region of the symmetry-related chain B (designated B') in an antiparallel orientation through coiled coil interactions, and these interactions probably are responsible for the structural stabilization and extension of the C-terminal helices in NTD-L62R. In NTD-L62R, the dimer of dimers contacts involves...
Lys68, Ala72, Glu76, and His79, which are knob residues (Fig. 5B). This assembly mode might be implicated in the oligomerization of Cyc8p-Tup1p complexes (see below).

DISCUSSION

We solved the Tup1p NTD, which is required for tetramerization of Tup1p and association with Cyc8p in the Cyc8p-Tup1p corepressor complex. NTD tetramerizes as a dimer of dimers with a novel arrangement, which is stabilized by nonpolar knob residues in coiled coils formed by intra- and interdimers. The knob residues are important for transcriptional repression and for Cyc8p association, but the acidic residues on the surface of NTD are not important. Replacement of the hydrophobic leucine at position 62 by an arginine causes a large conformational change, explaining why the mutant largely decreases the affinity of binding to TPR 1–3.

NTD Structure—A structural homology search by the Dali server (60) using tetrameric NTD as the query confirms that the structure of the aspartate receptor is most homologous to it (Z score = 13.6), although the receptor has dimensions of 65 × 30 × 30 Å, distinct from those of NTD. We further manually examined ~100 hits made by the Dali server manually, but no homologous structures were found. Homotetrameric four-helix bundles have been found in several proteins, which are involved in transcriptional repression, e.g. the tetramerization domains of histone deacetylase (65). The arrangement of the NTD helices differs from those of p53, the lac repressor, and the Mnt repressor (supplemental Fig. S3). Similar to those of NTD, the helices in the four-helix bundle of p53 and the Mnt repressor assemble as a dimer of dimers, but the dimer pairs are oriented at different angles. The oligomerization domains of histone deacetylase have dimensions of 108 × 20 × 20 Å similar to NTD, but the protomers are arranged symmetrically, which is distinct from NTD. Thus, NTD tetramerizes to form a novel antiparallel four-helix bundle.

Secondary structural and coiled coil predictions (51, 52) for yeast, Drosophila (Groucho), and human (TLE) homologues of Tup1p suggest that these proteins also contain N-terminal coiled coil structures (Fig. 2A and supplemental Fig. S4). Of the CC-1 and CC-2 residues involved in intradimer contacts, several are moderately conserved among the yeast homologues, Groucho, and TLE (Fig. 2A), despite low sequence identity. Given the functional and structural resemblance of Tup1p, Groucho, and TLE, it is quite possible that the tetrameric assembly of these homologues is structurally conserved.

NTD-L62R Structure—The oligomeric state of NTD-L62R had been found to be tetrameric according to an analytical ultracentrifugation study (59); therefore, the L62R mutation was not thought to influence the wild-type quaternary structure. Consequently, we were surprised to find that the mutation induced a large conformational alteration so that the quaternary structure changed entirely. The molecular surface structure of NTD-L62R is also different from that of NTD (supplemental Fig. S5). The NTD-L62R and NTD tetramers are stabilized by interactions in their coiled coils, but the interactions differ. The L62R mutation also prevents transcriptional repression and association with TPR1–3. A loss of function must therefore correlate with the conformational change. Interestingly, the major structural differences between the

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**TABLE 2**

| Data set | $k_a$ | $k_d$ | $K_D$ |
|----------|-------|-------|-------|
| WT       | $1.75 \times 10^5$ | $7.00 \times 10^{-3}$ | $4.10 \times 10^{-8}$ |
| E22A     | $1.79 \times 10^4$ | $5.08 \times 10^{-3}$ | $5.55 \times 10^{-8}$ |
| E51A     | $2.61 \times 10^4$ | $6.63 \times 10^{-3}$ | $6.91 \times 10^{-8}$ |
| L62A     | $3.87 \times 10^{-7}$ | $1.51 \times 10^{-6}$ | |
| L62R     | $1.51 \times 10^{-6}$ | | |

