Importance of Homodimerization for the in Vivo Function of Yeast RNA Triphosphatase*

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Saccharomyces cerevisiae RNA triphosphatase Cet1 is an essential component of the yeast mRNA capping apparatus. The active site of Cet1 resides within a topologically closed hydrophilic β-barrel (the triphosphate tunnel) that is supported by a globular hydrophobic core. The homodimeric quaternary structure of Cet1 is formed by a network of contacts between the partner protomers. By studying the effects of alanine-cluster mutations, we highlight the contributions of two separate facets of the crystallographic dimer interface to Cet1 function in vivo. One essential facet of the interface entails hydrophobic cross-dimer interactions of Cys 230 and Val 331 and a cross-dimer hydrogen bond of Asp 280 with the backbone amide of Gln 329. The second functionally relevant dimer interface involves hydrophobic side-chain interactions of Phe 272 and Leu 273. Ala-cluster mutations involving these residues elicited lethal or severe temperature-sensitive phenotypes that were suppressed completely by fusion of the mutated triphosphatases to the guanylyltransferase domain of mammalian capping enzyme. The recombinant D279A-D280A and F272A-L273A proteins retained phosphohydrolase activity but sedimented as monomers. These results indicate that a disruption of the dimer interface is uniquely deleterious when the yeast RNA triphosphatase must function in concert with the endogenous yeast guanylyltransferase. We also identify key residue pairs in the hydrophobic core of the Cet1 protomer that support the active site tunnel and stabilize the triphosphatase in vivo.

RNA triphosphatase catalyzes the first step in mRNA cap formation, the cleavage of the β-γ phosphoanhydride bond of 5'-triphosphate RNA to yield a diphosphate end that is then capped with GMP by RNA guanylyltransferase (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 heteromeric capping enzyme complex. Yeast cell growth depends on the catalytic activity of both enzymes and their physical interaction.

The binding of yeast Cet1 to Ceg1 elicits two apparently beneficial outcomes. First, Cet1-Ceg1 interaction stabilizes the intrinsically labile guanylyltransferase activity of Ceg1 against thermal inactivation at physiological temperatures. Second, the physical tethering of Cet1 to Ceg1 may facilitate recruitment of the triphosphatase to the RNA polymerase II elongation complex. Ceg1 binds in vitro and in vivo to the phosphorylated carboxyl-terminal domain (CTD) of the largest subunit of RNA polymerase II, whereas Cet1 by itself does not interact in vitro with the phosphorylated CTD (3–6).

Cet1 consists of three domains: (i) a 230-aa amino-terminal segment that is dispensable for catalysis in vitro and for Cet1 function in vivo; (ii) a protease-sensitive segment from residues 230 to 275 that is dispensable for catalysis but essential for Cet1 function in vivo; and (iii) a catalytic domain from residues 275 to 549 (7). A homodimeric quaternary structure for the biologically active Cet1 protein was inferred from analysis of the purified recombinant enzyme by gel gradient sedimentation and then confirmed by x-ray crystallography (7, 8). A Cet1-Ceg1 capping enzyme complex reconstituted in vitro from separately purified components is surmised from velocity sedimentation analysis to be a heterotrimer consisting of two molecules of triphosphatase and one molecule of guanylyltransferase (7).

Is homodimer formation essential for Cet1 function? Deletion analysis shows that the carboxyl-terminal domain Cet1(276–549) has a monomeric quaternary structure, yet it retains full catalytic activity in vitro (7). Thus, homodimerization is not essential for catalysis. 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 amino-terminal truncation to position 275 also removes the guanylyltransferase-binding site 243WAKQW247, which is located on the protein surface (9) and is responsible for Cet1-mediated stabilization of the guanylyltransferase Ceg1.2 Remarkably, the in vivo function of Cet1(276–549) is completely restored when the monomeric triphosphatase domain is fused in cis to the guanylyltransferase domain of the mouse capping enzyme (7). The mouse domain, Mcc1(211–597), binds avidly to the phosphorylated CTD (10) and can thereby act as a vehicle to deliver the fused RNA triphosphatase to the RNA polymerase II elongation complex (7, 11). Also, because the mouse guanylyltransferase is thermostable (unlike Ceg1), the chimeric capping enzyme bypasses the need for the Ceg1-stabilization function of the 243WAKQW247 peptide of Cet1.2

