Structure–function analysis of yeast tRNA ligase

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ABSTRACT

Trl1 is an essential 827-amino-acid enzyme that executes the end-healing and end-sealing steps of tRNA splicing in *Saccharomyces cerevisiae*. Trl1 consists of two catalytic domains—an N-terminal adenylyltransferase/ligase component (amino acids 1–388) and a C-terminal 5′-kinase/cyclic phosphodiesterase component (amino acids 389–827)—that can function in tRNA splicing *in vivo* when expressed as separate polypeptides. Sedimentation analysis indicates that the ligase and kinase/CPD domains are monomeric proteins that do not form a stable complex *in trans*. To understand the structural requirements for the RNA ligase component, we performed a mutational analysis of amino acids that are conserved in Trl1 homologs from other fungi. Alanine scanning identified 23 new residues as essential for Trl1-(1–388) activity *in vivo*. Structure–activity relationships at these positions, and four essential residues defined previously, were clarified by introducing 50 different conservative substitutions. Lethal mutations of Lys114, Glu184, Glu266, and Lys284 abolished Trl1 adenylyltransferase activity *in vitro*. The essential elements embrace (1) putative equivalents of nucleotidyltransferase motifs I, Ia, III, IV, and V found in DNA ligases, T4 RNA ligase 2, and mRNA capping enzymes; (2) an N-terminal segment shared with the T4 RNA ligase 1 subfamily only; and (3) a constellation of conserved residues specific to fungal tRNA splicing enzymes. We identify yeastlike tRNA ligases in the proteomes of *Leishmania* and *Trypanosoma*. These findings recommend tRNA ligase as a target for antifungal and antiprotozoal drug discovery.

Keywords: tRNA splicing; polynucleotide ligase; adenylyltransferase; antifungal target

INTRODUCTION

Yeast tRNA splicing occurs in three genetically and biochemically distinct stages (Abelson et al. 1998). First, a splicing endonuclease breaks the phosphodiester backbone of pre-tRNA at the exon–intron boundaries to yield 2′,3′ cyclic phosphate and 5′-OH termini at both incision sites. Second, the ends of the broken tRNA halves are healed and then sealed by yeast tRNA ligase (Trl1) to form a spliced tRNA containing a 2′-PO₄, 3′-5′ phosphodiester at the splice junction. Third, the 2′-PO₄ at the splice junction is removed by the 2′-phosphotransferase Tpt1.

The healing and sealing phases of the yeast tRNA splicing pathway consists of three enzymatic reactions catalyzed by Trl1: (1) the 2′,3′ cyclic phosphate terminus is hydrolyzed to a 3′-OH, 2′-PO₄ terminus by a 2′,3′ cyclic phosphodiesterase (CPD) activity; (2) the 5′-OH terminus is phosphorylated by a GTP-dependent polynucleotide kinase activity; and (3) the resulting 3′-OH, 2′-PO₄ and 5′-PO₄ ends are sealed by an ATP-dependent RNA ligase activity (Greer et al. 1983; Apostol

et al. 1991; Westaway et al. 1993; Sawaya et al. 2003). Trl1 consists of an N-terminal ligase module, a central polynucleotide kinase module, and a C-terminal CPD module (Fig. 1). The mechanism of the ligase component of yeast tRNA ligase generally resembles that of bacteriophage T4 RNA ligase 1 (Rnl1), whereby RNA joining entails three nucleotidyl transfer steps: (1) ligase reacts with ATP to form a covalent ligase-(lysyl-N)-AMP intermediate plus pyrophosphate; (2) AMP is transferred from ligase-adenylate to the 5′-PO₄ RNA end to form an RNA-adenylate intermediate (AppRNA); and (3) ligase catalyzes attack by an RNA 3′-OH on the RNA-adenylate to seal the two ends via a phosphodiester bond and release AMP (Cranston et al. 1974; Sugino et al. 1977). tRNA ligase and T4 Rnl1 are functionally and structurally homologous proteins (Sawaya et al. 2003; Wang et al. 2003) dedicated to the repair of programmed tRNA breaks *in vivo* (Amitsur et al. 1987; Phizicky et al. 1992). A key distinction between the two enzymes is that tRNA ligase apparently requires the terminal 2′-PO₄ on the proximal tRNA half-molecule, whereas T4 Rnl1 does not (Schwer et al. 2004).

tRNA ligase and Rnl1 exemplify a distinct branch of the covalent nucleotidyltransferase enzyme superfamily. The superfamily is composed of ATP-dependent and NAD⁺-dependent DNA ligases, ATP-dependent RNA ligases, and GTP-dependent mRNA capping enzymes, all of which catalyze the nucleotidylation of polynucleotide 5′ ends via a

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Article and publication are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2170305>.