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The lac repressor (63), the Mnt repressor (64), and histone deacetylase (65). The arrangement of the NTD helices differs from those of p53, the lac repressor, and the Mnt repressor (supplemental Fig. S3). Similar to those of NTD, the helices in the four-helix bundle of p53 and the Mnt repressor assemble as a dimer of dimers, but the dimer pairs are oriented at different angles. The oligomerization domains of histone deacetylase have dimensions of 108 × 20 × 20 Å similar to NTD, but the protomers are aligned symmetrically, which is distinct from NTD. Thus, NTD tetramerizes to form a novel antiparallel four-helix bundle.
NTD and NTD-L62R tetramers occur in the C-terminal region (residue 80–92), which is structured in NTD-L62R but disordered in NTD, which suggests that NTD might also assemble as a meta-stable structure similar to that of NTD-L62R.

**Cyc8p-Tup1p Interactions**—Examination of the NTD tetramer shows that large areas between protomers interact, which should result in tight binding at the protomer interfaces, and explain how Tup1p maintains a stable tetrameric structure. The mechanism of transcriptional repression may be a reflection of the stable tetrameric structure of NTD, even though Cyc8p is functionally important and interacts with the DNA-binding protein Matα2p (17). This hypothesis is consistent with loss of function studies that have shown that when CYC8 and TUP1 are both inactivated, expression of Tup1p, but not of Cyc8p, leads to recovery of repression (14).

Interestingly, other *S. cerevisiae* WD40 motif-containing protein-TPR complexes, similar to Cyc8p-Tup1p, exist, e.g. Ski3p-Ski8p (for normal 3′-directional degradation of mRNA) (66), Utp6p-Utp21p (for processing and assembly of rRNA) (67), and Fis1p-Mdv1p (for mitochondrial membrane fission) (68). Similar human protein complexes have been found, e.g. PEX5-PEX7 (for import of PTS1 proteins into peroxisomes) (69, 70), XAB2-CSA (for nucleotide excision repair) (71, 72), and CDC27-CDC20 (for ubiquitin ligase activity) (73–76). Several of these WD40 repeat proteins and TPR-type proteins also interact with other proteins. However, we did not find structures in these WD40 repeat proteins that were similar to those of the Tup1p NTD, suggesting that these WD40 repeat proteins interact with their TPR protein partners in different manners. This suggestion is consistent with our previous observation that the N-terminal domain of *S. pombe* Tup11p, which has a sequence that is somewhat similar to that of *S. cerevisiae* Tup1p (22% identity), did not interact with *S. cerevisiae* Cyc8p (9).

**Model for Cyc8p-Tup1p Oligomerization**—Tup1p and its homologues (Groucho/TLE) mediate long range repression ([77, 78]). Oligomerization mechanisms for Cyc8p-Tup1p have been proposed to explain how the complex accomplishes long range transcriptional repression ([77–79]). CC proteins inherently tend to assemble as an extended fiber (80), supporting the hypothesis that the Tup1p tetramer may oligomerize. Therefore, the question arises as to how oligomerized Tup1p tetramers are organized. The NTD-L62R structure might help answer this question. In NTD-L62R, the C-terminal regions of the protomers associate in an antiparallel orientation, thereby extending the α-helices in the C-terminal direction. Although we cannot exclude the possibility that the structure of NTD-L62R is an artifact, NTD might have the potential to adopt the C-terminal antiparallel CC. We hypothesize that the interactions between C-terminal antiparallel CCs might cause Tup1p tetramers to oligomerize as shown in Fig. 6. Because Tup1p assembles as a dimer of dimers, each dimer might be able to associate with a dimer in an adjacent Tup1p tetramer (Fig. 6).
Diederick and Simpson (77) used a chromatin immunoprecipitation assay to show that, in vivo, Tup1p is associated with repressed STE6 chromatin in an 1:2 Cyc8p-Tup1p nucleosome ratio. Our model, in which one Cyc8p-Tup1p associates with two nucleosomes, is consistent with their data. Because we also found a few tetramer-tetramer interactions in the wild-type NTD crystal (supplemental Fig. S6), we cannot exclude the possibility that Tup1p oligomerization might be mediated via such intertetramer interactions.

