An Essential Function of *Saccharomyces cerevisiae* RNA Triphosphatase Cet1 Is to Stabilize RNA Guanylyltransferase Ceg1 against Thermal Inactivation*

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*S. cerevisiae* RNA triphosphatase (Cet1) and RNA guanylyltransferase (Ceg1) interact *in vivo* and *in vitro* to form a bifunctional mRNA capping enzyme complex. Here we show that the guanylyltransferase activity of Ceg1 is highly thermolabile *in vitro* (98% loss of activity after treatment for 10 min at 35 °C) and that binding to recombinant Cet1 protein, or a synthetic peptide Cet1(232–265), protects Ceg1 from heat inactivation at physiological temperatures. *Candida albicans* guanylyltransferase Cgt1 is also thermolabile and is stabilized by binding to Cet1(232–265). In contrast, *Schizosaccharomyces pombe* and mammalian guanylyltransferases are intrinsically thermostable *in vitro* and they are unaffected by Cet1(232–265). We show that the requirement for the Ceg1-binding domain of Cet1 for yeast cell growth can be circumvented by overexpression in high gene dosage of a catalytically active mutant lacking the Ceg1-binding site (Cet1(269–549)) provided that Ceg1 is also overexpressed. However, such cells are unable to grow at 37 °C. In contrast, cells overexpressing Cet1(269–549) in single copy grow at all temperatures if they express either the *S. pombe* or mammalian guanylyltransferase in lieu of Ceg1. Thus, the cell growth phenotype correlates with the inherent thermal stability of the guanylyltransferase. We propose that an essential function of the Cet1-Ceg1 interaction is to stabilize Ceg1 guanylyltransferase activity rather than to allosterically regulate its activity. We used protein-affinity chromatography to identify the COOH-terminal segment of Ceg1 (from amino acids 245–459) as an autonomous Cet1-binding domain. Genetic experiments implicate two peptide segments, 287KPVSLYW295 and 337WQLNKNLEQPLN348, as likely constituents of the Cet1-binding site on Ceg1.

RNA triphosphatase catalyzes the first step in mRNA cap formation entailing the cleavage of the β-γ phosphohydridine bond of 5′ triphosphate RNA to yield a 5′ diphosphate end that is then capped with GMP by RNA guanylyltransferase. The genetic and physical organization of these two capping enzymes differs in higher versus lower eukaryotes (1). Mammals encode a bifunctional capping enzyme (Mec1; 597 aa) consisting of an NH2-terminal triphosphatase domain Mec1(1–210) fused to a COOH-terminal guanylyltransferase domain Mec1(211–597). 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.

The binding of yeast Cet1 to Ceg1 elicits two apparently beneficial outcomes. First, Cet1-Ceg1 interaction stimulates the guanylyltransferase activity of Ceg1 by increasing the extent of formation of the covalent Ceg1-GMP reaction intermediate (2, 3). Second, the physical tethering of Cet1 to Ceg1 may facilitate recruitment of the capping apparatus 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 (4–7), whereas Cet1 by itself does not interact *in vitro* with the phosphorylated CTD (8). It has also been suggested that Cet1 binding to Ceg1 antagonizes negative effects of CTD-P03 binding on the guanylyltransferase activity of Ceg1 (8).

Cet1 consists of three domains: (i) a 230-aa NH2-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 276 to 539 (9). The catalytic domain by itself is a monomeric protein and does not support yeast cell growth, whereas the biologically active triphosphatase has a homodimeric quaternary structure (9, 10). Mutational disruption of the Cet1 homodimer interface is uniquely deleterious *in vivo* when the yeast RNA triphosphatase functions in concert with the endogenous yeast guanylyltransferase Ceg1. Lethal or severe temperature-sensitive (ts) growth phenotypes elicited by mutations of the Cet1 homodimer interface are suppressed by fusion of the mutated triphosphatase to the guanylyltransferase domain of mammalian capping enzyme (11).

Genetic evidence indicates that the Cet1-Ceg1 interaction is important. *cet1-ts* mutations are suppressed in an allele-specific manner by overexpression of *CET1* (2, 8). In turn, *cet1-ts* mutations can be suppressed by overexpression of *CEG1* (9). The guanylyltransferase-binding and guanylyltransferase-stimulation functions of Cet1 localize to a 21-amino acid segment from residues 239 to 259 (3). The guanylyltransferase-binding domain is located on the protein surface (10) and is conserved in the *Can dida albicans* RNA triphosphatase CaCet1 (3), but not in the RNA triphosphatase Pct1 from the fission yeast *Schizosaccharomyces pombe* (12). Alanine-cluster mutations of a WAQKW motif within the Ceg1-binding domain of Cet1 abolish guanylyltransferase binding *in vitro* and Cet1 function *in vivo*, but do not affect

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‡The abbreviations used are: aa, amino acid(s); PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; GST, glutathione s-transferase; ORF, open reading frame; 5-FOA, 5-fluorooorotic acid; CTD, COOH-terminal domain.
Interactions of Yeast mRNA Capping Enzyme Subunits

EXPERIMENTAL PROCEDURES

Recombinant Capping Enzymes—S. cerevisiae guanylyltransferase Ceg1, C. albicans guanylyltransferase Cgt1, S. pombe guanylyltransferase Pce1, mammalian guanylyltransferase Mce1(1–210). S. cerevisiae RNA triphosphatase Cet1(201–549) were produced in Escherichia coli as NH2-terminal His6-tagged fusions and purified from soluble bacterial lysates by Ni-agarose chromatography as described previously (3, 9, 14, 15). The enzyme preparations were dialyzed against buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, 5% glycerol, 0.03% Triton X-100 and then stored at −80 °C. Protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard.

Guanylyltransferase Assay—Guanylyltransferase activity was assayed by the formation of the covalent enzyme-GMP intermediate. Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 0.17 μM [32P]GTP, and enzyme as specified were incubated for 10 min at 22 °C. The reaction was halted by adding SDS to 1% final concentration. The samples were analyzed by SDS-PAGE. The guanylyltransferase-32P[GMP] complex was resolved and visualized by autoradiography of the dried gel and quantitated by scanning the gel with a FUJIX BAS2500 PhosphorImager.