To focus specifically on the role of homodimerization in Cet1 function in vivo, we have initiated a mutational analysis of the amino acids comprising the homodimer interface revealed by the crystal structure (8). Alanine-cluster mutations were introduced into the biologically active protein Cet1(201–549), which contains both the guanylyltransferase-binding and catalytic domains (7). The results of this analysis indicate that ho-

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1 The abbreviations used are: aa, amino acids; CTD, carboxyl-terminal domain; 5-POA, 5-fluoroacetate acid; DTT, dithiothreitol; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis.

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modimerization of yeast RNA triphosphatase is important for its function in vivo.

EXPERIMENTAL PROCEDURES

Mutagenesis of Yeast RNA Triphosphatase—Alanine-cluster mutations were introduced into the CET1(201–549) gene by polymerase chain reaction (12). The mutated genes were inserted into the yeast CEN TRP1 plasmid pCET1–59, where expression of the inserted gene is under the control of the natural CET1 promoter (13). The inserts were sequenced completely to exclude the acquisition of unwanted mutations during amplification and cloning. The genes were excised from their respective pCET1–59 plasmids with NdeI and BamHI and inserted into the yeast expression vector pYN132 (CEN TRP1). In this vector, expression of CET1(201–549) is driven by the strong constitutive yeast TPI1 promoter. NdeI/BamHI restriction fragments containing the mutated genes were also cloned into the multicopy expression plasmid pYX232 (2 μm TRP1) with triphosphatase expression being driven by the TPI1 promoter. The in vivo activity of the mutated CET1 alleles was tested by plasmid shuffle. Yeast strain YBS20 (trp1Δ trp1 ura3 leu2ΔΔΔΔ) was transformed with pYN132 (CEN URA3 CET1 :: CEN TRP1) containing 50 mM Tris HCl, pH 8.0, 1 M NaCl, 2 mM DTT, 10% glycerol, 0.05% Triton X-100, and then stored at ~80°C. Trichlorophosphatase reaction mixtures (10 μl) containing 50 mM Tris HCl (pH 7.5), 5 mM DTT, 2 mM MnCl2, 1 μM [γ-32P]ATP, and CET1(201–549) 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, which was developed with 0.5 M LiCl and 1 M formic acid. The release of 32P from [γ-32P]ATP was quantitated by scanning the TLC plate with a PhosphorImager.

Glycerol Gradient Sedimentation—Aliquots (30 μg) of the nickel-agarose preparations of the wild-type and mutant CET1(201–549) proteins were mixed with BSA (25 μg), and cytochrome c (25 μg) in 0.2 ml of buffer G (50 mM Tris HCl, pH 8.0, 100 mM NaCl, 2 mM DTT, 15–30% glycerol gradients containing buffer G. The gradients were centrifuged in a Beckman SW50 rotor at 50,000 rpm for 25 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 along with samples of the input protein mixtures for each gradient. Polypeptides were visualized by staining with Coomassie Blue dye. Aliquots (1 μl) of each fraction were assayed for triphosphatase activity as described above.

RESULTS

Structure-based Mutational Analysis of the CET1 Homodimer Interface—Yeast RNA triphosphatase crystallized as a homodimer (Fig. 1A). Each protomer is composed of four α helices and 11 β strands. The secondary structure elements are displayed over the CET1 protein sequence in Fig. 2. The striking feature of the tertiary structure is the formation of a topologically closed tunnel composed of 8 antiparallel β strands. In the dimer, the two tunnels are parallel and oriented in the same direction. A surface view of the monomer is shown in Fig. 1A looking into the tunnel. Rotation of the molecule provides a side view looking into the tunnel entrance. The Phe272 side chain is shown in green.
view that highlights a platform-like structure in front of the tunnel entrance (Fig. 1B). The triphosphatase active site resides within the tunnel. The guanylyltransferase-binding site is located on the surface and is colored red in Fig. 1.

