Homodimeric Quaternary Structure Is Required for the in Vivo Function and Thermal Stability of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* RNA Triphosphatases*‡

Received for publication, March 25, 2003, and in revised form, May 22, 2003 Published, JBC Papers in Press, June 3, 2003, DOI 10.1074/jbc.M303060200

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*Saccharomyces cerevisiae* Cet1 and *Schizosaccharomyces pombe* Pct1 are the essential RNA triphosphatase components of the mRNA capping apparatus of budding and fission yeast, respectively. Cet1 and Pct1 share a baroque active site architecture and a homodimeric quaternary structure. The active site is located within a topologically closed hydrophobic β-barrel (the triphosphate tunnel) that rests on a globular core domain (the pedestal) composed of elements from both protomers of the homodimer. Earlier studies of the effects of alanine cluster mutations at the crystallographic dimer interface of Cet1 suggested that homodimerization is important for triphosphatase function in vivo, albeit not for catalysis. Here, we studied the effects of 14 single-alanine mutations on Cet1 activity and thereby pinpointed Asp280 as a critical side chain required for dimer formation. We find that disruption of the dimer interface is lethal in vivo and renders Cet1 activity thermolabile at physiological temperatures in vitro. In addition, we identify individual residues within the pedestal domain (Ile970, Leu971, Ile972, Phe973, Leu974, and Ile979) that stabilize Cet1 in vivo and in vitro. In the case of Pct1, we show that dimerization depends on the peptide segment ILE41VPKIEMNFLN50 located immediately prior to the start of the Pct1 catalytic domain. Deletion of this peptide converts Pct1 into a catalytically active monomer that is defective in vivo in *S. pombe* and hypersensitive to thermal inactivation in vitro. Our findings suggest an explanation for the conservation of quaternary structure in fungal RNA triphosphatases, whereby the delicate tunnel architecture of the active site is stabilized by the homodimeric pedestal domain.

RNA triphosphatase catalyzes the first step in mRNA cap formation, the cleavage of the β-γ phosphodiester bond of 5′-triphosphate RNA to yield a diphosphate end. In the second step of the pathway, the RNA diphosphate is capped with GMP by RNA guanylyltransferase to yield GpppRNA (1). The budding yeast *Saccharomyces cerevisiae* encodes separate triphosphatase (Cet1; 549 aa) and guanylyltransferase (Ceg1; 459 aa) proteins that interact in trans to form a stable capping enzyme complex consisting of one Ceg1 protomer bound to a dimer of Cet1 (2–5). Although the fission yeast *Schizosaccharomyces pombe* also encodes separate triphosphatase (Pct1; 303 aa) and guanylyltransferase (Pce1; 402 aa) enzymes, they do not interact with each other (6–8).

The Cet1-Ceg1 interaction stabilizes the intrinsically labile guanylyltransferase activity of Ceg1 against thermal inactivation at physiological temperatures (9). In addition, the physical tethering of Cet1 to Ceg1 facilitates recruitment of the triphosphatase to the RNA polymerase II elongation complex, via Ceg1 binding to the phosphorylated C-terminal domain (CTD) of the largest subunit of RNA polymerase II (10–13). Cet1 by itself does not interact with the CTD. In contrast, the *S. pombe* guanylyltransferase Pce1 is inherently thermostable, and its stability is unaffected by the presence of the triphosphatase Pct1 (9). Also, *S. pombe* employs a distinctive strategy of targeting capping to polymerase II transcripts, whereby the Pct1 and Pce1 enzymes bind independently to the phosphorylated CTD (8). Thus, the fission yeast has elided on both counts the need for a triphosphatase-guanylyltransferase complex. It is therefore not surprising that Pct1 has no counterpart of the surface domain of Cet1 that mediates binding to the guanylyltransferase.

Although the fungal triphosphatase components display species-specific differences in their protein-protein interactions, they are nonetheless conserved with respect to their active site architecture, catalytic mechanism, and quaternary structure. The yeast triphosphatases belong to a family of metal-dependent phosphohydrolases that embraces the RNA triphosphatase components of the capping enzymes of unicellular eukaryotes and certain DNA viruses (7, 14). The family is defined by the presence of two conserved glutamate-containing motifs (61 and β11 in Fig. 1) and the signature property of hydrolyzing NTPs to NDPs in the presence of manganese or cobalt (7,14). The crystal structure of the *S. cerevisiae* RNA triphosphatase Cet1 revealed that the enzyme is a homodimer with active sites located within parallel topologically closed tunnels composed of eight β strands (16) (Fig. 1). The “triphosphate tunnel” architecture is supported by an intricate network of hydrogen bonds and electrostatic interactions within the cavity, most of which are required for catalytic activity (17,18). The tunnel floor rests on a globular “pedestal” domain. Amino acid sequence comparisons and mutational analyses of the RNA triphosphatases from other fungi (e.g. *Candida albicans* and *S. pombe*), microsporidia, protozoa, and Chlorella virus underscore the conservation of the β strands that comprise the triphosphate tunnel (15). Mutational analyses of the *C. albicans* and *S. pombe* RNA triphosphatases indicate that their active sites and catalytic mechanism adhere closely to that of Cet1 (7, 19).