ATPase Assay—Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 7.5), 5 mM dithiothreitol, 2 mM MnCl2, 1 mM γ-[32P]ATP, and enzyme were incubated for 15 min at 30 °C. The reactions were quenched by adding 2 μl of 5 M formic acid. Aliquots (2 μl) of the mixture were added to a polyethyleneimine-cellulose TLC plate, which was developed with 1M formic acid, 0.5 M LiCl. The extent of 32Pi release was quantitated by scanning the chromatogram with a FUJIX BAS2500 PhosphorImager.

Thermal Inactivation of Fungal and Mammalian Guanylyltransferases—Purified guanylyltransferase (2 μM of Ceg1, Cgt1, Pce1, or Mce1(1–210–597)) was incubated for 15 min on ice either alone or with 12 μM purified Cet1(201–549), with 29 μM Ceg1(232–265), 180 μM peptide, or the mutant peptide Cet1(232–265)4xAla in 50 μl of binding buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol, 5% glycerol, 0.03% Triton X-100). Aliquots (5 μl) of the guanylyltransferase alone or guanylyltransferase plus Cet1(201–549) or Cet1(232–265) mixtures were preincubated for 10 min at 22, 30, 35, 40, 45, or 50 °C and then quenched on ice. Control aliquots were kept on ice throughout the pretreatment. An aliquot (2 μl) of each sample was then assayed at 22 °C for enzyme-GMP complex formation. The signal intensities of the enzyme-32P[GMP] complexes formed by the preheated enzyme mixtures were normalized to that of the unheated control (defined as 100%). The normalized activities were plotted as a function of preincubation temperature (Fig. 1, A-E).

Thermal Inactivation of Cet1—Purified Cet1(201–549) (2.2 μM) was incubated for 15 min on ice either alone or with 12 μM purified Ceg1. Aliquots (5 μl) of the mixtures were preincubated for 10 min at 30, 35, 40, 45, 50, or 55 °C and then quenched on ice. Control aliquots were kept on ice throughout the pretreatment. An aliquot (2 μl) of each sample was then assayed at 30 °C for manganese-dependent ATP hydrolysis. The extent of ATP hydrolysis by preheated enzyme was normalized to that of the unheated control enzyme (14 nmol of 32P release; defined as 100%). The normalized activities were plotted as a function of preincubation temperature in Fig. 1F.

Yeast Vectors Encoding Triphosphatase Guanylyltransferase Fusion Proteins—Yeast 2μ plasmids encoding chimeric capping enzymes composed of an NH2-terminal segment derived from S. cerevisiae Cet1 fused to either S. cerevisiae Ceg1 or S. pombe Pce1 were constructed as follows. CET1 gene fragments coding Cet1(201–549), 269–294, and 276–549 were PCR amplified using an antisense primer that changed the Val548 codon to alanine and introduced an NcoI restriction site at the codons for the COOH-terminal Cet1 dipeptide Ala547-Met548. The PCR products were digested with NcoI and then inserted into the NcoI site of pYX232-CEG1 (2μ TRP1) and pYX232-PE1 (2μ TRP1) to yield plasmids encoding in-frame fusion proteins Cet1(201–547–Ceg1, Cet1(269–547–Ceg1, Cet1(276–547–Ceg1, Cet1(201–547–Pce1, Cet1(269–547–Pce1, and Cet1(276–547–Pce1. Expression of the chimeric genes in these plasmids is under control of the yeast TPI1 promoter. The CET1 inserts were sequenced completely to confirm the in-frame fusion to CEG1 or PCE1 and to exclude the introduction of unwanted coding changes during primer design.

Yeast 2μ plasmids encoding chimeric capping enzymes composed of mammalian RNA triphosphatase Mce1(1–210) fused to either Ceg1 or Pce1 were constructed as follows. The MCE1(1–210) reading frame was subjected to two-stage PCR amplification using primers designed to eliminate an internal NcoI site (without altering the protein sequence), to translate the start codon, and to change the Glu511 codon to alanine and introduce an NcoI restriction site at the codons for the Ser210-Ala211 dipeptide. The PCR product was digested with NcoI and inserted into the NcoI site of pYX232-CEG1 (2μ TRP1) or pYX232-PCE1 (2μ TRP1) to yield plasmids encoding the in-frame fusion proteins Mce1(1–210–Ceg1 and Mce1(1–210–Pce1. Expression of the chimeric mammalian-fungal capping enzymes in these plasmids is under the control of the yeast TPI1 promoter. The MCE1(1–210) inserts were sequenced completely to confirm the in-frame fusion to CEG1 or PCE1 and to exclude the introduction of unwanted coding changes during amplification and cloning.

Yeast CEN plasmids containing the chimeric genes were constructed by excising from the 2μ plasmids an AniI-Nhel fragment containing the fusion gene and transferring the fragment into pYX102 (CEN TRP1). Expression of the fusion genes in the CEN plasmids is also under control of the TPI1 promoter.

Expression and Purification of GST-Ceg1 Fusion Proteins—The ORF encoding full-length Ceg1(1–459) fused to an NH2-terminal His6 tag leader peptide was inserted into the pGEX-KG vector between the NcoI and BamHI sites. The resulting plasmid encodes a glutathione S-transferase (GST)-His6-Ceg1(1–549) fusion protein. Plasmids for expression of Ceg1 domains and domain fragments fused to GST were engineered as follows. An ORF encoding the NH2-terminal domain Ceg1(1–244) was amplified by PCR using an antisense primer that introduced a translation stop codon in lieu of the codon for Leu549 and a BamHI site 3’ of the stop codon. An ORF encoding the COOH-terminal domain Ceg1(245–459) was amplified by PCR using a sense primer that introduced a methionine codon in lieu of the codon for Thr549. The PCR products were digested with NcoI and BamHI and then inserted into pGEX-KG to generate plasmids encoding fusion proteins GST-Ceg1(1–244), GST-Ceg1(245–459), GST-Ceg1(245–360), and GST-Ceg1(361–459).