The Cet1 dimer interface (colored green in Fig. 1) is extensive, with a buried surface area of 1860 Å² per protomer. 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. The molecular contacts of the dimer interface are detailed in Table I. These entail multiple hydrophobic interactions and a network of side-chain and main-chain hydrogen bonds. The hydrophobic core of the dimer interface is stabilized by interactions of residues Ile268, Phe272, Leu273, and Pro277 (located immediately proximal to α1) with hydrophobic residues of the dimer partner located in the segments from 354–368, 463–467, and 519–530 (Fig. 3). Indeed, the aromatic side chain of Phe272 inserts into a small hydrophobic pocket on the surface of the dimer partner (Fig. 1C). Additional hydrophobic interactions occur between side chains of strand β2 of one protomer (325–330) and strand β3 in the partner (330–334) (Table I). Main-chain hydrogen bonds between β2 and β3 form an antiparallel sheet at the dimer interface.

To gauge the role of homodimerization in Cet1 function, we performed alanine-cluster mutagenesis of pairs of vicinal amino acids. The double-Ala mutations were introduced into the biologically active Cet1(201–549) protein. A total of 42 residues (17% of the polypeptide) were changed to alanine in this analysis, 24 of which were constituents of the crystallographic dimer interface. The cluster mutations also embraced residues in helices α1 and α4 that comprise the hydrophobic core of the Cet1 protomer upon which the triphosphate tunnel rests.

**Mutational Effects in Vivo**—The CET1(201–549)-Ala/Ala genes were cloned into a CEN TRPI vector under the control of the natural CET1 promoter and then tested by plasmid shuffle for their ability to complement a cet1Δ strain of S. cerevisiae. The results are summarized in Table I. Growth of cet1Δ is contingent upon maintenance of a wild-type CET1 allele on a CEN URA3 plasmid. Therefore, the cet1Δ strain is unable to grow on agar medium containing 5-FOA (5-fluoroorotic acid, a drug which selects against the URA3 plasmid) unless it is first transformed with a biologically active RNA triphosphatase gene on the TRPI plasmid. Trp⁺ CET1(201–549)-Ala/Ala transformants were tested for growth on 5-FOA. Triphosphatase mutations were judged to be lethal if they failed to support colony formation on 5-FOA after prolonged incubation at four different temperatures (14, 25, 30, and 37 °C). Four of the Ala-cluster alleles were lethal by this criterion: D279A-D280A, C330A-V331A, L519A-I520A, and F523A-L524A.

Four other Ala-cluster mutants displayed a conditional phenotype, whereby they gave rise to 5-FOA-resistant colonies at low temperatures but failed to yield colonies at high temperatures. These CET1(201–549)-Ala/Ala strains were isolated from 5-FOA plates at permissive temperature and then tested for growth on rich medium (YPD) at 14, 25, 30, and 37 °C. The D287A-W288A strain grew as well as wild-type yeast at 14 °C (scored as ++ +), but formed small colonies at 25 °C (scored as ++), pinpoint colonies at 30 °C (scored as +), and failed to grow at 37 °C (−growth). The F272A-L273A mutant grew slowly at 14 °C and not at all at 30 °C or higher. The I470A-A472A and I529A-I530A mutations elicited the most severe conditional phenotypes, with weak growth at 14 °C and no growth at 25 °C or higher (Table I).