FIGURE 1. Fungal tRNA ligases. The N-terminal ligase domain and C-terminal kinase-CPD domain of Trl1 are depicted as light and dark horizontal bars. The amino acid sequence of the ligase domain of *S. cerevisiae* (Sce) Trl1 from residues 51–360 is aligned to the sequences of the homologous proteins of *Saccharomyces bayanus* (Sba), *Candida albicans* (Cal), *Magnaporthe grisea* (Mgr), *Candida glabrata* (Cgl), *Gibberella zeae* (Gze), *Ustilago maydis* (Uma), *Neurospora crassa* (Ncr), *Coccidioides posadasii* (Cpo), and *Schizosaccharomyces pombe* (Spo). Positions of side chain identity or similarity in all 11 proteins are denoted by ^ below the alignment. Nucleotidyltransferase motifs I, Ia, IV, and V and the newly identified motif III are highlighted in shaded boxes. Residues identified as essential by alanine scanning are indicated by |. Nonessential residues are indicated by +. Positions Asn212 and Phe216 at which alanine mutations conferred a *ts* phenotype are indicated by Δ above the alignment.

covalent enzyme-(lysyl-N ζ)-NMP intermediate (Shuman and Lima 2004). The typical nucleotide-binding pocket of covalent nucleotidyltransferases is composed of six peptide motifs (I, Ia, III, IIIa, IV, and V) that contribute essential constituents of the active site. Structure–function studies of T4 Rnl1 and yeast tRNA ligase identified the putative equivalents of motifs I, Ia, IV, and V (Fig. 1), but revealed no obvious counterparts of motifs III or IIIa, or of the OB-fold domain located immediately downstream of motif V in DNA ligases and mRNA capping enzymes (Sawaya et al. 2003; Wang et al. 2003). Thus, the evolutionary relationship between tRNA ligase and other covalent nucleotidyltransferases remains unclear.

The ligase/adenylyltransferase functions of Trl1 reside within the N-terminal segment. Alignment of the N-terminal domains of Trl1 orthologs from 11 species of fungi reveals 120 positions of identity/similarity in all 11 polypeptides (indicated by ^ in Fig. 1). It is remarkable that no homolog of the Trl1 ligase domain can be detected in the available proteomes of any metazoan species, which makes tRNA ligase a plausible target for antifungal drug discovery. The value of tRNA ligase as a therapeutic target would be enhanced if one knew more about the structural basis for catalysis and substrate specificity. An initial alanine scan of eight residues in the ligase domain showed that Lys114 in motif I, which is the site of covalent

adenylation (Xu et al. 1990), Glu266 and Gly267 in motif IV, and Lys284 and Lys286 in motif V are essential for Trl1 activity, whereas alanine substitutions at Asn116 (in motif I), Glu152, and Glu153 are benign (Sawaya et al. 2003). Here we conduct a more extensive structure–function analysis of the N-terminal domain of Trl1, entailing alanine scanning and conservative substitutions at 46 positions. The results confirm an evolutionary connection between tRNA ligase and other covalent nucleotidyltransferases, but reveal a large number of essential components that are unique to the fungal tRNA ligase clade.