Plasmids encoding GST and the GST-Ceg1 fusion proteins were transformed into E. coli BL21 (pYSE5). Exponentially growing cultures were induced with 0.4 mM IPTG at 4 h and then incubated with 2% ethanol for 20 h at 17 °C. GST and the GST-Ceg1 fusion proteins were purified from soluble lysates by affinity chromatography on a glutathione-Sepharose 4B resin according to the instructions of the vendor (Amersham Pharmacia Biotech). GST and GST-Ceg1 fusion proteins were eluted from the resin with buffer containing 10 mM glutathione, 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 10% glycerol. The eluates were dialyzed against binding buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM dithiothreitol, 5% glycerol, 0.03% Triton X-100) and then stored at −80 °C.

Protein-Protein Interaction Assays—Purified GST and the GST-Ceg1 fusion proteins (either 10–15 μg of the proteins specified in Fig. 5A or 30–40 μg of the proteins in Fig. 5B) were incubated for 1 h at 4 °C with glutathione-Sepharose beads (either 20–25 μl of beads in Fig. 6A or 50–60 μl of beads in Fig. 6B) in 350 μl of binding buffer. The beads were then washed three times with 1 ml of binding buffer to remove any unbound protein. Affinity chromatography was performed by mixing the GSH beads containing bound GST-Ceg1 fusion proteins or GST with purified yeast triphosphatase Cet1(201–549) (either 4 μg in Fig. 6A or 8 μg in Fig. 6B) in 50 μl of binding buffer. After incubation for 1 h on ice, the products were concentrated and centrifuged if necessary. The bound product was washed with 150 μl of washing buffer and subjected to three rounds of concentration and washing. After the third wash, the protein bound to the beads was eluted by beads were resuspended in 50 μl of binding buffer containing 10 mM glutathione. Aliquots (20 μl) of the input Cet1(201–549) sample, the first supernatant fraction containing the “free” protein, and the bead-
bound fraction were mixed with 5 μl of SDS sample buffer, heated at 90 °C for 3 min, and then analyzed by SDS-PAGE. Polypeptides were visualized by staining the gel with Coomassie Blue dye.

**Mutagenesis of Yeast Guanylyltransferase—**Aniline substitution mutations were introduced into the CEG1 gene by using the PCR-based two-stage overlap extension method as described (16). The mutated CEG1 genes were inserted into the yeast plasmid pGYCE-358 (CEN TRP1) (17), where expression of the CEG1 gene is under the control of the natural CEG1 promoter. The insertions were sequenced completely to confirm the presence of the programmed changes and to exclude the introduction of unwanted coding changes during amplification and cloning.

**Yeast Strains—**Yeast strain YBS30 (MATa ura3 ade2 trp1 his3 leu2 can1 ceg1::hisG pGYCE-360) is deleted at the chromosomal CEG1 locus. Growth of YBS30 depends on maintenance of plasmid pGYCE-360 (CEN URA3 CEG1). Yeast strain YBS50 (MATa leu2 ade2 trp1 his3 ura3 can1 ceg1::hisG cet1::LEU2 p360-CET1/CEG1) is deleted at the chromosomal CET1 and CEG1 loci. Growth of YBS50 is contingent on the maintenance of plasmid p360-CET1/CEG1 (CEN URA3 CET1 CEG1).

**RESULTS**

**Cet1 Binding to Ceg1 Stabilizes Ceg1 against Thermal Inactivation—**Prior studies showed that the binding of full-length Cet1 or the truncated enzyme Cet1(201–549) to Ceg1 stimulated the guanylyltransferase activity of Ceg1 by an order of magnitude when guanylyltransferase activity was measured at 37 °C (2). An equivalent stimulation was elicited by a synthetic peptide Cet1(232–265), which binds quantitatively and with high affinity to Ceg1 in vitro (3). We show here that the purified recombinant Ceg1 protein is extremely thermolabile in vitro (Fig. 1A). Guanylyltransferase activity was abolished by preincubation of the protein for 10 min at 35 °C or higher and reduced by a factor of 5 after 10 min at 30 °C. Thus, Ceg1 is rapidly inactivated at physiological temperatures.

The instructive finding was that mixture of Ceg1 with recombinant Cet1(201–549) protein effected a dramatic shift to the right in the guanylyltransferase thermal inactivation profile, such that the Cet1(201–549)-Ceg1 complex was impervious to preincubation for 10 min at 30 °C and retained 50% of the original activity after incubation at 35 °C (Fig. 1A). The synthetic peptide Cet1(232–265) also had a profound protective effect against thermal inactivation of Ceg1, whereby the Cet1(232–265)-Ceg1 complex retained 70% of the original activity after incubation for 10 min at 35 °C (Fig. 1B). A synthetic Cet1(232–265) peptide containing a quadruple alanine-cluster mutation of the WAQKW motif had no salutary effect on the stability of Ceg1 (Fig. 1B). The 4xAla mutant peptide does not bind Ceg1 and does not stimulate its activity at 37 °C (3). We conclude that the stimulation of Ceg1 by Cet1 is attributable to protein stabilization.

In contrast to the instability of Ceg1, we found that the triphosphatase activity of Cet1(201–549) was stable to preincubation at 30–35 °C and its thermal inactivation profile was unaffected by prior binding to the yeast guanylyltransferase Ceg1 (Fig. 1F). Thus, the protective effects of Cet1-Ceg1 complex formation on enzyme stability in vitro are not reciprocal.

To test whether the inactivation of Ceg1 at physiological temperatures can be reversed *ex post facto* by Cet1, we varied the order of addition of the protective Cet1(232–265) peptide with respect to the heat treatment. Mixture of Ceg1 with the Cet1 peptide on ice before heating the protein for 10 min at 35 °C resulted in 11-fold higher guanylyltransferase activity compared with heat-treated Ceg1 that had not been exposed to Cet1(232–265). Yet, mixing the already heat-treated Ceg1 with the Cet1 peptide on ice had no restorative effect on the guanylyltransferase activity (Fig. 2). Thus, Cet1 could not reverse the inactivation of Ceg1, implying that Cet1 is not serving as a chaperone that promotes refolding of Ceg1 or resumption of an active conformation. Rather, Cet1 binding to Ceg1 stabilizes the guanylyltransferase only prospectively against thermal inactivation.