The remaining 13 double-Ala mutants yielded 5-FOA-resistant colonies at all temperatures and grew as well as the isogenic CET1(201–549) "wild-type" strain on YPD agar at 14, 25, 30, and 37 °C. We infer that the 26 side chains that were substituted in the fully viable mutants are unimportant per se for Cet1 function in vivo. These nonessential residues (highlighted by plus signs over the Cet1 sequence in Fig. 2) included...
15 amino acids that comprise the crystallographic dimer interface (Ile283, Asp286, Pro270, Pro271, Ser284, Pro325, Val326, Ser327, Ser228, Phe329, Thr330, Ile331, Lys367, Lys368, and Asn526). The Ala-cluster mutants that were fully viable were not analyzed further.

**Effects of Increased Gene Expression and Gene Dosage on the Mutant Phenotypes**—The 16 residues substituted in the 8 Ala-cluster alleles that were lethal or ts when expressed in single copy from the natural CET1 promoter are highlighted by dots above the Cet1 sequence in Fig. 2. Six of the eight Ala-clusters entailed substitution of at least one component of the crystallographic dimer interface. If homodimerization is important in vivo, we reasoned that the growth phenotypes elicited by mutations that diminish the dimerization equilibrium constant might be ameliorated by increasing the expression of the mutant protein, in effect attaining a threshold level of dimeric Cet1 via mass action. Also, if some of the mutations reduced triphosphatase activity to a level below the threshold required for growth, then increased gene expression might restore activity to a supra-threshold level. To explore these scenarios, the lethal and ts Ala-cluster alleles were retested for biological activity in single-copy when their expression was driven by the strong constitutive yeast TPI1 promoter instead of the CET1 promoter.

Increased promoter strength completely suppressed the ts phenotype of D287A W288A such that the mutant strain grew as well as the CET1 strain on YPD agar at 37 °C (Table II). Changing promoters partially suppressed the ts phenotypes of F272A L273A, I470A I472A, and I529A I530A. Partial suppression was manifest as an upward shift in the restrictive growth window. For example, I470A I472A under TPI1 control displayed + + + growth at 30 °C, compared with no growth under its natural promoter. F272A L273A grew at 30 and 37 °C (albeit slowly) and I529A I530A was able to grow at 30 °C (Table II). Increased promoter strength did not reverse the lethality of the D279A D280A, C330A V331A, or F523A L524A mutations. However, it did restore partial function to the L519A I520A allele, which was capable of supporting growth at 14 °C, albeit not at 25 °C or higher (Table II).

In light of these effects, we proceeded to transfer the TPI-CET1-Ala/Ala alleles to high-copy 2μ vectors and to test the effects of increased gene dosage on their *in vivo* activities. This maneuver abated the lethality of F523A L524A, which displayed + + + growth at 14 °C, but no growth at 25 °C or higher; it also improved the growth of L519A I520A at 14° and 25 °C (Table II). The other mutational effects were unaffected by increased gene dosage; in particular, the D279A D280 and C330A V331A alleles were lethal even in high copy.

**Rescue of Yeast RNA Triphosphatase Mutations by Fusion to Mouse Guanylyltransferase**—Even a monomeric version of yeast RNA triphosphatase is functional in vivo when it is fused to the guanylyltransferase domain of the mouse capping enzyme, provided that it retains phosphohydrolase catalytic activity (7). Thus, a functional test of chimeric capping enzymes containing Cet1(201–549)-Ala/Ala mutants fused to the mouse guanylyltransferase can discriminate genetically whether the complete or partial loss of function elicited by the Ala-cluster mutations is caused by a catalytic defect (be it a global folding problem or a direct perturbation of the active site) or a defect in ancillary functions uniquely required in yeast cells containing only the endogenous guanylyltransferase Ceg1. In the latter scenario, we would expect the fusion maneuver to restore function to the Cet1(201–549)-Ala/Ala mutant, whereas no salutary effects are expected in the former case.