The *S. cerevisiae* and *S. pombe* RNA triphosphatases are
both homodimers (4, 7, 16). Available evidence indicates that homodimer formation is essential for Cet1 function in vivo but not for catalytic activity. Deletion analysis showed that the C-terminal domain Cet1(276–549) has a monomeric quaternary structure and retains activity in vitro (4). However, the monomeric domain by itself cannot support yeast cell growth, even when it is overexpressed at high gene dosage under the control of a strong promoter. Interpretation of the deletion data is complicated by the fact that an N-terminal truncation to position 275 also removes the guanylyltransferase-binding site W247AQQW251, which is located on the protein surface (4, 16, 20, 21) and is responsible for Cet1-mediated stabilization of the guanylyltransferase Ceg1 (9). The in vivo function of Cet1(276–549) is restored when the monomeric triphosphatase is fused to either S. pombe guanylyltransferase (Pce1) or the guanylyltransferase domain of mammalian capping enzyme (4, 9). The phosphorylated CTD and can thereby act as the 247WAQKW251 peptide of Cet1 (9).

enzymes bypass the need for the Ceg1-stabilization function of S. cerevisiae 549) is restored when the monomeric triphosphatase is fused to alanine mutations on Cet1 activity in vivo  and probe the role of quaternary structure in triphosphatase and thereby pin down the need for the Ceg1-stabilization function of the 247WAQKW251 peptide of Cet1 (9).

To focus specifically on the role of homodimerization in Cet1 function in vivo, we previously performed an alanine cluster mutational analysis guided by the Cet1 crystal structure (24). Double-alanine mutations at vicinal amino acids were introduced into the biologically active protein Cet1(201–549), which contains both the guanylyltransferase-binding and catalytic domains. A total of 42 residues were changed to alanine, 24 of which were constituents of the crystallographic dimer interface. Four of the Ala cluster alleles were lethal in vivo. Three other Ala cluster mutants displayed temperature-sensitive (ts) growth defects, even when the mutant alleles were present in high copy under the control of a strong promoter. Several of the lethal and ts mutations were suppressed by fusion of the Cet1-Ala/Ala protein to the mammalian guanylyltransferase. Moreover, two of the lethal mutant proteins were characterized in vitro and found to be catalytically active monomers (24). These results indicated that homodimerization of the budding yeast RNA triphosphatase is critical in vivo when Cet1 functions in concert with the endogenous yeast guanylyltransferase.

It remains unclear why homodimerization of yeast RNA triphosphatase is important in vivo. If the dimer is critical for the functional interactions of S. cerevisiae RNA triphosphatase and RNA guanylyltransferase, then it is not obvious why a homodimeric quaternary structure for RNA triphosphatase would be conserved in the fusion yeast S. pombe, where the triphosphatase and guanylyltransferase components do not interact physically. On the other hand, homodimerization may confer added value to the triphosphatase in other ways that are independent of the guanylyltransferase component of the capping apparatus.

Here we conduct a series of experiments to define the individual essential constituents of the Cet1 homodimer interface and probe the role of quaternary structure in triphosphatase function in vivo and in vitro. Guided by the initial results of the Ala cluster mutagenesis, we tested the effects of 14 single-alanine mutations on Cet1 activity in vivo and thereby pinpointed Asp300 as a critical side chain required for dimer formation. We find that disruption of the dimer interface renders Cet1 thermolabile in vitro. We engineered a catalytically active monomeric version of S. pombe Pct1 and show that it is thermolabile in vitro. Introduction of the monomeric triphosphatase into a S. pombe pctl Δ strain confers a dosage-suppresive lethal phenotype in vivo. We propose a model whereby homodimerization of the globular pedestal domain is critical to stabilize the delicate active site tunnel architecture of the fungal RNA triphosphatases.

EXPERIMENTAL PROCEDURES

Mutagenesis of S. cerevisiae RNA Triphosphatase—Alanine mutations were introduced into the CET1(201–549) gene by PCR (25). The mutated genes were inserted into the yeast CEN TRP1 plasmid pCET1–5′3′, where expression of the inserted gene is under the control of the natural CET1 promoter (26). The inserts were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The in vivo activity of the mutated CET1 alleles was tested by plasmid yeast. Strain YBS20 (trpl ura3 leu2 cet1Δ::LEU2 pSC80-CET1[CEN URA3 CET1]) was transformed with TRP1 plasmids containing the wild-type and mutant alleles of CET1(201–549). Trp+ isolates were selected and then streaked on agar plates containing 0.75 mg/ml 5-fluoroorotic acid (5-FOA). Growth was scored after 7 days of incubation at 18°, 25°, 30°, and 37° C. Lethal mutants were those that failed to form colonies on 5-FOA at any temperature. Individual colonies of the viable CET1 mutants were picked from the FOA plate at permissive temperature and patched to YPD agar. Two isolates of each mutant were tested for growth on YPD agar at 18°, 25°, 30°, and 37° C. Growth was assessed as follows: ++ indicates colony size indistinguishable from strains bearing wild-type CET1(201–549); ++ denotes slightly reduced colony size; + indicates that only pinpoint colonies were formed.