**The Stabilizing Effect of Cet1 on Fungal Guanylyltransferase Is Species-specific—**The guanylyltransferase-binding peptide domain is conserved in *C. albicans* RNA triphosphatase Caet1 and we have shown that the *C. albicans* guanylyltransferase Cgt1 binds avidly to the Cet1(232–265) peptide in vitro (3). To gauge if stabilization of guanylyltransferase by triphosphatase is a general phenomenon in fungal systems, we examined the thermal stability of Cgt1 in the absence and presence of purified Ceg1 as determined described under “Experimental Procedures.” The normalized triphosphatase activity is plotted as a function of the preincubation temperature (A–E). The thermal inactivation profile of Cet1(201–549) in the absence or presence of purified Ceg1 was determined as described under “Experimental Procedures.” The normalized triphosphatase activity is plotted as a function of the preincubation temperature (F).

**Fig. 1.** Cet1 protects Ceg1 against thermal inactivation in vitro. Thermal inactivation profiles for the purified guanylyltransferases Ceg1 (A, B), Cgt1 (C), Pce1 (D), and Mec1(211–507) (E) alone and in the presence of purified Cet1(201–549) (A) or Cet1(232–265) (B-E) were determined as described under “Experimental Procedures.” The normalized guanylyltransferase activities are plotted as a function of the preincubation temperature (A–E). The thermal inactivation profile of Cet1(201–549) in the absence or presence of purified Ceg1 was determined as described under “Experimental Procedures.”
The Interaction of Cet1 with Ceg1 Stabilizes Ceg1 in Vivo

The catalytic domain Cet1(276–549) lacks the high-affinity guanylyltransferase-binding site and does not interact with Ceg1 in vitro (9). Cet1(269–549) also lacks the Ceg1-binding site. Neither CET1(269–549) nor CET1(276–549) was able to complement growth of S. cerevisiae cet1Δ cells, even when the truncated enzymes were expressed in high gene dosage under the control of the strong constitutive yeast TPI1 promoter (Ref. 9 and data not shown). Remarkably, the in vivo function of CET1(276–549) was restored when it was fused to the guanylyltransferase domain of the mammalian capping enzyme (9). We proposed that the mammalian domain, Mec1(211–597), which binds avidly to the phosphorylated CTD (15, 20), can act as a vehicle to deliver the fused RNA triphosphatase to the RNA polymerase II elongation complex (9, 21). Also, because Mec1(211–597) is thermostable (unlike Ceg1; see Fig. 1), the chimeric capping enzyme likely bypasses the need for the Ceg1-stabilization function of the 232–259 domain of Cet1.

We reasoned that if the only defect of the truncated Cet1 proteins missing the Ceg1-binding site was a lack of targeting to the CTD, then fusing them in cis to Ceg1 would restore their function in vivo, just as the fusion to Mec1(211–597) does. However, if the Cet1(232–259) peptide is as important for stabilizing Ceg1 in vivo as it is in vitro, then the chimeric Cet1-Ceg1 proteins lacking that domain would either not function in vivo or else would evoke a temperature-sensitive growth defect. To test if Ceg1 would tolerate a large fusion peptide at its N terminus, we expressed a chimeric yeast-yeast capping enzyme in which the biologically active truncated triphosphatase Cet1(201–547) was linked to full-length Ceg1. The CET1(201–547)-CEG1 fusion complemented the growth of cet1Δ ceg1Δ cells in a plasmid shuffle assay when the chimeric gene was expressed on either a 2µ plasmid or a CEN plasmid (Fig. 3). Moreover, 2µ CET1(201–547)-CEG1 and CEN CET1(201–547)-CEG1 cells grew as well as wild type CET1 CEG1 cells on YPD agar at 22, 30, and 37 °C (scored as + + + growth in Fig. 3). Thus, an NH2-terminal fusion per se did not perturb the in vivo activity of Ceg1.

cet1Δ ceg1Δ cells expressing the Cet1(269–547)-Ceg1 chimera at high gene dosage grew as well as wild-type cells on YPD at 30 °C (+ + +), but failed to grow at all at 37 °C (ts) (Fig. 3). Cells expressing Cet1(269–547)-Ceg1 from a CEN plasmid formed pinpoint colonies on YPD at 30 °C (scored as + + + growth) and failed to grow at 37 °C (ts). Thus, the fusion to Ceg1 bypassed the requirement for a high-affinity Ceg1-binding site on Cet1, but conferred a profound ts growth defect that was exacerbated when the chimera was expressed at low gene dosage. Given that cells expressing a Cet1(269–547)-Pce1 fusion grew well at 37 °C (see below), we attribute the ts growth to a defect in the Ceg1 component of the chimeric capping enzyme.

cet1Δ ceg1Δ cells expressing the more extensively truncated Cet1(276–547)-Ceg1 fusion protein from a 2µ plasmid formed smaller colonies than wild-type cells on YPD at 30 °C (+ + +). These experiments show that the stabilizing effect of RNA triphosphatase on guanylyltransferase-binding site and does not interact with the fungal triphosphatase component of mammalian cap-mRNA capping enzyme Subunits

In contrast, the S. pombe RNA guanylyltransferase Pce1 (18) was clearly more thermostable than either Ceg1 or Cgt1. Pce1 was unaffected by pretreatment at up to 35 °C (which abolished Ceg1 activity), retained 70% of basal activity after heating at 40 °C (compared with 5% activity for Cgt1) and 25% activity after treatment at a 45 °C. Moreover, its thermal inactivation profile was unaffected by the Cet1(232–265) peptide (Fig. 1E). We found that the Cet1(232–265) peptide interacts very weakly with Pce1 in vitro as gauged by peptide-affinity chromatography (data not shown) (3). These experiments show that the stabilizing effect of RNA triphosphatase on guanylyltransferase is conserved in two species of budding yeast, but not in fission yeast.