Cet1(201–549)-Ala/Ala (Table II). The saline findings were that the D279A D280A and C330A V331A alleles, which were lethal even when expressed at high gene dosage, were fully functional at all temperatures when fused to the mouse guanylyltransferase (Table II). We surmise that these two Ala-cluster mutations do not significantly affect phosphohydrolase catalytic activity in vivo. This conclusion is consistent with the distant location of the

| CET1 Mutations | Growth
|---------------|-------|
| K267A I268A   | + + + |
| D279A P270A   | + + + |
| F272A L273A   | + + + |
| I276A P277A   | + + + |
| D279A D280A   | + + + |
| T282A L283A   | + + + |
| S284A V285A   | + + + |
| D287A W288A   | + + + |
| V289A Y290A   | + + + |
| F293A V294A   | + + + |
| P325A V326A   | + + + |
| S327A S328A   | + + + |
| C330A V331A   | + + + |
| F332A T333A   | + + + |
| Y354A I358A   | + + + |
| K367A F368A   | + + + |
| S466A C467A   | + + + |
| I470A I472A   | + + + |
| L519A I520A   | + + + |
| F523A L524A   | + + + |
| N525A N526A   | + + + |
| I529A I530A   | + + + |

### Table I

| CET1 Mutations | Growth
|---------------|-------|
| F272A L273A   | + + + |
| D279A D280A   | + + + |
| D287A W288A   | + + + |
| C330A V331A   | + + + |
| I470A I472A   | + + + |
| L519A I520A   | + + + |
| F523A L524A   | + + + |
| I529A I530A   | + + + |

### Table II

| CET1 Mutations | Growth
|---------------|-------|
| F272A L273A   | + + + |
| D279A D280A   | + + + |
| D287A W288A   | + + + |
| C330A V331A   | + + + |
| I470A I472A   | + + + |
| L519A I520A   | + + + |
| F523A L524A   | + + + |
| I529A I530A   | + + + |

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**Homodimerization of Yeast RNA Triphosphatase**

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**Effects of alanine-cluster mutations on Cet1 function in vivo**

+++ colony size indistinguishable from strains bearing wild-type CET1(201–549); ++, slightly reduced colony size; +, only pinpoint colonies formed; −, colonies failed to grow.

| CET1 Mutations | Growth
|---------------|-------|
| K267A I268A   | + + + |
| D279A P270A   | + + + |
| F272A L273A   | + + + |
| I276A P277A   | + + + |
| D279A D280A   | + + + |
| T282A L283A   | + + + |
| S284A V285A   | + + + |
| D287A W288A   | + + + |
| V289A Y290A   | + + + |
| F293A V294A   | + + + |
| P325A V326A   | + + + |
| S327A S328A   | + + + |
| C330A V331A   | + + + |
| F332A T333A   | + + + |
| Y354A I358A   | + + + |
| K367A F368A   | + + + |
| S466A C467A   | + + + |
| I470A I472A   | + + + |
| L519A I520A   | + + + |
| F523A L524A   | + + + |
| N525A N526A   | + + + |
| I529A I530A   | + + + |

**Suppression of Cet1 mutational effects by overexpression or fusion to mouse guanylyltransferase**

+++ colony size indistinguishable from strains bearing wild-type CET1(201–549); ++, slightly reduced colony size; +, only pinpoint colonies formed; −, colonies failed to grow.

| CET1 Mutations | Growth
|---------------|-------|
| F272A L273A   | + + + |
| D279A D280A   | + + + |
| D287A W288A   | + + + |
| C330A V331A   | + + + |
| I470A I472A   | + + + |
| L519A I520A   | + + + |
| F523A L524A   | + + + |
| I529A I530A   | + + + |
four mutated residues from the enzyme's active site in the crystal structure (8). Because the Asp<sup>280</sup>, Cys<sup>330</sup>, and Val<sup>331</sup> side chains are components of the homodimer interface, we infer that the lethal phenotypes of the cluster mutations reflect a requirement for Cet1 homodimerization for RNA processing by the yeast capping apparatus.