RESULTS AND DISCUSSION

Velocity sedimentation of the ligase and kinase-CPD domains of Trl1

Trl1 consists of an N-terminal adenylyltransferase/ligase domain, a central polynucleotide kinase domain, and a C-terminal CPD domain (Fig. 1). All three domains are essential *in vivo*, though they need not be linked covalently in the same polypeptide. For example, complementation of a lethal *trl1*Δ mutation by the plasmid shuffle method can be achieved by expressing the ligase domain Trl1-(1–388) and the kinase-CPD domain Trl1-(389–827) as separate polypeptides (Sawaya et al. 2003). However, it is possible that the domains normally interact physically with one another, either in *cis* within a single Trl1 protomer or in *trans* to form a Trl1 homo-oligomer, in which case the complementation by separately expressed domains might entail physical interaction between the ligase and kinase-CPD proteins. Here we analyzed the native size of the ligase domain Trl1-(1–388) and the kinase-CPD domain Trl1-(389–827) by zonal velocity sedimentation in a 15–30% glycerol gradient. Marker proteins catalase (248 kDa), BSA (66 kDa), and cytochrome c (12 kDa) were included as internal standards in the gradient. After centrifugation, the polypeptide compositions of the odd-numbered gradient fractions were analyzed by SDS-PAGE. The ligase domain (calculated to be a 45-kDa polypeptide) and the kinase-CPD domain (a 50-kDa polypeptide) both sedimented as a discrete peak overlapping the “light” side of the BSA peak (Fig. 2A). The adenylyltransferase activity profile of the ligase domain, gauged by reaction of the gradient fractions with

[α^{32} P]ATP to form a radiolabeled covalent ligase-AMP adduct, was coincident with the sedimentation profile of the Trl1-(1–388) protein (Fig. 2A). The polynucleotide kinase activity profile, assayed by label-transfer from [γ^{32} P]GTP to a 5'-OH 18-mer oligoribonucleotide, coincided with the abundance of the kinase-CPD polypeptide (Fig. 2B). These results are consistent with monomeric quaternary structures for the ligase and kinase-CPD domains. We tested interdomain complex formation by preincubating equal amounts of the ligase and kinase-CPD domains prior to glycerol gradient sedimentation. The sedimentation behavior of each component of the domain mixture was indistinguishable from what was seen when the domains were analyzed separately (not shown). That is, there was no evidence of formation of a heavier complex of the two proteins.

Mutational analysis of the N-terminal ligase domain

An initial alanine scan of eight positions in the adenylyltransferase domain identified five amino acids (Lys114, Glu266,

FIGURE 2. Sedimentation analysis of the ligase and kinase-CPD domains. Sedimentation of the ligase domain (A) and kinase-CPD domain (B) was performed as described in Materials and Methods. Aliquots (20 μ L) of the odd-numbered gradient fractions were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions of the recombinant Trl1 proteins and the internal standards catalase, BSA, and cytochrome c are indicated. The adenylyltransferase and polynucleotide kinase activity profiles are shown below the polypeptide profiles. Adenylyltransferase reaction mixtures (20 μ L) containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 2 mM DTT, 20 μ M [α^{32} P]ATP, and 1 μ L of the indicated glycerol gradient fractions were incubated for 12 min at 37°C. The products were analyzed by SDS-PAGE. The protein-[32 P]AMP adduct was quantified by scanning the gel with a Fujix BAS2500 imaging apparatus. Polynucleotide kinase reaction mixtures (10 μ L) containing 70 mM Tris-HCl (pH 7.6), 5 mM DTT, 10 mM $MgCl_2$, 50 pmol of 18-mer 5'-OH oligoribonucleotide r(AUUCGGAUAGUGACUACA), 40 μ M [γ^{32} P]GTP, and 1 μ L of a 1:20 dilution of the indicated glycerol gradient fractions were incubated for 20 min at 37°C. The products were analyzed by electrophoresis through a 15-cm 20% polyacrylamide gel containing 7 M urea in 45 mM Tris-borate 1.25 mM EDTA. The radiolabeled oligonucleotide products were quantified by scanning the gel.

Gly267, Lys284, and Lys286) as essential for Trl1 activity (Sawaya et al. 2003); these essential residues are located within putative counterparts of nucleotidyltransferase motifs I (¹¹⁴KANG¹¹⁷), IV (²⁶⁶EGFVI²⁷⁰), and V (²⁸²FFKYK²⁸⁶). Here, we used the alignment of fungal tRNA ligases in Figure 1 to guide a more comprehensive mutational analysis of Trl1-(1–388). The strategy was to replace individual residues of interest with alanine and then test the *TRL1*-(1–388)-*Ala* alleles for in vivo activity by complementation of *S. cerevisiae trl1Δ*. We selected 42 residues for alanine scanning. We focused on (1) conserved Arg, Lys, and His residues that we regarded as candidates for a direct role in catalysis of phosphoryl transfer or substrate binding; (2) conserved Glu and Asp residues as candidates for binding the requisite divalent cation cofactor(s); (3) conserved Gln, Asn, Ser, Thr, and Tyr side chains that might engage in hydrogen bonding with ATP or tRNA; and (4) conserved Phe residues that might engage in base-stacking interactions with ATP or tRNA.