The guanylyltransferase component of the mammalian capping enzyme, Mec1(211–597), was also much more thermostable than S. cerevisiae Ceg1. The mammalian capping enzyme was unaffected by preincubation at up to 35 °C (which abolished Ceg1 activity), retained 70% of basal activity after heating at 40 °C (Fig. 1D) and incubated on ice for 10 min. Aliquots (2 µl) of each sample were assayed for guanylyltransferase activity. The PhosphorImager signal intensity (PSL) of the enzyme-[32P]GMP complex is shown. The order of additions and heating are indicated by an arrow on the left.

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cet1Δ ceg1Δ cells expressing the more extensively truncated Cet1(276–547)-Ceg1 fusion protein from a 2µ plasmid formed smaller colonies than wild-type cells on YPD at 30 °C (+ + +).
growth) and did not form colonies at 37 °C (ts). Expression of Cet1(276–547)-Ceg1 from a CEN plasmid resulted in failure to recover viable colonies during the 5-FOA selection step of the plasmid shuffle conducted at either 18, 22, or 30 °C. Thus, the CEN CET1(276–547)-CEG1 allele was lethal in single copy (scored as − in Fig. 3). Comparing the profound ts and lethal phenotypes of the Cet1(276–547) fusion to Ceg1 to the normal function in vivo of the Cet1(276–547) fusion to mammalian guanylyltransferase (9) again prompts the conclusion that it is the Ceg1 component that is thermolabile or defective in vivo.

The more severe phenotypes observed for Cet1(276–547)-Ceg1 fusions versus Ceg1(289–547)-Ceg1 fusions may reflect the fact that the shorter derivative of Cet1 has lost the ability to homodimerize (9) because resides Phe272 and Leu273 are key components of the dimer interface (10, 11). This theme is underscored by the effects of fusing the monomeric mammalian RNA triphosphatase domain Mec1(1–210) to Ceg1 (Fig. 3), whereby expression of the Mec1(1–210)-Ceg1 chimera from a 2μ plasmid resulted in slow growth at 30 °C (++) and no growth at 37 °C (ts), while expression of Mec1(1–210)-Ceg1 from a CEN plasmid was lethal. Thus, the identical growth defects were elicited by fusing Ceg1 to catalytically active monomeric triphosphatases from yeast or mammals. This suggests that indirect dimerization of Ceg1 (via the homodimeric yeast triphosphatase) enhances Ceg1 function at 37 °C in vivo.

We extended this analysis to chimeric capping enzymes consisting of an NH2-terminal truncated Cet1 polypeptide linked in cis to the full-length S. pombe guanylyltransferase Pce1. The chimeric enzyme Cet1(201–547)-Pce1 complemented growth of cet1Δ ceg1Δ cells when provided on either 2μ or CEN plasmids and CET1(201–547)-PCE1 cells grew as well as wide-type yeast at 22, 30, and 37 °C (++) in Fig. 3. Thus, Pce1 function was not compromised by the NH2-terminal fusion. The salient finding was that expression of CET1(269–547)-Pce1 from a 2μ or a CEN plasmid fully complemented growth of cet1Δ ceg1Δ and that the fusion to the S. pombe guanylyltransferase was functional in vivo at 37 °C (+++ growth), unlike the fusion of CET1(269–547) to Ceg1, which was growth impaired when expressed in single copy and was defective in vivo at 37 °C whether expressed at low or high gene dosage (Fig. 3). Thus, when the essential Ceg1-binding domain of the triphosphatase is eliminated, the activity of the chimeric capping enzymes in vivo reflects faithfully the thermal stability of the guanylyltransferase component in vitro (i.e. unstable for Ceg1 versus stable for Pce1).

cet1Δ ceg1Δ cells expressing the Cet1(276–547)-Pce1 fusion protein from a 2μ plasmid grew at 30 °C (++) and 37 °C (+), unlike the equivalent fusion to Ceg1 which failed to grow at 37 °C. Moreover, expression of CET1(276–547)-Pce1 from a CEN plasmid resulted normal (++) growth at 30 °C, unlike the lethal CET1(276–547)-Ceg1 fusion. CET1(276–547)-PCE1 cells grew slowly at 37 °C (+ growth), suggesting that the monomeric triphosphatase catalytic domain may be partially thermolabile in vivo. On the other hand, the fusion of the mammalian RNA triphosphatase Mec1(1–210) to S. pombe Pce1 was fully functional at 30 and 37 °C, whether expressed from a 2μ or a CEN plasmid. This result contrasts with the ts and lethal phenotypes observed when the Mec1(1–210)-Ceg1 chimera was expressed from 2μ and CEN plasmids, respectively (Fig. 3).

Stabilization of Ceg1 by Cet1 Versus Targeting of Cet1 by Ceg1—The preceding analysis of chimeric capping enzymes (Fig. 3) provides evidence that an essential function of the Cet1-Ceg1 interaction in vivo is the stabilization of the inherently labile guanylyltransferase activity of Ceg1. Yet the experiments do not probe the putative role of the Cet1-Ceg1 interaction in helping to target the triphosphatase to the RNA polymerase II transcription complex. To approach this issue, we expressed RNA triphosphatase and RNA guanylyltransferase in trans from separate CEN plasmids, marked either with TRP1 (for the triphosphatase) or ADE2 (for the guanylyltransferase). Expression of both components was under the control of the strong constitutive yeast TPI1 promoter, the same promoter used to drive expression of the fused capping enzymes. The coexpression of the biologically active domain CET1(269–549) with Ceg1 supported normal growth of cet1Δ ceg1Δ cells at 30 and 37 °C, as expected (Fig. 4). In contrast, cet1Δ ceg1Δ cells co-transformed with the TPI1-CET1(269–549) and TPI1-CEG1 alleles yielded few viable FOA-resistant colonies, which then grew very slowly on YPD agar at 30 °C (growth) and failed to grow on YPD at 37 °C (Fig. 4). Thus, the slow growth and ts phenotypes of cells expressing CET1(269–549) and Ceg1 as separate proteins were the same as those observed for the CET1(269–549)-Ceg1 fusion protein (Fig. 3). Note that the TPI1-CET1(269–549) allele, which encodes a protein that lacks the Ceg1-binding domain, is lethal in single copy or in high copy in yeast cells that express CEG1 in single copy under the control of its natural promoter. Thus, we surmise that the gain of function of TPI1-CET1(269–549) in the TPI1-CEG1 background is attributable to overexpression of the guanylyltransferase, which, although it is thermolabile as a consequence of the loss of the stabilizing influence of Cet1 binding, is able via overexpression to attain a threshold level of active guanylyltransferase to support very slow cell growth at 30 °C.