Purification of Recombinant S. cerevisiae RNA Triphosphatase–NdeI BamHI fragments encoding mutated versions of CET1(201–549) were excised from the respective pCET1–5′3′ plasmids and inserted into pET16b. Wild-type CET1(201–549) and the CET1(201–549)-Ala mutants were expressed in Escherichia coli BL21(DE3) at 18°C by isopropyl-1-thio-β-galactopyranoside induction for 20 h in the presence of 2% ethanol (14). The purified proteins were purified from soluble bacterial lysates by nickel-agarose chromatography as described previously (4, 14). The 0.2 M imidazole eluate fractions containing CET1(201–549) were dialyzed against 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM DTT, 10% glycerol, 0.05% Triton X-100, then stored at −80°C.

Mutagenesis of S. pombe RNA Triphosphatase—Gene fragments encoding N-terminal-truncated versions of Pct1 were generated by PCR amplification using sense primers that introduced an NdeI site at the codons for amino acids 41 or 51. The antisense primers introduced a BamHI site immediately downstream of the stop codon. The PCR products were digested with NdeI and BamHI and then inserted into pET16b. Full-length Pct1 and the N40 and N50 mutants were produced in E. coli as N-terminal His6-tagged fusions and purified from soluble bacterial lysates by nickel-agarose chromatography as described previously (3, 8).

Glycerol Gradient Sedimentation—Aliquots (45 μg) of the nickel-agarose preparations of wild-type CET1(201–549) and the D280A mutant were mixed with BSA (40 μg) and cytochrome c (40 μg) in 0.2 ml of buffer G (50 mM Tris HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.05% Triton X-100). Aliquots (50 μg) of the nickel-agarose preparations of wild-type Pct1 and the N40 and N50 mutants were mixed with BSA (50 μg), ovalbumin (50 μg), and cytochrome c (50 μg) in 0.2 ml of buffer G. The mixtures were layered onto 4.8 ml of 15–30% glycerol gradients containing buffer G. The gradients were centrifuged in a Beckman SW50 rotor at 50,000 rpm for 24 h at 4°C. Fractions (−0.2 ml) were collected from the bottoms of the tubes. Aliquots (20 μl) of odd-numbered fractions were analyzed by SDS-PAGE. Polypeptides were visualized by staining with Coomassie Blue dye.

Assay of Triphosphatase Activity—Reaction mixtures (10 μl) containing 50 mM Tris HCl (pH 7.5), 5 mM DTT, 2 mM MnCl2, 1 mM [γ-32P]ATP, and Cet1 or Pct1 as specified were incubated for 15 min at 30°C. The reactions were quenched by adding 2.5 μl of 5 M formic acid. An aliquot of the mixture was applied to a polyethyleneimine-cellulose TLC plate, visualized with Coomassie blue, and autoradiographed with a phosphorimager.

Test of RNA Triphosphatase Function in Vivo in S. pombe—The full-length pctlΔ and truncated pctl-N340 and pctl-N150 cDNAs were cloned into the S. pombe expression vectors pREP6IX, pREP4IX, and pREP3X (LEU2 amber) so that the under the control of the nmt1*, nmt1*, and nmt1 promoters, respectively (27, 28). The plasmids were transformed into a heterozygous pctlΔ/pctlΔ marxΔ diploid (29) using the lithium acetate method (30). The Leu+ diploid transformants were selected and then sporulated on MR plates at room temper
perature. A loopful of cells was inoculated into 500 μl of sterile water, and the mixture was incubated overnight at 28 °C with 10 μl of β-glucuronidase (Sigma G7770). The spores were plated on EMM(-Leu) agar medium and incubated at 30 °C. Individual colonies were then re-streaked onto YES agar and on YES agar containing 200 μg/ml G418. Growth was scored after incubation for 5–7 days at 30° and 37 °C.

RESULTS

Structure-based Mutational Analysis of the Cet1 Homodimer Interface—The Cet1 homodimer interface is extensive, with a buried surface area of 1860 Å² per protomer (16). Elements that comprise the dimer interface are strands β2 and β3, helices α1 and α4, the loop immediately preceding α1, the loop between β9 and β10, and the loop between α3 and α4 (see Fig. 1). The molecular contacts of the dimer interface entail multiple hydrophobic interactions and a network of side-chain and main-chain hydrogen bonds. We previously tested the effects of 21 double-alanine mutations of vicinal amino acids on Cet1 function in vivo (24). Twenty-four of the mutated residues were constituents of the crystallographic dimer interface. The Ala cluster mutations also targeted residues in helices α1 and α4 that compose the hydrophobic core of the pedestal upon which the triphosphate tunnel rests. Four of the Ala cluster alleles were lethal in vivo: D279A-D280A, C330A-V331A, L519A-I520A, and F523A-L524A. Three Ala cluster mutants displayed temperature-sensitive (ts) growth defects, even at high gene dosage: F272A-L273A, I470A-I472A, and I529A-I530A.