TRL1-(1–388)-*Ala* alleles were cloned into a *CEN TRP1* plasmid so as to place their expression under the control of the native *TRL1* promoter. The plasmids were then cotransformed with a *TRL1*-(389–827) plasmid into a *S. cerevisiae trl1Δ* strain. Growth of *trl1Δ* is contingent on maintenance of a wild-type *TRL1* allele on a *CEN URA3* plasmid (Sawaya et al. 2003). Therefore, the *trl1Δ* 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 genes encoding biologically active ligase and kinase-CPD enzymes.

Twenty-three of the *TRL1*-(1–388)-*Ala* transformants failed to give rise to 5-FOA-resistant colonies after 7 d of incubation at 18°C, 25°C, or 30°C; thus these 23 alanine mutations were deemed lethal in vivo. The new residues defined as essential by the alanine scan were Arg66, Arg82, Lys86, Phe87, Phe88, Ser134, Lys135, His136, Ser137, His148, Glu184, Asp187, Asp188, Glu191, Glu192, His193, His207, Asn210, Arg271, Arg298, Glu299, Lys302, and Arg352. The essential amino acids are denoted by | over the aligned protein sequences in Figure 1. Failure of in vivo complementation could result from loss of catalytic function of the mutated proteins or mutational effects on intracellular Trl1-(1–388) protein concentration (via decreased synthesis or accelerated turnover) or localization. Lacking an antibody to Trl1, we did not determine the steady-state levels of the mutant proteins. Thus, we cannot assign the basis for in vivo lethality for every defective mutant. However, an analysis of the catalytic activity of recombinant versions of selected mutant Trl1-(1–388) proteins was informative (see below).

Nineteen other *TRL1*-(1–388)-*Ala* mutants supported colony formation during selection on 5-FOA at either 25°C or 30°C. The viable *TRL1*-(1–388)-*Ala* strains were tested for growth on rich medium (YPD agar) at 18°C, 25°C, 30°C, and 37°C. Two of the mutants, *N212A* and *F216A*, displayed a temperature-sensitive phenotype, whereby *N212A* and

F216A cells grew as well as “wild-type” *TRL1*-(1–388) *TRL1*-(389–827) cells at 18°C, 25°C, and 30°C, but failed to grow at 37°C (scored as *ts* in Table 2 and denoted by Δ in Fig. 1). Seventeen other *TRL1*-(1–388)-*Ala* strains grew at all temperatures and their colony sizes were similar to that of wild-type *TRL1*-(1–388) *TRL1*-(389–827) cells (Tables 1 and 2). We surmise that Asp85, Asn89, Glu92, Thr96, Asp110, Thr212, Cys133, Thr180, Asn242, Glu289, Arg295, Glu296, Thr301, Lys313, Arg315, Lys316, and Thr321 are not essential for tRNA ligase activity in vivo. The nonessential residues are denoted by + in Figure 1.

We tested the effects of conservative substitutions at the 23 positions defined as essential by the present alanine scan and at the four essential nonglycine residues identified previously. Arginine was replaced by lysine and glutamine, lysine by arginine and glutamine, histidine by asparagine and glutamine, glutamate by aspartate and glutamine, aspartate by glutamate and asparagine, asparagine by aspartate and glutamine, serine by threonine, and phenylalanine by leucine. A total of 50 conservative mutants were tested by plasmid shuffle for *trl1Δ* complementation; the results are shown in Tables 1 and 2.