In addition, we found that the other Ala-cluster mutants that displayed <i>ts</i> growth defects when expressed by themselves in single copy under the control of the <i>TP11</i> promoter were suppressed completely or partially by fusion to the mouse guanylyltransferase. Of special note was F272A-L273A, which was fully functional at all temperatures as a chimeric enzyme. The Phe<sup>272</sup> and Leu<sup>272</sup> side chains are components of the hydrophobic dimer interface (Fig. 1C), and we surmise from the genetic evidence that the phenotype of this mutant is likely caused by a dimerization defect. On the other hand, the I529A-I530A chimera supported growth at 30 °C, but not at 37 °C, whereas growth of the L519A-I520A and F523A-L524A chimeras was still severely <i>ts</i>. Apparently, these Ala-cluster mutants are defective in aspects other than, or in addition to, Cet1 homodimerization.

**Biochemical Characterization of Mutant Enzymes**—We produced the mutated Cet1(201–549) proteins F272A-L273A and D279A-D280A in bacteria as His<sub>10</sub>-tagged fusions and purified them from soluble bacterial lysates by nickel-agarose chromatography. Wild-type His<sub>10</sub>-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. 4A). Phosphohydrolase activity was assayed by the release of <sup>32</sup>P from [γ<sup>32</sup>P]ATP in the presence of manganese chloride (2). The extents of ATP hydrolysis increased as a function of input enzyme for each protein (Fig. 4B). A specific activity for the wild-type Cet1(201–549) of 0.58 nmol of ATP hydrolysed per nanogram of protein in 15 min was calculated from the slope of the titration curve in the linear range. This value translates into a turnover number of 29 s<sup>−1</sup>. The specific activity of F272A-L273A was virtually identical to that of the wild-type enzyme, whereas D279A-D280A was 30% as active as wild-type. The remaining lethal or <i>ts</i> Ala-cluster mutants were insoluble when produced in bacteria and thus not amenable to biochemical characterization.

The native sizes of the recombinant triphosphatases were investigated by sedimentation through 15–30% glycerol gradients. Marker proteins BSA and cytochrome <i>c</i> were included as internal standards. After centrifugation, the polypeptide compositions of the odd-numbered gradient fractions were analyzed by SDS-PAGE. Coomassie Blue-stained gels are shown. Gradient fraction numbers are specified above the lanes. The input protein mixtures are analyzed in lane L. The identities of the polypeptides are indicated on the left.
most critical constituents. This interface also embraces Asp\textsuperscript{290} (in a1), which engages in a cross-dimer hydrogen bond with the backbone amide of Gln\textsuperscript{329} (in the turn connecting b2 and b3). Two Ala-cluster mutations involving these residues (D279A-D280A and C330A-V331A) elicited lethal phenotypes that could not be suppressed by overexpression of the mutated triphosphatases but were suppressed completely by fusion of the mutated enzymes to the mammalian RNA guanylyltransferase. These results indicate that a disruption of the dimer interface is uniquely deleterious when the yeast RNA triphosphatase must function in concert with the yeast guanylyltransferase Ceg1. Biochemical analysis of the recombinant D279A-D280A protein confirms that its phosphohydrolase activity was intact and that the mutant protein sedimented as a monomer. Thus, we attribute the \textit{in vivo} phenotypes of D279A-D280A to an isolated defect in homodimerization. Although biochemical analysis of C330A-V331A was hampered by insolubility of the recombinant mutant protein, the \textit{in vivo} phenotypes are consistent with a dimerization defect. Mutations of several other side chains (Pro\textsuperscript{245}, Val\textsuperscript{266}, Ser\textsuperscript{267}, Ser\textsuperscript{282}, Phe\textsuperscript{312}, and Thr\textsuperscript{333}) that make up this segment of the crystallographic dimer interface did not elicit effects on cell growth, implying that their individual contributions are subtle at best. Of course, some of these residues also engage in main-chain contacts to the dimer partner, but the significance of these interactions may not be revealed by replacing the side chain with alanine.

