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.

Significance: Our data highlight the importance of the architecture of the Tup1p N-terminal domain for self-association.

Results: The yeast Cyc8p-Tup1p protein complex is a general transcriptional corepressor of genes involved in many different physiological processes. Herein, we present the crystal structure of the Tup1p N-terminal domain (residues 1–92), essential for Tup1p self-assembly and interaction with Cyc8p. This domain tetramerizes to form a novel antiparallel four-helix bundle. Coiled coil interactions near the helical ends hold each dimer together, whereas interdimeric association involves only two sets of two residues located toward the chain centers. A mutagenesis study confirmed that the nonpolar residues responsible for the association of the protomers as dimers are also required for transcriptional repression. An additional structural study demonstrated that the domain containing an Leu→Arg mutation that had been shown not to bind Cyc8p exhibits an altered structure, distinct from the wild type. This altered structure explains why the mutant cannot bind Cyc8p. The data presented herein highlight the importance of the Tup1p N-terminal domain for self-association.

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, Tup11p and Tup12p, exist in the fission yeast Schizosaccharomyces pombe (9). Cyc8p (also named Ssn6p) 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.

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 underacylated histone H3 and H4 isoforms at their N-terminal regions in vitro (26). Notably, genes repressed by Tup1p are associated with underacylated 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).

Repression by Cyc8p, when recruited to a promoter through a bacterial LexA DNA-binding domain, is Tup1p-dependent, whereas repression by Tup1p-LexA can occur in the absence of Cyc8p (28, 29). Tup1p may therefore be responsible for repressor activity, with Cyc8p possibly serving as a bridge between Tup1p and other proteins. S. cerevisiae CYC8, not TUP1, is
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-oligomerization, 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 ura3-52 trp1 his3 phospho4-3,112::[LEU2, STE6-PHOS]) was used for complementation assays of Tup1p 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-TPR1–3 for the expression of His-tagged TPR1–3 was constructed by inserting into pCDF-1b (Novagen) the 0.3-kbp KpnI-SacI fragment encoding TPR1–3 that had been PCR-amplified using the primers CYC8TPRIF and CYC8TPRIR. The YCp50-based plasmid pYMCI119, previously referred to as CCC (9), was used for expression of Tup1p in *S. cerevisiae*.

**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*\(_{590}\) 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\(_2\)HPO\(_4\), 1.47 mm KH\(_2\)PO\(_4\), 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\(_2\)HPO\(_4\), 1.47 mm KH\(_2\)PO\(_4\), 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 concentr-
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 A_{600} 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 x g for 30 min at 4 °C, and then the supernatant was loaded onto an nickel-nitrilotriacetic 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% (v/v) Triton X-100, 500 mM imidazole. The eluted, pooled fractions were chromatographed through a Hiloak 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 50 mL of MilliQ water, 1 M ammonium sulfoate and 1.6 M ammonium sulfate, pH 6.5, 20% (w/v) glycerol, and 0.01% (w/v) bromophenol 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 M NaCl. K_p, k_{on}, and k_{off} 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% (R_{free} = 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 confirmed that only full-length NTD was present (supplemental Fig. S1A); therefore, the remaining residues are disordered in the crystal.

**Quaternary Structure of NTD**—The NTD tetramer is a rod-like, antiparallel four-helix bundle, with dimensions of ∼130 × 25 × 25 Å (Figs. 1, B–D). Its interchain interactions are those involving the side chains of the two protomers in a dimer (supplemental Fig. S1B); the interface areas between two adjacent chains range from 567 to 1570 Å². Contacts between chains A and B (interface area, 1560 Å²) and C and D (interface area, 1570 Å²) are more extensive than those between chains A and C (interface area, 982 Å²), B and D (interface area, 988 Å²), A and D (interface area, 567 Å²), and B and C (interface area, 703 Å²). The structure of chain A is most similar to that of chain C (root mean square deviation 0.57 Å for 74 Ca positions), and that of chain B is most similar to that of chain D (root mean square deviation 0.60 Å for 74 Co positions). The other pairwise root mean square deviations, those for A-B or B-C, etc., have values >1.30 Å. The tetramer is therefore organized as a dimer of dimers, with the dimers related by an approximate 2-fold symmetry axis (Fig. 1D).

**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)₆, 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, Leu¹², 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 Thr³⁶, Gln⁵⁶, 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 (Phe²¹ and Arg²⁴) are responsible for the association of the neighboring AB and
CD dimers (supplemental Fig. S1D). These residues are knob residues at positions a and e (Fig. 2A). Additionally, His<sup>66</sup> of chain A (or B) is stacked on the side chain of His<sup>66</sup> of chain D (or C), respectively (supplemental Fig. S1E). This residue seems to stabilize the tetrameric organization.

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 <i>S. cerevisiae</i> strain YMH427, which harbors a deletion in <i>TUP1</i> (strain <i>S. cerevisiae</i> K1787, domain organization of Tup1p. 

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, <i>TUP1</i> in a centromeric YCp50 plasmid was randomly PCR mutagenized, and the resulting plasmid library was introduced into YMH427, which also contained <i>STE6-PHOS</i>, 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 <i>STE6-PHOS</i>; 82 positive clones were isolated. Sequencing of the mutated <i>TUP1</i>s 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 <i>ANB1</i>, <i>STE2</i>, and <i>SUC2</i> 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 structure reduced repression, consistent with those residues being 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 <i>S. cerevisiae</i> 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 <i>E. coli</i>, 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 Tup1p.

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% ($R_{free} = 25.8%$; Table 1). The asymmetry 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 $170 \times 20 \times 20$ Å, and each protomer is an $\alpha$-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 $\alpha$-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 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 ($\sim 100$ Å in length) consist of two intertwined $\alpha$-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

FIGURE 3. Quantitative RT-PCR complementation assays. A, Tup1p point mutants in which one, two, or four acidic residues had been replaced. B, Tup1p point mutants in which a hydrophobic residue in a coiled coil had been replaced. Samples of the S. cerevisiae strain YMH427, which harbors a deletion in TUP1 (Δtup1), were transformed with one of the plasmids that harbored a mutated TUP1. The ability of the expressed, mutated Tup1ps to repress ANB1 (blue bars), STE2 (yellow bars), and SUC2 (red bars) expression was measured by quantitative RT-PCR.

FIGURE 4. Association of wild-type or mutant NTD and TPR1–3. A, electrophoretic mobility shift assay. The interactions were measured using a 1:1, 1:4, or 4:1 molar ratio of an NTD construct and TPR1–3. B, surface plasmon resonance versus time for interaction of NTD constructs with TPR1–3 at 25 °C.
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 the human tumor suppressor protein p53 (61, 62), 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 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

**TABLE 2**

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FIGURE 5. **NTD-L62R structure.** **A,** the two chains (A and B) in the asymmetric unit are colored **green** and **cyan,** and the two symmetry-related chains (A’ and B’) are colored **magenta** and **yellow.** **B,** knob into hole interactions between the protomers of the NYD-L62R dimer (region boxed in **A**). Knob residues are identified by name and residue position, and their side chains are shown as stick models.
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* Tup1lp, 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).
Acknowledgments—We thank Dr. Takahiko Yamamoto, Mr. Taka-fumi Yamaguchi, and Ms. Yumiko Iga for early work on the protein purification and crystallization.

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AUGUST 3, 2012•VOLUME 287•NUMBER 32 JOURNAL OF BIOLOGICAL CHEMISTRY 26537
Dimer of Dimers of Corepressor Tup1p Tetramer

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