The instructive finding was that the TPI1-CET1(269–549) allele was fully functional at both 30 and 37 °C when present in trans with TPI1-PCE1 (Fig. 4). TPI1-CET1(269–549) was also fully functional at 30 and 37 °C in the presence of a separate CEN plasmid expressing the mammalian guanylyltransferase gene TPI1-MCE1(211–597) (not shown). These results underscore two key points: (i) that the ts growth phenotype elicited by removal of the Ceg1-binding domain of Cet1 correlates with the inherent thermolability of the coexisting guanylyltrans-
plasmids containing the wild-type and mutant alleles of CEG1 were viable at 30 °C/H11001/Growth was assessed as follows:. The motifs V and VI that were mutated in the present study are highlighted in shaded boxes. N348A, failed to yield colonies on 5-FOA after 7 days of incubation at 18, 22, and 30 °C. TPI1-MCE1(211–597) was lethal in conjunction with the TPI1-CEG1 RNA triphosphatase mutants deleted in the conserved guanyl-binding domain. Similar gain of function results have been pre-mRNAs without the benefit of its guanylyltransferase–RNA triphosphatase superfamily (16, 24) (Fig. 5A). Domain 1 includes motifs I, III, IIIa, IV, and the proximal half of motif V; domain 2 includes the distal part of motif V as well as motif VI.

Initial studies of the Ceg1 side of the yeast triphosphatase–guanylyltransferase interface entailed proteolytic footprinting of Ceg1 in the presence and absence of Cet1 (3). The principal tryptic cleavage sites in Ceg1, located within domain 2 at Arg504 and Lys508 (denoted by arrowheads in Fig. 5A), were protected from trypsin digestion by Cet1(201–549), whereas secondary tryptic sites located close to the NH2 terminus in domain 1 were not shielded. The Cet1(232–265) peptide also protected Ceg1 from proteolysis by trypsin, whereas the Cet1(232–265)4xAla mutant peptide did not (3). These results suggested that at least part of a Cet1-binding site on Ceg1 is located within domain 2, but they did not address whether Ceg1 contains a discrete triphosphatase-binding epitope or whether the domain 1 is required for interaction with Cet1.

Here we used affinity chromatography to address these issues. Fusion proteins containing GST linked to full-length Ceg1 (aa 1–459), Ceg1 domain 1 (aa 1–244), or Ceg1 domain 2 (aa 245–459) were produced in bacteria and purified. The GST fusions proteins were immobilized on glutathione-Sepharose beads, which were then mixed with purified Cet1(201–549) protein (shown in Fig. 6A, lane L). The material that did not bind to the beads (free fraction, F) was analyzed by SDS-PAGE along with the material that had bound to the resin and was subsequently stripped off with glutathione and SDS (bound fraction, B). The Cet1(201–549) protein was retained quantitatively on the beads containing GST-Ceg1(1–459), but not at all on beads containing GST alone or GST-Ceg1(1–244) (Fig 6A). A majority of the input Cet1(201–549) did adsorb to the resin when the affinity ligand was GST-Ceg1(245–459). We conclude that domain 2 comprises an autonomous Cet1-binding module. The fine structure of the binding site within domain 2 is likely to be complex, insofar as the splitting of domain 2 fusion into

Effects of alanine substitutions in domain 2 on Ceg1 function in vivo. A, the 459-amino acid Ceg1 polypeptide is depicted as a horizontal line with the six nucleotidyl transferase motifs (I, III, IIIa, IV, V, and VI) shown as boxes. B, the Ceg1 amino acid sequence between residues 225 and 399 is aligned to the sequences of the guanylyltransferases of C. albicans (cal), mouse (mus), S. pombe (spo), and Chlorella virus PCBV-1 (chv) based on the location of essential Ceg1 amino acid side chains within the nucleotidyl transferase motifs (16, 18; denoted by 1 above the sequence). The secondary structure elements of the Chlorella virus guanylyltransferase are shown below the amino acid sequence; β strands are depicted as arrows; and α helices as bars. Ceg1 residues between motifs V and VI that were mutated in the present study are highlighted in shaded boxes. The boxes also embrace side chain identity or similarity in the other aligned guanylyltransferase sequences at the positions selected for mutagenesis. B, ceg1Δ cells were transformed with CEN TRP1 plasmids containing the wild-type and mutant alleles of CEG1 under the control of the CEG1 promoter. A control transformation was performed using the TRP1 vector. Tryp’ isolates were selected and then streaked on agar plates containing 0.75 mg/ml 5-FOA. Only one mutant, L347A N348A, failed to yield colonies on 5-FOA after 7 days of incubation at 18, 22, and 30 °C; this mutant was scored as lethal (−). Individual FOA-resistant colonies were picked and patched on YPD agar. Two isolates of each mutant were then streaked on YPD agar at 30 and 37 °C. Growth was assessed as follows: + + + indicates colony size indistinguishable from wild-type; + + indicates colony size smaller than the wild-type; + indicates pinpoint colonies. Temperature-sensitive (ts) indicates pinpoint colonies. Temperature-sensitive (ts) indicates pinpoint colonies.
Effects of Domain 2 Mutations on Ceg1 Function in Vivo—The trypsin-sensitive sites of Ceg1, Arg304 and Lys306, that are shielded from proteolysis when Cet1 is bound to Ceg1, are identified by alanine-cluster mutagenesis of pairs of vicinal amino acids and also introduced single alanine substitutions of four residues and also introduced single alanine substitutions of four residues and also introduced single alanine substitutions of four residues and also introduced single alanine substitutions of four residues, which were changed to alanine in this analysis (Fig. 5B). The CEG1-Ala genes were cloned into a CEN vector under the control of the natural CEG1 promoter and then tested by plasmid shuffle for their ability to complement a ceg1Δ mutant. The mutational effects are tabulated in Fig. 5B.