Structure–activity relationships at essential residues of motifs Ia, IV, and V

Four of the essential residues are located within or immediately adjacent to nucleotidyltransferase motifs Ia, IV, and V. Crystal structures of exemplary nucleotidyltransferase family members show that motif Ia is located atop a β-hairpin loop on the surface of the enzyme; the Arg or Lys chain of the Ser-(Arg/Lys) dipeptide is proposed to contact the γ phosphate of the NTP substrate and/or the reactive 5'-PO₄ end of the polynucleotide to be sealed (Håkansson et al. 1997; Odell et al. 2000; Fabrega et al. 2003; Deng et al. 2004; Ho et al. 2004; Pascal et al. 2004). Mutations of the motif Ia Lys/Arg residue resulted in loss of activity in the case of T4 Rnl1, T4 Rnl2, *Chlorella* virus DNA ligase, and mammalian RNA capping enzyme (Odell et al. 2000; Sawaya and Shuman 2003; Wang et al. 2003; Yin et al. 2003). Here we found that Lys135 in motif Ia of Trl1 was strictly essential, i.e., Arg and Gln substitutions were lethal (Table 1). The two flanking serine residues of motif Ia, Ser134 and Ser137, were also strictly essential—i.e., threonine substitutions were lethal. Thus, the hydroxyl group is critical at positions 134 and 137 and the extra bulk of the threonine methyl group is deleterious. Replacing the essential His136 side chain of motif Ia with glutamine was lethal, but the asparagine substitution restored Trl1 function in vivo. These findings argue against a role for His136 as a general acid–base catalyst; rather they suggest that hydrogen bonding of the histidine Nδ atom (on which the Nδ of Asn can superimpose) is the relevant functional contribution of His136.

The motif IV carboxylate side chain is essential for all members of the covalent nucleotidyltransferase superfamily (Sriskanda and Shuman 2002a; Sawaya and Shuman 2003;

TABLE 1. Effect of missense mutations on Trl1 ligase activity in vivo

Trl1 (1–388) mutation	<i>trl1</i> complementation
R66A	lethal
R66K	lethal
R66Q	lethal
R82A	lethal
R82K	lethal
R82Q	lethal
D85A	+++
K86A	lethal
K86R	lethal
K86Q	lethal
F87A	lethal
F87L	lethal
F88A	lethal
F88L	lethal
N89A	+++
E92A	+++
T96A	+++
D110A	+++
T112A	+++
K114R	lethal
K114Q	lethal
C133A	+++
S134A	lethal
S134T	lethal
K135A	lethal
K135R	lethal
K135Q	lethal
H136A	lethal
H136N	+++
H136Q	lethal
S137A	lethal
S137T	lethal
H148A	lethal
H148N	lethal
H148Q	lethal
T180A	+++
E184A	lethal
E184D	lethal
E184Q	lethal
D187A	lethal
D187N	+++
D187E	lethal
D188A	lethal
D188N	lethal
D188E	lethal
E191A	lethal
E191Q	lethal
E191D	lethal
E192A	lethal
E192Q	+++
E192D	lethal
H193A	lethal
H193N	lethal
H193Q	lethal
H207A	lethal
H207N	lethal
H207Q	lethal

(++) Wild-type growth

Wang et al. 2003; Yin et al. 2003). Here we found that replacing the motif IV Glu266 side chain of Trl1 by either glutamine or aspartate resulted in loss of function. We surmise that a carboxylate moiety is essential in Trl1 and that activity depends on the proper distance of the carboxylate from the main chain. The motif IV carboxylate coordinates a divalent cation in the crystal structures of DNA ligases (Odell et al. 2000; Pascal et al. 2004); we invoke a similar role for Glu266 in Trl1. The Arg271 side chain immediately flanking motif IV can be functionally replaced by lysine, but not glutamine (Table 2). Many, but not all, nucleotidyltransferases have an arginine or lysine at the position corresponding to Arg271 of Trl1. The equivalent residue is a lysine in T4 Rnl2; mutation of this lysine to Ala abolishes Rnl2 activity (Yin et al. 2003). Exemplary crystal structures show that the motif IV Arg/Lys side chain comprises half of an ion pair that stabilizes the architecture of the active site (Odell et al. 2000; Deng et al. 2004; Ho et al. 2004).

The two motif V lysines are critical for the function of ATP-dependent DNA and RNA ligases and GTP-dependent mRNA capping enzymes (Srisankanda and Shuman 2002b; Sawaya and Shuman 2003; Yin et al. 2003). Here we find that neither arginine nor glutamine can function in lieu of Lys284 and Lys286 of Trl1 (Table 2). The motif V lysines contact the phosphates of the NTP substrate, the covalent ligase-adenylate intermediate, or the polynucleotide-adenylate intermediate in various crystal structures of nucleotidyltransferase superfamily members (Subramanya et al. 1996; Håkansson et al. 1997; Odell et al. 2000; Pascal et al. 2004).