The second functionally relevant dimer interface involves hydrophobic side-chain interactions between a4 and residues Phe\textsuperscript{272} and Leu\textsuperscript{273} in the loop preceding a1. Replacement of the Phe\textsuperscript{272} and Leu\textsuperscript{273} side chains results in a catalytically active monomeric enzyme that confers a severe \textit{ts} growth phenotype when expressed under its native promoter. The growth defect is ameliorated in part by increasing the expression level and expression of the dimer partner, but the significance of these interactions may not be revealed by replacing the side chain with alanine.

Alanine mutations of neighboring dimer interface residues Ile\textsuperscript{268}, Asp\textsuperscript{269}, Pro\textsuperscript{270}, and Pro\textsuperscript{271} did not affect cell growth. However, as noted above, we cannot judge from these results whether or not the main chain contacts of residues 269 and 270 contribute to Cet1 function \textit{in vivo}. A related issue is whether the growth defect of the F272A-L273A mutant might result from an indirect effect of alanine substitution on the cross-dimer hydrogen bond of the main chain amide of residue 272 (Fig. 3). If this were the case, then we would expect the loss of the hydrogen-bonding partner (the side chain O\textsubscript{6} of Asp\textsuperscript{296}) to phenocopy F272A-L273A. Because the replacement of Asp\textsuperscript{296} by alanine elicited no phenotype, we surmise that the F272A-L273A phenotypes reflect the contributions of the hydrophobic side chains to the dimer interface rather than those of the main chain.

The receiving hydrophobic pocket on the dimer partner for the pre-a1 loop is composed primarily of residues in a4. Although the N525A-N526A cluster mutation of a4 did not affect cell growth, the three other cluster mutants in a4 (L519A-I520A, F523A-L524A, and I529A-I530A) had either lethal or severely conditional phenotypes. To what extent are these mutant phenotypes attributable to defects in homodimerization? The side chains of Ile\textsuperscript{529} and Ile\textsuperscript{530} make intimate cross-Strands of the tunnel wall. The aromatic residues Glu\textsuperscript{492} and Glu\textsuperscript{494} of b11 bind the metal cofactor in the active site of Cet1. Thus, we suspect that the I530A mutation imposes conformational effects on the structure of the tunnel in addition to its effects on homodimerization and that the homodimerization problem is overcome in the yeast-mouse chimera whereas the conformational effects on the tunnel are not. This model is consistent with our isolation in a genetic screen of a temperature-sensitive yeast mutant (\textit{cet}-1) that contains a single mutation of Ile\textsuperscript{530} to threonine (7). Furthermore, directed mutagenesis of b11 showed that the single alanine mutations of Val\textsuperscript{493} or Leu\textsuperscript{495} (the residues contacted intramolecularly by Ile\textsuperscript{530}) resulted \textit{per se} in a \textit{ts} growth defect at 37 °C (11).

The I470A-I472A (b10) mutant phenotype is also likely to reflect more than one underlying process, including (i) diminished dimerization affinity caused by the alanine substitution for Ile\textsuperscript{470} (which interacts with Phe\textsuperscript{527} on the dimer partner) and (ii) intramolecular effects of the I472A mutation on the conformation of b10, which comprises part of the floor and lateral wall of the triphosphate tunnel. Ile\textsuperscript{472} makes van der Waals contacts with Phe\textsuperscript{484} and Ile\textsuperscript{355} in a2, and this helix in turn stabilizes the b strands of the tunnel wall. B10 contains three hydrophilic side chains (Arg\textsuperscript{269}, Asp\textsuperscript{471}, and Thr\textsuperscript{473}) immediately surrounding Ile\textsuperscript{472} and Ile\textsuperscript{474}, which are essential for the phosphohydrolase activity of Cet1.

We propose that the L519A-I520A (a4) phenotype, i.e. lethality when expressed by its own promoter and a severe \textit{ts} growth defect when overexpressed and in the context of the mouse guanylyltransferase fusion, is caused predominantly by intramolecular effects of one or both alanine changes on the stability of the triphosphate tunnel. Leu\textsuperscript{519} makes van der Waals contacts with Ile\textsuperscript{497} in b11 in the same protomer. The cross-dimer interaction of Leu\textsuperscript{519} with Ile\textsuperscript{355} is probably not relevant to the \textit{ts} phenotype insofar as alanine substitution for Ile\textsuperscript{355} was itself without effect. Note that the temperature-sensitive \textit{cet}-1/5 strain isolated previously in a genetic screen contains a single mutation of Leu\textsuperscript{519} to proline (7).