The cluster mutagenesis approach provided useful information in two respects: (i) it showed that 15 of the amino acids...
Plasmid shuffle of the indicated CET1(201–549) alleles in the yeast cet1Δ strain as performed as described under “Experimental Procedures.” Trp° isolates were selected and then streaked on agar plates containing 0.75 mg/ml 5-FOA. Growth was scored after 7 days of incubation at 25, 30, and 37 °C or 12 days at 18 °C. Lethal mutants D280A and I520A failed to form colonies on 5-FOA at any temperature. Individual colonies of the viable CET1 mutants were picked from the 5-FOA plates at permissive temperature and patched to −Trp agar. Two isolates of each mutant were tested for growth on YPD agar at 18, 25, 30, and 37 °C. Growth was assessed as follows: + + + indicates colony size indistinguishable from strains bearing CET1; + + denotes slightly reduced colony size; + indicates that only pinpoint colonies were formed; − denotes no growth. The cross-dimer and intra-protomer contacts of the 14 side chains see in the Cet1 crystal structure are indicated in the middle and right columns, respectively.

| CET1 mutant | Growth | Cross-dimer contacts | Intra-protomer contacts |
|-------------|--------|----------------------|------------------------|
| F272A       | ++     | ++       | ++       | ++     | Phe272 Ile275 |
| L273A       | ++     | ++       | ++       | ++     | ++     |
| D279A       | ++     | ++       | ++       | ++     | ++     |
| D280A       | +      | +        | +        | +      | +      |
| C330A       | ++     | ++       | ++       | ++     | ++     |
| V331A       | ++     | ++       | ++       | ++     | ++     |
| I470A       | ++     | ++       | ++       | ++     | ++     |
| I472A       | ++     | ++       | ++       | ++     | ++     |
| L519A       | ++     | ++       | ++       | ++     | ++     |
| L524A       | ++     | +        | +        | +      | +      |
| I529A       | ++     | ++       | ++       | ++     | ++     |
| I530A       | ++     | ++       | ++       | ++     | ++     |

Three of the residues at which single-alanine substitutions elicited significant growth defects are components of the crystallographic homodimer interface: Asp280, Ile470, and Ile472. The Asp280 side chain is the only one of these that is strictly essential for cell viability. Thus, Asp280 appears to be an important constituent of the homodimer interface. None of the other three amino acids (Ile470, Phe472, and Leu524) at which alanine mutations resulted in significant growth defects are involved in cross-dimer contacts; rather, they are components of the hydrophobic core of the pedestal domains of the individual protomers.

### Biochemical Characterization of Mutant Enzymes

We produced the 14 CET1(201–549)-Ala proteins in bacteria as His10-tagged fusions and purified them from soluble bacterial lysates by nickel–agarose chromatography. Wild-type CET1(201–549) was purified in parallel. SDS-PAGE analysis showed that the ~44-kDa Cet1(201–549) protein was the predominant species in each enzyme preparation (Fig. 2A). Phosphohydrolase activity was assayed by the release of 32P from [γ-32P]ATP in the presence of manganese chloride (7). The extents of ATP hydrolysis increased as a function of input enzyme for each protein (Fig. 2B). A specific activity for the wild-type CET1(201–549) of 0.31 nmol of ATP hydrolyzed per ng of protein in 15 min at 30 °C was calculated from the slope of the titration curve in the linear range. (This value translates into a turnover number of 16 s⁻¹.) The specific activities of 11 of the CET1(201–549)-Ala mutants were similar (within a factor of 2) to that of the wild-type enzyme, as follows: F272A (79% of wild-type), L273A (96%), D279A (79%), D280A (62%), C330A (100%), V331A (64%), I470A (57%), I472A (77%), L519A (86%), I529A (76%), and I530A (77%). The retention of phosphatase activity by the F272A, L273A, D279A, C330A, V331A, I470A, I472A, L519A, I529A, and I530A proteins is consistent with the observations that each of these ten mutants supported yeast cell growth at two or more of the temperatures tested (Table I). The instructive finding was that the D280A mutation, which was lethal in vivo, had no significant impact on Cet1 phosphohydrolase activity in vitro.

Three of the CET1(201–549)-Ala mutants were catalytically defective; I520A, F523A, and L524A were 2, 5, and 7% as active as wild-type, respectively (Fig. 2B, right panel). The hierarchy of mutational effects on phosphatase activity in vitro paralleled the lethal (I520A) and severe (F523A and L524A) ef-
fected on Cet1 function in vivo (Table I), suggesting a causal relationship between the loss of phosphohydrolase activity and defective cell growth.

Asp280 Is Essential for Homodimerization—The native sizes of the wild-type and D280A proteins were investigated by zonal velocity sedimentation in a glycerol gradient. Marker proteins BSA and cytochrome c were included as internal standards. After centrifugation, the polypeptide compositions of the odd-numbered gradient fractions were analyzed by SDS-PAGE. The sedimentation profile for wild-type Cet1(201–549) is shown in Fig. 3 (top panel). The triphosphatase (44 kDa) sedimented as a discrete peak coincident with BSA (66 kDa), consistent with the wild-type enzyme being an asymmetric homodimer (4,16). The triphosphatase activity profile paralleled the distribution of the Cet1 polypeptide (Fig. 3, bottom panel).