An essential Rnl1-like module located proximal to motif I

Five of the essential side chains of Trl1 mapped to a bipartite module upstream of the AMP attachment site, of consensus sequence RGLF(X)₉₋₁₇RGYDKFFN (essential residues underlined), that is conserved in bacteriophage T4 Rnl1 and the Rnl1-like ligases encoded by other phages and by certain baculoviruses (Wang et al. 2003; Martins and Shuman 2004; Blondal et al. 2005), but not in other branches of the covalent nucleotidyltransferase superfamily. Mutational analysis of T4 Rnl1 showed that the arginine of the RGLF peptide and the lysine and second phenylalanine of the KFFN peptide were essential for RNA strand joining in vitro (Wang et al. 2003). Here we find that Arg66 and Arg82 are strictly essential for Trl1 function; lysine or glutamine substitutions were lethal at both positions (Table 1). Lys86 is also strictly essential, insofar as arginine or glutamine substitutions were lethal (Table 1). We surmise that the aromaticity of Phe87 and Phe88 are essential, because leucine substitutions were lethal at either position (Table 1).

A candidate motif III counterpart in tRNA ligase

Analysis of Trl1 primary structure did not reveal any obvious counterparts of nucleotidyltransferase motifs III or IIIa found

TABLE 2. Effect of missense mutations on Trl1 ligase activity in vivo

Trl1 (1–388) mutation	<i>trl1Δ</i> complementation
N210A	lethal
N210D	<i>ts</i>
N210Q	<i>ts</i>
N212A	<i>ts</i>
F216A	<i>ts</i>
N242A	+++
E266Q	lethal
E266D	lethal
R271A	lethal
R271K	+++
R271Q	lethal
K284R	lethal
K284Q	lethal
K286R	lethal
K286Q	lethal
E289A	+++
R295A	+++
Q296A	+++
R298A	lethal
R298K	lethal
R298Q	lethal
E299A	lethal
E299Q	<i>cs</i>
E299D	lethal
T301A	+++
K302A	lethal
K302R	lethal
K302Q	lethal
K313A	+++
R315A	+++
K316A	+++
T321A	+++
R352A	lethal
R352K	lethal
R352Q	lethal

(+++) Wild-type growth
(*ts*) Temperature-sensitive
(*cs*) Cold-sensitive

in other branches of the ligase/capping enzyme superfamily. The consensus motif III sequence is $\phi\phi\phi\text{DGE}\phi\phi$ (where ϕ is an aliphatic side chain). In our initial small-scale alanine scan of Trl1, we targeted the $^{152}\text{Gly-Glu}^{153}$ dipeptide as a possible counterpart of motif III, but found that Trl1 activity in vivo was unaffected by the G152A or E153A mutations (Sawaya et al. 2003). This raises two possibilities: either (1) Trl1 has no counterpart of motif III or (2) it has a counterpart of motif III, but its sequence has diverged from the consensus. We noted previously that the aspartate preceding the glycine, though essential when present in ligases or capping enzymes, is not strictly conserved in all nucleotidyltransferases (Wang et al. 1997). Also, the glycine is replaced in some ligases and capping

enzymes by alanine, serine, cysteine, or threonine. The glutamate is the only strictly conserved motif III residue, and it is essential for ligase or capping enzyme activity in all cases tested (Skriskanda and Shuman 2002a; Sawaya and Shuman 2003; Zhu and Shuman 2005). The present mutational analysis of Trl1 identified three essential glutamate side chains in the segment between motif Ia and motif IV. Of these, Glu184 is located within the sequence $^{179}\text{VTAVA}\underline{\text{EYC}}^{186}$, which we propose is the motif III equivalent of Trl1 (Fig. 1). We find that Glu184 is strictly essential, because conservative substitutions with glutamine or aspartate were lethal in vivo (Table 1). The motif III glutamate contacts the ribose sugar of the NTP substrate, the covalent enzyme-NMP intermediate, or the polynucleotide-adenylate intermediate in various crystal structures of nucleotidyltransferase superfamily members (Subramanya et al. 1996; Håkansson et al. 1997; Pascal et al. 2004). We propose a similar role for Glu184 in Trl1.