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REFERENCES

1. Mukai, Y., Harashima, S., and Oshima, Y. (1991) AAR1/TUP1 protein, with a structure similar to that of the β subunit of G proteins, is required for α1-α2 and α2 repression in cell type control of Saccharomyces cerevisiae. Mol. Cell. Biol. 11, 3773–3779
2. Williams, F. E., and Trumbly, R. J. (1990) Characterization of TUP1, a mediator of glucose repression in Saccharomyces cerevisiae. Mol. Cell. Biol. 10, 6500–6511
3. Balasubramanian, B., Lowry, C. V., and Zitomer, R. S. (1993) The Rox1 repressor of the Saccharomyces cerevisiae hypoxic genes is a specific DNA-binding protein with a high-mobility-group motif. Mol. Cell. Biol. 13, 6071–6078
4. Zhou, Z., and Elledge, S. Y. (1992) Isolation of Ssn6 mutants constitutive for expression in Saccharomyces cerevisiae. Genetics 131, 851–866
5. Marquez, J. A., Pascual-Ahuir, A., Prof, M., and Serrano, R. (1998) The Ssn6-Tup1 repressor complex of Saccharomyces cerevisiae is involved in the osmotic induction of Hog1-dependent and -independent genes. EMBO J. 17, 2543–2553
6. Malavé, T. M., and Dent, S. Y. (2006) Transcriptional repression by Tup1p. Saccharomyces. Cell Biol. 94, 437–443
7. Fisher, A. L., and Caudy, M. (1998) Groucho transcriptional corepressors for specific subsets of DNA-binding transcription factors in vertebrates and invertebrates. Genes Dev. 12, 1931–1940
8. Stifani, S., Blummueller, C. M., Redhead, N. I., Hill, R. E., and Artavanis-Tsakonas, S. (1992) Human homologs of a Drosophila enhancer of split gene product define a novel family of nuclear proteins. Nat. Genet. 2, 119–127
9. Mukai, Y., Matsuo, E., Roth, S. Y., and Harashima, S. (1999) Conservation of histone and transcriptional repressor functions in a Schizosaccharomyces pombe Tup1p homolog. Mol. Cell. Biol. 19, 8461–8468
10. Grbavec, D., Lo, R., Liu, Y., Greenfield, A., and Stifani, S. (1999) Groucho/transducin-like enhancer of split (TLE) family members interact with the yeast transcriptional corepressor Ssn6 and mammalian Ssn6-related proteins. Implications for evolutionary conservation of transcription repression mechanisms. Biochem. J. 337, 13–17
11. Lahn, B. T., and Page, D. C. (1997) Functional coherence of the human Y chromosome. Science 278, 675–680
12. Conlan, R. S., and Tzamarias, D. (2001) Sfl1 functions via the co-repressor Ssn6 and the cAMP-dependent protein kinase Tpk2. J. Mol. Biol. 309, 1007–1015
13. Huang, M., Zhou, Z., and Elledge, S. I. (1998) The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell 94, 595–605
14. Komachi, K., Redd, M. J., and Johnson, A. D. (1994) The WD repeats of Tup1p interact with the homeo domain protein α2. Genes Dev. 8, 2857–2867
15. Park, S. H., Koh, S. S., Chun, J. H., Hwang, H. J., and Kang, H. S. (1999) Nrg1 is a transcriptional repressor for glucose repression of STα1 gene expression in Saccharomyces cerevisiae. Mol. Cell. Biol. 19, 2044–2050
16. Prof, M., Pascual-Ahuir, A., de Nadal, E., Arito, J., Serrano, R., and Posas, F. (2001) Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. EMBO J. 20, 1123–1133
17. Smith, R. L., Redd, M. J., and Johnson, A. D. (1995) The tetratricopeptide repeats of Ssn6 interact with the homeo domain of α2. Genes Dev. 9, 2903–2910
18. Treitel, M. A., and Carlson, M. (1995) Repression by Ssn6-Tup1 is directed by MIG1, a repressor/activator protein. Proc. Natl. Acad. Sci. U.S.A. 92, 3132–3136
19. Zitomer, R. S., and Lowry, C. V. (1992) Regulation of gene expression by oxygen in Saccharomyces cerevisiae. Microbiol. Rev. 56, 1–11
20. Gromoller, A., and Lehming, N. (2000) Srb7p is a physical and physiological target of Tup1p. EMBO J. 19, 6845–6852
21. Papamichos-Chronakis, M., Conlan, R. S., Gounalaki, N., Copf, T., and Tzamarias, D. (2000) Hrr1/Med3 is a Cyc8-Tup1p corepressor target in the RNA polymerase II holoenzyme. J. Biol. Chem. 275, 8397–8403
Dimer of Dimers of Corepressor Tup1p Tetramer