D280A sedimented between BSA and cytochrome c, suggesting that D280A is a monomer (Fig. 3, middle panel). The triphosphatase activity profile of D280A paralleled the distribution of the Cet1 polypeptide in the gradient (Fig. 3, bottom panel). The effects of the D280A single mutation of Cet1 quaternary structure (conversion to monomer) and Cet1 function in vivo (lethality) and in vitro (no significant effect on ATPase activity) are identical to those noted previously for the D279A-D280A cluster mutation (24). Given the present findings that the neighboring residue Asp279 is not important for Cet1 activity, we conclude that the Asp280 side chain per se is essential for Cet1 homodimerization and for Cet1 function in vivo. Control experiments showed that the sedimentation profiles of wild-type Cet1(201–549) and D280A were unaffected by inclusion of 0.2 mM ATP in the glycerol gradient (data not shown).

Mutational Effects on Cet1 Thermal Stability in Vitro—Several of the Cet1-Ala mutations studied here resulted in a ts growth defect in vivo (Table I). To evaluate the basis for the ts phenotype, we compared the thermal stability of wild-type Cet1(201–549) to that of the Cet1(201–549)-Ala mutants. The purified enzymes were preincubated for 10 min at 30 °C, 35 °C, 40 °C, 45 °C, or 50 °C, followed by quenching on ice. The protein samples were then assayed for ATPase activity at 22 °C. The data were expressed as the ratio of ATP hydrolysis by enzyme preincubated at a given test temperature to the activity of the respective unheated control. The thermal inactivation curves are plotted in Fig. 4. The activity of wild-type Cet1(201–549) was stable to preincubation at 30 °C and reduced only 15% by treatment at 40 °C. The activity declined sharply after heating at 45 °C (to 55% of the unheated control value) and 50 °C (to 10% of the control value). The I470A, F523A, L524A, and I530A proteins, which were temperature-sensitive in vivo, were clearly thermolabile in vitro (Fig. 4). The inactivation curves for I470A, F523A, L524A, and I530A were shifted ~15 °C to the left relative to the wild-type enzyme. The L519A mutation, which elicited a less severe ts defect in vivo, shifted the thermal inactivation curve ~10 °C to the left (Fig. 4).

An instructive finding was that the D280A change, which disrupted homodimerization, rendered yeast RNA triphosphatase thermolabile in vitro. Heating for 10 min at 40° and 45 °C reduced D280A phosphohydrolase activity by 82 and 88%, respectively. Thus, homodimerization enhances the stability of yeast RNA triphosphatase at physiological temperatures. Control experiments showed that the heat inactivation profiles of wild-type Cet1(201–549) and D280A were unaffected by inclusion of 0.2 mM ATP in the buffer during the preincubation step (data not shown).
Deletion Analysis of S. pombe RNA Triphosphatase Identifies a Monomeric Catalytic Domain—The primary structure of S. pombe RNA triphosphatase Pct1 resembles the catalytic domains of budding yeast RNA triphosphatases Cet1 and CaCet1 across the segment extending from strand β1 to the C terminus (7). Reference to the crystal structure of Cet1 indicates that the essential active site residues in the β strands that compose the triphosphate tunnel are strictly conserved (denoted by the symbol ^ in Fig. 1). Pct1 (303 aa) is considerably smaller than Cet1(549 aa) or CaCet1 (520 aa), because Cet1 and CaCet1 contain nonessential N-terminal extensions that are missing from Pct1.

To evaluate the contributions of the N-terminal segment to Pct1 structure and function, we constructed two N-terminal deletions, Pct1(41–303) [NΔ40] and Pct1(51–303) [NΔ50]. (The N termini of the NΔ40 and NΔ50 polypeptides are denoted by arrowsheads below the Pct1 sequence in Fig. 1.) The NΔ40 and NΔ50 proteins were produced in bacteria as His10 fusions and purified from soluble bacterial lysates by nickel-agarose chromatography. SDS-PAGE analysis of the imidazole eluate fractions of the full-length and truncated Pct1 proteins revealed similar extents of purification and the expected increments in electrophoretic mobility (Fig. 5A). The phosphohydrolase specific activity of NΔ40 was identical to that of wild-type Pct1, whereas NΔ50 was 61% as active as the full-length enzyme (Fig. 5B). Note that the N terminus of the catalytically active Pct1(51–303) protein corresponds to the proximal margin of the monomeric catalytic domain of S. cerevisiae Cet1.

The native sizes of the Pct1 NΔ40 and NΔ50 proteins were analyzed by glycerol gradient sedimentation (Fig. 6). Marker proteins BSA, ovalbumin, and cytochrome c were included as internal standards. Wild-type Pct1 and NΔ40 sedimented faster than ovalbumin and just slightly ahead of BSA, consistent with both proteins being homodimers. In contrast, NΔ50 sedimented between ovalbumin and cytochrome c, indicating that the deletion of the segment from amino acids 41–50 converted Pct1 into a monomeric enzyme.