Essential structural elements unique to tRNA ligases

The essential His148 side chain is conserved in all fungal tRNA ligases and is located between motifs Ia and III. Replacing His148 with either asparagine or glutamine was lethal in vivo (Table 1). These conservative replacements are mimetics of the hydrogen-binding capacity of the N δ and N ϵ atoms, respectively. Conceivably, the His149 side chain either (1) makes bivalent polar interactions via N δ and N ϵ , (2) makes ionic interactions as the protonated form, (3) serves as a general acid or general base catalyst during one or more steps of the ligation reaction, or (4) depends on its aromatic character for sustaining Trl1 function. It is noteworthy that Sidrauski et al. (1996) identified a viable mutant allele of *TRL1* with a single H148Y coding change that selectively impaired the function of Trl1 in *HAC1* mRNA splicing during the unfolded protein response without affecting tRNA splicing in vivo. The ability of tyrosine to function in lieu of His149 excludes options 1 and 2 above and weighs against a mechanism whereby this residue acts as an essential general base catalyst.

Seven essential side chains are located within the region downstream of motif III, five of which (Asp187, Asp188, Glu191, Glu192, and His193) are clustered within an acidic patch. Based on the crystal structures of other ligases, we would predict that this patch projects on the surface of the nucleotidyltransferase domain. Whereas Asp188 and Glu191 are strictly essential, the ligase retained function when either Asp187 or Glu192 were replaced by their isosteric amide derivatives (Table 1). We surmise that hydrogen bonding capacity is the relevant property of the Asp187 and Glu192 side chains. Neither His193 nor His207 tolerated replacement by asparagine or glutamine. The ligase domain was functional in vivo when Asn210 was replaced by either aspartate or glutamine. The *N210D* and *N210Q* strains grew at 25°C and 30°C, but not at 37°C (scored as *ts* in Table 2).

Five essential residues mapped to the ligase segment downstream of motif V. Changing Arg298 to lysine or glutamine

was lethal in vivo, suggesting that this residue makes an essential bidentate interaction. Replacing Lys302 with either arginine or glutamine was lethal as well. Whereas changing Glu299 to aspartate was lethal, the glutamine mutant grew at 25°C, 30°C, and 37°C, but not at 18°C (scored as *cs* is Table 2). The most distal essential residue, Arg352, also could not be replaced by Lys or Gln. The necessity of Arg352 explains the previous finding that deletion of the segment from amino acids 321–362 abolished ligase function in vivo (Sawaya et al. 2003).

Effects of selected mutations on adenylyltransferase activity in vitro

Recombinant Trl1-(1–388) proteins containing lethal conservative substitutions in motifs I, IV, and V were produced in *Escherichia coli* as His₁₀-tagged fusions and purified from soluble bacterial extracts by Ni-agarose chromatography in parallel with wild-type Trl1-(1–388) (Fig. 3, top panel). The adenylyltransferase activity of wild-type Trl1-(1–388) was evinced by label transfer from [α -³²P]ATP to form a covalent enzyme-[³²P]adenylate adduct detectable by SDS-PAGE and autoradiography (Fig. 3, bottom panel). The yield of Trl1-(1–388)-[³²P]AMP is proportional to input enzyme (Sawaya et al. 2003). Changing the motif I lysine (Lys114) to either arginine or glutamine abolished

FIGURE 3. Mutational effects on adenylyltransferase activity in vitro. (A) Aliquots (8 μ g) of recombinant wild-type and mutated Trl1-(1–388) proteins were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions and sizes (in kilodaltons) of marker polypeptides are indicated on the left. (B) Adenylyltransferase reaction mixtures (20 μ L) containing 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2 mM DTT, 20 μ M [α -³²P]ATP, and 1 μ g of the indicated ligase protein were incubated for 12 min at 37°C. The products were resolved by SDS-PAGE and visualized by autoradiography.

FIGURE 4. Effects of motif III glutamate mutations on adenylyltransferase activity. (A) Aliquots (8 μ g) of recombinant wild-type Trl1-(1–388) and mutant proteins E184A, E184D, and E184Q were analyzed by SDS-PAGE. The Coomassie blue-stained gel is shown. The positions and sizes (in kilodaltons) of marker polypeptides are indicated on the left. (B) Adenylyltransferase reaction mixtures (20 μ L) contained 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 2 mM DTT, 20 μ M [α -³²P]ATP, and 1 μ g of the indicated ligase protein. The products were resolved by SDS-PAGE and visualized by autoradiography.