Harbor Laboratory, Cold Spring Harbor, NY

41. Tob-E, A., and Oshima, Y. (1974) Characterization of a dominant, constitutive mutation, PHOO, for the repressible acid phosphatase synthesis in *Saccharomyces cerevisiae*. *J. Bacteriol.* 120, 608–617

42. Matsumura, H., Sugiyama, S., Hirose, M., Kakinouchi, K., Maruyama, M., Murai, R., Adachi, H., Takano, K., Murakami, S., Mori, Y., and Inoue, T. (2011) Approach for growth of high-quality and large protein crystals. *J. Synchrotron. Radiat.* 18, 16–19

43. Otwinowski, Z. (1993) in *Data Collection and Processing: Proceedings of the CCP4 Study Weekend: Data Collection and Processing* (Sawyer, L., Isaacs, N., and Bailey, S., eds) pp. 56–62, Daresbury Laboratory, Warrington, UK

44. Sheldrick, G. M. (2008) A short history of SHELX. *Acta Crystallogr.* A 64, 112–122

45. Pape, T., and Schneider, T. R. (2004) *Appl. Crystallogr.* 37, 843–844

46. Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) Crystallography & NMR system. A new software suite for macromolecular structure determination. *Acta Crystallogr.* D 54, 905–921

47. Adams, P. D., Grosse-Kunstleve, R. W., Hung, L. W., Joerger, T. R., McCoy, A. J., Moriarty, N. W., Read, R. J., Scacchetti, J. C., Sauter, N. K., and Terwilliger, T. C. (2002) PHENIX: Building new software for automated crystallographic structure determination. *Acta Crystallogr.* D Biol. Crystallogr. 58, 1948–1954

48. Emsley, P., and Cowtan, K. (2004) Coot: Model-building tools for molecular graphics. *Acta Crystallogr.* D Biol. Crystallogr. 60, 2126–2132

49. Laskowski, R., MacArthur, M., Moss, D., and Thornton, J. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J. Appl. Crystallogr.* 26, 283–291

50. Krasinski, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. *J. Mol. Biol.* 372, 774–797

51. McGuﬃn, L. J., Bryson, K., and Jones, D. T. (2000) The PSIPRED protein structure prediction server. *Bioinformatics* 16, 404–405

52. Lupas, A., Van Dyke, M., and Stock, J. (1991) Predicting coiled coils from protein sequences. *Science* 252, 1162–1164

53. Kraulis, P. J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* 24, 946–950

54. Merritt, E. A. (1994) Raster3D Version 2.0. A program for photorealistic molecular graphics. *Acta Crystallogr.* D Biol. Crystallogr. 50, 869–873