Only one mutant allele, L347A N348A, failed to support the growth of ceg1Δ cells on 5-FOA at all temperatures tested (18, 22, and 30 °C); thus, this mutation was lethal in vivo. The 347–348 dipeptide is predicted to comprise a short turn between two β strands in Ceg1 and other cellular capping enzymes; the β strands are connected by a loop in the Chlorella virus enzyme (Fig. 5A). The strand immediately distal to the essential dipeptide corresponds to motif V described by Wang et al. (16). This motif is located just upstream of the essential nucleotidyl transferase motif VI in all cellular capping enzymes and in the Chlorella virus guanylyltransferase (Fig. 5A). Mutation of the conserved motif V glutamate (Glu353) had no effect on the in vivo activity of Ceg1 (Fig. 5B). The double mutant E353A C354A grew normally at 30 °C, but colony size at 37 °C was smaller than wild-type (+ +). Several groups had previously isolated C354Y mutants in a screen for ceg1Δ alleles (25–27). We surmise in light of the present results that the tight growth defect of C354Y at 37 °C is caused not by the loss of the cysteine functional group (alanine being better tolerated), but rather by steric effects of introducing a bulky tyrosine at this position. The observation that the ts growth defect of C354Y was suppressed by overexpression of Cet1 (8) is consistent with a perturbation of the nearby triphosphatase-binding site by a Tyr354 side chain.

Alanine cluster mutants L343A E344A and Q345A P346A in the β strand immediately proximal to the essential 347–348 dipeptide had no effect on cell growth. Thus, we infer that the ts growth phenotype noted previously for the P346L mutant, which can be suppressed by overexpression of Cet1 (8, 27), reflects the perturbation of a triphosphatase-binding site by the bulky leucine rather than a specific contribution of proline to Cet1 binding (alanine being able to function in place of Pro346). Here, we noted ts phenotypes for the neighboring mutants, L340A K341A and W337A Q338A, located just upstream at the takeoff point of the proposed surface loop of the cellular guanylyltransferases (Fig. 5). L340A K341A cells failed to grow at 37 °C; however, growth at the restrictive temperature was restored by provision of CET1(201–549) on a multicopy (2 μ) plasmid (Fig. 7). These effects of simple side chain removal imply either that the Leu340/Lys341 dipeptide is a component of the Cet1-binding site or that the loss of these side chains affects the binding site indirectly via a local conformational change.

The Ceg1 segment immediately upstream is a strongly hy-
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drophilic region that is larger in Ceg1 than in the other gua-
nyltransferases (Fig. 5A). Alanine substitutions at 10 posi-
tions within this segment (Gln296, Gly297, Asp300, Val301, Arg304, Lys306, Ser313, Asp314, Glu335, and Glu336) had no effect on cell growth. Thus, although Arg304 and Lys306 were protected from trypsin digestion when Ceg1 was bound to Cet1, these two residues are not required for the essential protein-protein interaction in vivo.

Alanine substitutions of 10 residues upstream of the hydro-
philic segment had no apparent effect on cell growth (Fig. 5B). These residues were: Asp269, Pro270, Trp280, Tyr281, Tyr282, Asn283, Tyr285, Asp285, Val289, and Phe290. The finding that the V289A mutant, either alone or in concert with F290A, had no phenotype is of interest in light of the earlier observations that the V289A E364G double mutant was ts for growth and could be suppressed by CET1 overexpression (2), i.e. it now seems likely that E364G is responsible for the growth phenotype. We found that two cluster mutants within this region, K287A P288A and L292A Y293A, displayed tight ts growth defects (Fig. 5B). CET1(201–549) overexpression suppressed L292A Y293A, but not K287A P288A (not shown). Single mutants P288L and P288S were isolated previously in screens for ceg1-ts alleles and it was found that the P288S ts phenotype could be suppressed by Cet1 overexpression (8, 27). We infer that the removal of the vicinal Lys287 side chain exacerbated the defect imparted by the proline change, insofar as the 287–288 cluster mutation was no longer suppressed by overexpression of the triphosphatase. Finally, the nearby V294A W295A mutant had a slow growth phenotype at 30 °C that was exacerbated at 37 °C (Fig. 5B). These data implicate the discrete peptide segments 287KPVSLLYW292 and 337WQNLKLNQEPLN348, as constituents of a Cet1-binding site in domain 2 of the yeast guanylyltransferase.

DISCUSSION

We have shown here that the S. cerevisiae guanylyltrans-
ferase Ceg1 is inherently thermolabile and that it is protected from heat inactivation by prior binding to S. cerevisiae RNA triphosphatase Cet1. The stabilization effect explains the ap-
parent stimulation of Ceg1 guanylyltransferase activity by Cet1 at 37 °C, as reported previously by our laboratory (2, 3), and may also bear on proposals concerning the effects of the RNA polymerase II CTD on yeast guanylyltransferase activity.

Cho et al. (8) have argued that the Ceg1 guanylyltransferase activity is allosterically regulated by interaction with both Cet1 and the CTD of RNA polymerase II. Their model is based on the following observations: Ceg1 bound to glutathione beads containing immobilized GST-CTD-PO4 did not react with [α-32P]GTP to form the covalent Ceg1-GMP intermediate; Cet1-Ceg1 complex bound to GST-CTD-PO4 beads did react to form Ceg1-GMP. They concluded from these data that the phosphorylated CTD inhibits the Ceg1 guanylyltransferase ac-
tivity and "association with Cet1 reverses this inhibition." However, no evidence was presented for reversal, i.e. it was not shown that the inactive form of Ceg1 bound to GST-CTD-PO4 beads could be reactivated by adding back Cet1 to the beads after the Ceg1 was bound. Furthermore, it is not clear whether the apparent loss of guanylyltransferase activity is solely attrib-
utable to the fact that Ceg1 is bound to CTD-PO4, because the binding reaction entails a 60-min incubation of isolated Ceg1 at room temperature, followed by a 20-min assay at 30 °C for enzyme-GMP formation (5, 8).