Similarly, the lethal F523A-L534A mutation, which is minimally suppressed even in high copy, is almost certainly caused by effects on active site architecture rather than quaternary structure, because Phe\textsuperscript{527} and Leu\textsuperscript{534} are oriented toward the hydrophobic core of the Cet1 protomer and they make no contributions to the crystallographic dimer interface. Rather, Phe\textsuperscript{527} is situated in a hydrophobic-aromatic-hydrophobic sandwich between Met\textsuperscript{508} (in b1) and Ile\textsuperscript{497} (in b11) that likely imparts stability to the floor of the tunnel.

Finally, the conditional phenotype of D287A-W288A (a1), which was suppressed completely by increasing promoter strength alone, is probably unrelated to effects on dimerization, insofar as neither Asp\textsuperscript{297} nor Trp\textsuperscript{286} contributes to the crystallographic dimer interface. However, Trp\textsuperscript{286} is a key component of the hydrophobic core of the Cet1 protomer. The aromatic Trp\textsuperscript{286} side-chain stacks on Leu\textsuperscript{415} (in b7) and makes van der Waals contacts with Ala\textsuperscript{413} (b7) and Ile\textsuperscript{420} (b8) that likely stabilize the floor and lateral wall of the triphosphate tunnel. The temperature-sensitive \textit{cet}-1/4 strain isolated previously in a genetic screen contains a single mutation of Trp\textsuperscript{286} to arginine (7).

\textbf{Why Is Dimerization Important?—}Although homodimerization is clearly important for Cet1 function \textit{in vivo}, the rationale for assembly of a dimeric triphosphatase with two parallel tunnels is still not clear. We suspect that mRNA modification \textit{in vivo} does not require two functional triphosphatase active
sites in the same complex, insofar as overexpression in yeast of a catalytically inactive triphosphatase mutant that still homodimerizes and binds to the guanylyltransferase Ceg1 does not elicit a dominant negative phenotype. The mutations characterized here that specifically affect homodimerization would not alter the high affinity guanylyltransferase-binding site on the surface of Cet1. The peptide motif Cet1(232–265) is sufficient per se to bind Ceg1 in solution and to stabilize the guanylyltransferase against thermal inactivation.

There are two potential Ceg1-binding sites on the triphosphatase dimer located on opposite sides of the structure. Sedimentation analysis suggests that the dimeric triphosphatase binds 1 molecule of Ceg1 (which is itself a monomer in solution) to form a heterotrimeric capping enzyme complex (7). The available data do not exclude the possibility that the reconstituted complex is an asymmetrically shaped (Cet1)$_2$-(Ceg1)$_2$ tetramer, and it is conceivable that the dimeric triphosphatase could bind two Ceg1 monomers if Ceg1 were present in excess during the reconstitution. In a strictly heterotrimeric model, it is possible that binding of Ceg1 to one surface of the Cet1-Cet1 dimer sterically precludes binding of a second Ceg1p on the opposite face (7). It is also conceivable that Ceg1 bound to the 232–265 peptide in one protomer of Cet1 makes cross-dimer interactions with the dimer partner that are relevant to cap formation. Perhaps the interaction of the Cet1 dimer with Ceg1 creates a binding site for nascent RNA that permits transit of the 5’ terminus from the triphosphatase active site to the guanylyltransferase active site without complete release of the polynucleotide between the catalytic steps. We anticipate that a crystal structure of yeast triphosphatase bound to yeast guanylyltransferase will provide a definitive account of the stoichiometry of the Cet1-Ceg1 complex and shed some light on the rationale for the distinctive homodimeric structure of the triphosphatase component.

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