Monomeric Pct1 Is Thermolabile in Vitro—The wild-type, NΔ40 and NΔ50 Pct1 preparations were treated for 10 min at either 30, 35, 40, 45, or 50 °C, then quenched on ice and assayed for ATP hydrolysis at 30 °C (Fig. 5C). The phosphohydrolase activities of wild-type Pct1 and the NΔ40 deletion mutant were unaffected by preheating at 30–50 °C. In marked contrast, the NΔ50 enzyme was thermolabile. Brief preincubations at 35°, 40°, and 45 °C reduced enzyme activity by 42, 78, and 84%, respectively (Fig. 5C). The stability or lability of the Pct1 mutants correlated with their homodimeric versus monomeric quaternary structures. Thus, we surmise that homodimerization stabilizes S. pombe RNA triphosphatase at physiological temperatures, a feature shared with S. cerevisiae Cet1.

Homodimerization Is Important for Pct1 Function in Vivo in S. pombe—The full-length pct1+ and truncated pct1NΔ3 cDNAs
were cloned into the S. pombe expression vectors pREP81X, pREP41X, and pREP3x (LEU2 ars1′) so as to place them under the control of the nmt1** (low strength), nmt1* (medium strength), and nmt1 (full strength) promoters, respectively. The normalized constitutive expression levels provided by these three promoters are 1 × (nmt1**), 3.5 × (nmt1*), and 42 × (nmt1) (28). The expression plasmids were introduced into a heterozygous S. pombe pctl/pctl kanMX diploid strain in which one of the pctl alleles was replaced with a gene conferring G418 resistance (29). The Leu haploid transformants were selected and then sporulated. Random populations of 32 individual Leu haploids were tested for G418 resistance or sensitivity. We found that half of the Leu haploids derived from a pctl/pctl kanMX strain containing a pREP81X plasmid with the pctl cDNA under the control of the intermediate promoter also contained the pctl kanMX chromosomal allele and were resistant to G418 (Table II). In contrast, none of the Leu haploids obtained by sporulating the pctl pctl kanMX diploid containing an empty pREP vector were resistant to G418 (29). These results show that the pctl strain is viable if the chromosomal deletion is complemented by an extrachromosomal triphosphatase gene driven by a weak promoter. Complementation was also observed when the wild-type pctl cDNA was under the control of the intermediate-strength nmt1* promoter (29).

The pctl-NΔ40 allele complemented the pctlΔ null mutation when expression of NΔ40 was driven by the weakest nmt1** promoter (15/32 G418R haploids) or the intermediate-strength nmt1* promoter (16/32 G418R haploids). In contrast, the pctl-NΔ50 mutant was unable to support growth of S. pombe when its expression was driven by the intermediate-strength nmt1* promoter (0/32 G418R haploids) (Table II). However, the lethality of the NΔ50 mutant was suppressed by overexpression under the control of the strong nmt1 promoter (15/32 G418R haploids) (Table II). Although the nmt1** promoter can be repressed ~6-fold by inclusion of 5 μg/ml thiamine in the growth medium (28), we observed that the growth of the plasmid-dependent nmt1**-pctl and nmt1**-pctl-NΔ40 strains was not affected by exogenous thiamine (Fig 7B). We infer that the expression levels of the full-length Pctl1 and truncated NΔ40...
enzymes in these strains exceeded a threshold required for cell viability. In contrast, the growth of the nmt1-pct1-NΔ50 strain was inhibited by thiamine (Fig. 7B). These findings (Table II and Fig. 7B) provide two lines of evidence that the monomeric NΔ50 mutant, although catalytically active in vitro, is unable to perform all of the requisite functions of Pct1 in vivo.

Finally, the viable nmt1**-pct1*, nmt1**-pct1-NΔ40, and nmt1-pct1-NΔ50 strains were tested for growth on rich agar medium at 30°C and 37°C (Fig. 7A). Whereas nmt1**-pct1* and nmt1**-pct1-NΔ40 grew well at both temperatures, the nmt1-pct1-NΔ50 strain displayed an obvious ts growth defect that was in keeping with the thermolability of the NΔ50 enzyme in vitro.

DISCUSSION
Importance of Triphosphatase Homodimerization in Fungi—
The present study shows that a homodimeric quaternary structure is critical for the in vivo function of RNA triphosphatases in two highly divergent species of fungi: the budding yeast S. cerevisiae and the fission yeast S. pombe. The finding that homodimerization ensures the thermal stability of S. cerevisiae and S. pombe triphosphatase activity in vitro at physiological temperatures engenders an explanation for the conservation of quaternary structure, whereby the delicate tunnel architecture of the active site is stabilized by the homodimeric pedestal domain upon which it rests.

The fold of the pedestal domain of Cet1 is composed of multiple secondary structure elements and connecting loops that interdigitate across the homodimer interface (Fig. 1A) (16). The β strands that comprise the triphosphate tunnel project into the pedestal, where they or their interconnecting loops make cross dimer contacts to the partner protomer. Most of the side chains at the dimerization surface of Cet1 have now been subjected to alanine substitution, either in clusters or individually. The present alanine scan highlights the dominant contributions of Asp279 to the dimer interface of Cet1. No other single alanine mutation tested here resulted in unconditional lethality in vivo, although single mutations of several other side chains at the dimer interface resulted in conditional phenotypes in vivo and thermolability in vitro. These effects are discussed in detail below.