Takase et al. (13) proposed that the most important function of the Cet1 interaction is to allosterically activate Ceg1 bound to the phosphorylated CTD of pol II. Our data instate a more parsimonious model whereby at least one important function of the Cet1-Ceg1 interaction in vivo is to stabilize the Ceg1 gua-
nyltransferase activity against thermal inactivation.

The relative contributions of stabilization versus potential allosteric activation to cap formation in vivo in S. cerevisiae are difficult to gauge, but we suspect that stabilization is the pre-
dominant mechanism that best accounts for the available in vivo data. It is clear that elimination of the Ceg1-binding sur-
fact of Cet1 is lethal in vivo in a setting where guanylyltrans-
ferase expression is driven by the natural CEG1 promoter (2, 9, 13). Viability of cells lacking the Ceg1-binding domain of RNA triphosphatase (e.g. CET1(269–547) cells) can be restored by overexpressing the truncated triphosphatase and wild type Ceg1 guanylyltransferase from strong constitutive promoters, especially at high gene dosage (Figs. 3 and 4). Yet, as we have seen here, such cells fail to grow at 37 °C, a finding that correlates well with the profound loss of Ceg1 activity at this temperature in vitro when it is unprotected by the Cet1(232–265) peptide domain. The allosteric model posits that Ceg1 bound to the CTD is catalytically inactive unless it is bound to the Cet1(232–265) peptide. The rescue of the lethality of Cet1(265–549) by Mce1(211–597) when both proteins were overexpressed was interpreted as supportive of the allosteric model (13), because Mce1(211–547) is not inhibited by binding to the phosphorylated CTD (indeed it is stimulated by CTD-PO4 (20, 28)) and therefore the need for the Ceg1-binding do-
main would be obviated. Our results suggest an alternative interpretation, that the requirement for the Ceg1-binding do-
main in this genetic background is obviated because there is no longer a need to protect the mammalian or S. pombe gua-
nyltransferases, which are intrinsically more stable than Ceg1. Also, the allosteric model does not easily account for suppres-
sion of CET1(269–549) by overexpression of Ceg1, i.e. if gua-
nyltransferase bound to the CTD-PO4 is catalytically inac-
tive, then producing more guanylyltransferase will not obviously lead to increased activity when the proteins are CTD-
bound, unless one invokes secondary pathways for cap guany-
lation by Ceg1 protein either free in the nucleoplasm or bound to a docking site on the transcription elongation complex other than the CTD. Available evidence indicates that in yeast cells expressing normal levels of the capping enzymes, CTD phosphor-
ylation is required for recruitment of the guanylytransfer-
ase to the transcription complex (6, 7).

We now propose that a major role for the Cet1-Ceg1 inter-
action is to stabilize the guanylyltransferase, but the function of the interaction in recruitment of Cet1 to the pre-mRNA remains unsettled. The initial hypothesis in the wake of the discoveries that Ceg1, but not Cet1, bound to the phosphory-
lated CTD was that: (i) Ceg1 recruited Cet1 to the transcription complex via the CTD (8, 9); (ii) deletion of the Ceg1-binding site on Cet1 was lethal because it resulted in failure of Ceg1 to recruit triphosphatase to the elongation complex (9); and (iii) fusion to Mce1(211–597) simply restored the targeting function (9). This model no longer accounts for the new findings that fusion to Mce1(211–597) (or Pce1) is not required for rescue of the Cet1 deletion mutants when the components are over-
expressed (Ref. 13 and present study) and that Cet1(265–549) is associated with promoter-proximal DNA in yeast cells coex-
pressing Mce1(211–597) (13). Apparently, the yeast RNA triphosphatase can access its pre-mRNA substrate independent of Ceg1 binding when the triphosphatase is overexpressed. Its is not clear whether it does so via the same pathway taken when Cet1 is driven by its own promoter and Ceg1 is the source of the guanylyltransferase. Overexpressed Cet1 might access the transcriptional elongation complex by direct binding to the nascent RNA chain or by interaction with a docking site on the RNA polymerase or polymerase-associated proteins. It is not known if Cet1 association with transcribed genes in vivo is
normally contingent on CTD phosphorylation. We have observed that yeast cells expressing Mce1(211–597) as their only source of guanylyltransferase display a slow growth phenotype when Cet1 is expressed from its natural promoter and that normal growth is restored by overexpressing Cet1 (2, 3). Thus, we suspect that Cet1-Ceg1 complex formation does facilitate the function of the triphosphatase, presumably via assisting in targeting it to the CTD, even if the requirement for CTD targeting via Ceg1 is not absolute. The triphosphatase targeting function is more clearly established for the mammalian RNA triphosphatase domain, which does not sustain yeast cell growth unless fused to a guanylyltransferase (Figs. 3 and 4), either the natural mammalian guanylyltransferase domain or the heterologous guanylyltransferase of S. pombe.

Finally, biochemical and genetic experiments presented here implicate domain 2 of Ceg1 in Cet1 binding in vitro. Two segments within domain 2, 287-KPVSLYVW295 and 337-WQNLKNLEQPLN348, emerge as likely constituents of a Cet1-binding site on Ceg1. Finer analysis of the Cet1-Ceg1 interface now hinges on crystallization of the yeast guanylyltransferase complexed either to the native yeast triphosphatase or to the Ceg1-binding peptide of Cet1.

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An Essential Function of *Saccharomyces cerevisiae* RNA Triphosphatase Cet1 Is to Stabilize RNA Guanylyltransferase Ceg1 against Thermal Inactivation

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