55. Oakley, M. G., and Hollenbeck, J. J. (2001) The design of antiparallel coiled coils. *Curr. Opin. Struct. Biol.* 11, 450–457

56. Parry, D. A., Fraser, R. D., and Squire, J. M. (2008) Fifty years of coiled-coils. *Adv. Protein Chem.* 80, 15–369–376

57. Gatto, G. J., Jr., Geisbrecht, B. V., Gould, S. J., and Berg, J. M. (2000) Peroxiosomal targeting signal-1 recognition by the TPR domains of human PEX5. *Nat. Struct. Biol.* 7, 1091–1095

58. Henning, K. A., Li, L., Iyer, N., McDaniell, L. D., Reagan, M. S., Legerski, R. S., Schultz, R. A., Stefani, M., Lehmann, A. R., Mayne, L. V., and Friedberg, E. C. (1995) The Cockayne syndrome group A gene encodes a WD repeat protein that interacts with CSB protein and a subunit of RNA polymerase II TFIIH. *Cell* 82, 555–564

59. Nakatsu, Y., Asahina, H., Citterio, E., Rademakers, S., Vermeulen, W., Kamiuchi, S., Yeo, J. P., Khaw, M. C., Saijo, M., Kodo, N., Matsuda, T., Hooiemakers, J. H., and Tanaka, K. (2000) XAB2, a novel tetratricopeptide repeat protein involved in transcription-coupled DNA repair and transcription. *J. Biol. Chem.* 275, 34931–34937

60. D’Angiolella, V., Mari, C., Nocera, D., Rametti, L., and Grieco, D. (2003) The spindle checkpoint requires cyclin-dependent kinase activity. *Genes Dev.* 17, 2520–2525

61. Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Hatging, A., Pines, J., and Peters, J. M. (2003) Mitotic regulation of the human anaphase-promoting complex by phosphorylation. *EMBO J.* 22, 6598–6609

62. Tugendreich, S., Tomkij, I., Earnshaw, W., and Hieter, P. (1995) CDC27Hs colocalizes with CDC16Hs to the centrosome and mitotic spindle and is essential for the metaphase to anaphase transition. *Cell* 81, 261–268

63. Weinstein, J. (1997) Cell cycle-regulated expression, phosphorylation, and degradation of p55Cdc. A mammalian homolog of CDC20/Fizzy1p1. *J. Biol. Chem.* 272, 28501–28511

64. Duckcr, C. E., and Simpson, R. T. (2000) The organized chromatin domain of the repressed yeast a cell-specific gene STE6 contains two molecules of the corepressor Tup1p per nucleosome. *EMBO J.* 19, 400–409

65. Flores-Saib, R. D., and Coury, A. J. (2000) Analysis of Groucho-histone interactions suggests mechanistic similarities between Groucho- and Tup1-mediated repression. *Nucleic Acids Res.* 28, 4189–4196

66. Chen, G., Nguyen, P. H., and Courey, A. J. (1998) A role for Groucho tetramerization in transcriptional repression. *Mol. Cell. Biol.* 18, 7259–7268

67. Lupas, A. N., and Gruber, M. (2005) The structure of α-helical coiled coils. *Adv. Protein Chem.* 70, 37–78

68. Gouet, P., Courcelle, E., Stuart, D. I., and Métoz, F. (1999) ESPript: Analysis of multiple sequence alignments in PostScript. *Bioinformatics* 15, 305–308

69. Noll, M., Zimmer, S., Engel, A., and Dubochet, J. (1980) Self-assembly of single and closely spaced nucleosome core particles. *Nucleic Acids Res.* 8, 21–42

70. Zhao, H., Zhang, Y., Zhang, S. B., Jiang, C., He, Q. Y., Li, M. Q., and Qian, R. L. (1999) The structure of the nucleosome core particle of chromatin in chicken erythrocytes visualized by using atomic force microscopy. *Cell Res.* 9, 255–260