In the case of fission yeast triphosphatase Pct1, we show that dimerization, thermal stability, and in vivo activity depend on the peptide segment 41VPKIELNFNL250 located immediately prior to the start of the Pct1 catalytic domain. The 48FL250 dipeptide within this segment of Pct1 corresponds to the 27FL270 dipeptide of Cet1, which is an essential component of the Cet1 dimer interface, i.e. an F272AL272A double mutation of Cet1 converts it into a catalytically active monomer that is thermosensitive in vivo (24) We suspect therefore that the homodimer interface of S. pombe Pct1 is at least partially similar to that of S. cerevisiae Cet1.

Initial speculations as to why S. cerevisiae RNA triphosphatase is a homodimer focused on a possible role for dimerization in the context of the heterotrimeric triphosphatase-guanylyltransferase complex, whereby the guanylyltransferase Ceg1 bound to the surface peptide 24WAGKW251 in one protomer of Cet1 might make interactions with the other Cet1 protomer that are relevant to cap formation (24). Although not excluding this idea, the present finding that triphosphatase homodimerization is conserved and essential in S. pombe, where the triphosphatase and guanylyltransferase components do not interact physically, suggests either that: (i) homodimerization confers added value to the fungal triphosphatases in a common manner that is independent of the guanylyltransferases, or (ii) triphosphatase homodimerization is required for different reasons in budding yeast and fission yeast. For reasons of parsimony, we invoke the former model and posit, based on concordant biochemical properties of the monomeric versions of Cet1 and Pct1, that stabilization of the triphosphatase fold is the principal value added by dimerization.

This is not to say that stabilization is the only benefit of homodimerization. For example, the S. pombe triphosphatase Pct1 interacts directly with several protein components of the transcription elongation complex, including: (i) the phosphorylated CTD of S. pombe polymerase II (8); (ii) the S. pombe ortholog of transcription elongation factor Spt5 (31); and (iii) S. pombe Cdk9, a cyclin-dependent protein kinase that phosphorylates S. pombe Spt5 and the polymerase II CTD (32). Any one or several of these protein-protein interactions of Pct1 might be affected by the dissociation of the Pct1 homodimer, either because the interactions entail contacts with both protomers of the dimer or Pct1 needs to interact simultaneously in vivo with more than one of its binding partners, each one being tethered to a different Pct1 protomer. Recent studies in S. cerevisiae have imputed new functions to the Cet1 in transcriptional repression in vitro (33) and in vivo (34), and it is conceivable that Cet1 dimerization impacts on those functions. Nonetheless, it is improbable that the adverse effects of monomerization of Cet1 on S. cerevisiae cell growth can be attributed to ancillary functions other than cap formation, because the lethal cet1Δ null mutation is rescued completely by the mammalian capping enzyme, whose triphosphatase component has no structural or mechanistic similarity whatsoever to yeast Cet1 (26, 35, 36).

In summary, the available evidence points to protein stabilization as a force behind the conservation of homodimeric quaternary structure among known fungal RNA triphosphatases involved in mRNA cap formation. It will be of interest to determine if nonfungal members of the tunnel family of RNA triphosphatases also rely on dimerization for stability. To date, we know that the RNA triphosphatase of the microsporidian parasite Encephalotozoon cuniculi is also a homodimeric tunnel family enzyme (37), but its dimer interface is undefined. *Microsporidia* are believed to be phylogenetically close to fungi. Tunnel family RNA triphosphatases are also found in protozoan parasites, including *Plasmodium* and *Trypanosoma* (38–40), but their quaternary structures have not been determined.

Structural Interpretations of the Cet1 Mutational Effects—
Cluster mutagenesis results had located two functionally important facets of the Cet1 homodimer interface (24). One of these entails hydrophobic interactions between the side chains of strand β2 of one protomer and strand β3 in the other protomer. This facet of the interface also embraces Asp279 (in α1), which engages in a cross-dimer hydrogen bond with the backbone amide of Gln229 in the turn connecting β2 and β3 (Fig. 8). We showed previously that two Ala cluster mutations involving or flanking these residues (D279A-D280A and C230A-V231A) elicited lethal phenotypes in vivo (24). Here we find that single-alanine mutations of Cys310, Val311, and Asp279 did not elicit
significant effects on cell growth or triphosphatase activity in vitro, implying that their individual contributions to Cet1 function are either negligible or subtle at best. The single D280A mutation was lethal in vivo. Biochemical analysis of the recombinant D280A protein confirmed that its phosphohydrolase activity was intact and that the mutant protein sedimented as a monomer. Thus, we attribute the lethal in vivo phenotype of D280A to an isolated defect in homodimerization.

The Asp$^{280}$ side chain emerges from this analysis as a critical determinant of Cet1 homodimerization. The Asp$^{280}$ carboxylate coordinates two separate structural elements on the partner protomer: (i) O$^{\text{H9254}}$ accepts a hydrogen bond from the backbone amide of Gln$^{329}$ at the top of the loop connecting the $\beta_2$ and $\beta_3$ strands, and (ii) O$^{\text{H9252}}$ forms a bifurcated salt bridge with two arginine side chains (Arg$^{531}$ and Arg$^{525}$) in helix $\alpha_4$ of the partner protomer (Fig. 8).

A second functionally relevant dimer interface involves hydrophobic side-chain interactions between $\alpha_4$ and residues Phe$^{272}$ and Leu$^{273}$ in the loop preceding $\alpha_1$. We showed previously that simultaneous replacement of the Phe$^{272}$ and Leu$^{273}$ side chains by alanine resulted in a catalytically active monomeric enzyme and a severe ts growth phenotype in vivo. In the current study, we find that the single L273A mutation had no effect on activity in vivo or in vitro, whereas the single F272A mutation conferred a weak ts phenotype in vivo without affecting catalysis in vitro. Thus, neither Phe$^{272}$ nor Leu$^{273}$ per se is essential for function.

Phe$^{272}$ and Leu$^{273}$ make van der Waals contacts with Ile$^{470}$, Ile$^{529}$, and Ile$^{530}$ in the partner protomer. We found earlier that cluster mutants I470A-I472A and I529A-I530A were unable to grow at 25, 30, or 37 °C and barely grew at 14 °C (24). Here we see that the single I529A mutation had no impact on Cet1 function and the I472A change conferred only a weak ts phenotype. In contrast, the single I470A and I530A mutations resulted in tight ts growth defects that correlated with the thermolability of the I470A and I530A proteins in vitro. The thermolability of I470A is likely to result from a combination of factors, including: (i) effects on dimerization caused by the loss of contact between Ile$^{470}$ and Phe$^{272}$ on the partner protomer and (ii) effects of the I470A mutation on the conformation of the $\beta_1$ strand caused by loss of the intra-protomer contact between Ile$^{470}$ and Leu$^{495}$ (see below).

Similarly, the I530A temperature sensitivity may also be multifactorial, insofar as the Ile$^{530}$ side chain, which contacts Phe$^{272}$ in the partner protomer, makes additional intramolecular contacts within the hydrophobic core of the pedestal involving Phe$^{312}$ in $\beta_1$ and Val$^{495}$ and Leu$^{495}$ in $\beta_11$ that support the floor of the triphosphate tunnel (Table I). Neighboring residues Glu$^{305}$ and Glu$^{307}$ in $\beta_1$ and Glu$^{494}$ and Glu$^{496}$ in $\beta_11$ bind the essential metal cofactor and are required for catalysis.

**Fig. 7.** Effects of N-terminal deletions on Pct1 function in vivo. *pct1Δ* haploid strains containing plasmid-borne *nmt1*-pct1*Δ* (WT), *nmt1*-pct1-N$^{40}$, or *nmt1*-pct1-N$^{50}$ alleles were patched on EMM(-Leu) agar medium and then streaked: A, on YES agar medium at 30 °C (top) or 37 °C (bottom); B, at 30 °C on EMM(-Leu) agar medium containing no thiamine (top) or 5 μg/ml thiamine (bottom). The plates were photographed after 5 days.

**Fig. 8.** Cross-dimer interactions of Asp$^{280}$.
Because the I530A phenotype is more severe than the F272A mutation in its cross-dimer contact (Table I), we surmise that the Ile530 change may directly destabilize the structure of the tunnel. This hypothesis is consistent with the previous findings that a single alanine mutation of Phe315 in b1 was lethal and single alanine mutations of Val483 or Leu585 in b11 (the other residues contacted intramolecularly by Ile530) resulted in ts growth defects at 37 °C and thermostabilization of Cet1 triphosphatase activity in vitro (17).

The L519A mutation resulted in a weak ts growth phenotype and had no effect on catalysis in vitro, although the L519A protein was sensitized to thermal inactivation. Although Leu519 makes a cross-dimer contact with Ile268, we suspect that the ts defects do not reflect the simple loss of this contact, because mutation of Ile268 to alanine had no effect on growth (26). Leu519 makes other intramolecular contacts to Cys467, Ile497, and Leu502 that tether the floor of the tunnel to the pedestal.

Three of the single alanine mutations studied here resulted in lethal (I520A) or tight ts (F523A and L524A) growth phenotypes that correlated with major defects in catalysis of γ phosphatase hydrolysis. These phenotypes are likely caused by effects on active site architecture rather than quaternary structure, because Ile520, Phe523, and Leu534 are oriented toward the hydrophobic core of the Cet1 protomer, and they make no contributions to the crystallographic dimer interface, although they make extensive contacts within the Cet1 protomer (Table I). Ile520 makes van der Waals contacts to Val285 and Val289 in helix a1 and also to Tyr716 and Leu824 in helix a4. This network of hydrophobic interactions stabilizes the helix packing within the pedestal, and its disruption by mutation would likely have global effects on Cet1 folding. Leu524 interacts with Val395, Ile428, and Ile520. Phe523 is situated in a hydrophobic-aromatic-hydrophobic sandwich between Met308 (in b1), Ile428 (in b8), and Ile497 (in b11) that imparts stability to the floor of the tunnel.

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J. Biol. Chem. 2003, 278:30487-30496.
doi: 10.1074/jbc.M303060200 originally published online June 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303060200

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