adenylyltransferase activity. Thus, arginine is not a suitable nucleophile for attack by Trl1 on the α phosphate of ATP. Replacing the motif IV glutamate (Glu266) with either aspartate or glutamine also abolished ligase adenylylation; this finding is consistent with the proposed metal-binding function of Glu266. Conservative mutations of motif V Lys284 eliminated adenylyltransferase activity as well. Thus, the in vivo lethality of these mutations is readily explained by inability to perform the first chemical step of the three-step RNA ligation reaction. In contrast, the motif V K286R mutant retained adenylyltransferase activity (the yield of K286R-AMP complex being 21% of the wild-type level), suggesting that lethality in vivo is caused by a defect downstream of step 1 of the ligation pathway. The K286Q mutant was 5% as active as wild-type ligase in the autoadenylation reaction (Fig. 3). We also purified mutants of the putative motif III glutamate, E184A, E184D, and E184Q (Fig. 4A). The E184A and E184Q mutations abolished adenylyltransferase activity; the E184D mutant was 13% as active as wild-type Trl1-(1–388) (Fig. 4B).

Candidate trifunctional tRNA ligases from *Leishmania* and *Trypanosoma*

The phylogenetic distribution of Trl1-like proteins is surprisingly narrow, given the wide occurrence of tRNA introns in archaea and eukarya. Trl1 homologs are found in all genera of fungi for which genome sequences are available. Trl1-like proteins are absent from the proteomes of archaea and metazoans. This may be because archaea and metazoans use a different end-joining mechanism for tRNA splicing than do fungi (Filipowicz and Shatkin 1983; Laski et al. 1983; Zofalova et al. 2000). None of the specific

proteins responsible for tRNA ligation in archaea or metazoans have been identified nor have their genes been cloned as yet. This scenario, in which the Trl1-like pathway in metazoans is either redundant or performed by enzymes without recognizable structural similarity to fungal tRNA ligases, recommends Trl1 as an excellent target for antifungal drug discovery.

One dividend of assembling a comprehensive structure–function map of Trl1 is that it improves our ability to confidently identify homologous tRNA splicing enzymes from novel sources, by focusing on conservation of the amino acids shown to be essential, even where global primary structure similarity is not high. This approach proved extremely fruitful in the case of another essential fungal RNA processing enzyme, the RNA triphosphatase component of the mRNA capping apparatus (Shuman 2002). Fungi and metazoans diverge completely with respect to the structure and mechanism of their RNA triphosphatase enzymes. The fungal triphosphatases belong to a superfamily of metal-dependent phosphohydrolases that includes the RNA triphosphatases of many nonfungal unicellular eukarya, including *Plasmodium*, *Trypanosoma*, and *Giardia*. A cap-centric scheme of eukaryotic phylogeny suggests a common ancestry for the mRNA processing machinery of fungi and protozoa (Shuman 2002).

To see whether this relationship extends to tRNA splicing, we searched for Trl1 homologs encoded by protozoan organisms and thereby identified candidate tRNA ligases from the kinetoplastid protozoa *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania major* (Fig. 5). The 870-amino-acid *T. brucei* protein (designated TbrTrl1) is composed of an N-terminal adenyltransferase/ligase domain, a central kinase module, and a C-terminal CPD module, each of which resembles the equivalent functional domain of yeast Trl1. The ligase domain of TbrTrl1 contains putative counterparts of motifs I, Ia, III, IV, and V (shaded in Fig. 5), as well as a T4 Rnl1-like segment upstream of the predicted AMP attachment site. Eighteen individual amino acids found in the protozoan ligase domains that we predict correspond to essential residues of Trl1 identified in the present study are indicated by dots over the sequences in Figure 5. A Blast search using the ligase domain of TbrTrl1 readily identified the fungal tRNA ligases as the closest homologs, followed by bacteriophage Rnl1. The central kinase domain of the protozoan tRNA ligases contains the GxGKT and RxxxR motifs found in yeast Trl1 and T4 polynucleotide kinase (Wang et al. 2002; Sawaya et al. 2003). The C-terminal CPD motif of the protozoan proteins contains the H Φ T Φ motifs of the 2H phosphotransferase enzymes that are present in fungal tRNA ligases (Mazumder et al. 2002; Sawaya et al. 2003). We found no credible homologs of the protozoan proteins in metazoan proteomes. Given that *Trypanosoma* and *Leishmania* are significant human pathogens, we regard the family of Trl1-like ligases as targets for the discovery of new broad-spectrum antifungal and antiprotozoal drugs.

Finally, as we were preparing this study for submission, Englert and Beier (2005) reported the identification of

Tbr	MEVLVERCLFKREWMGANVEDIVECAHSGKAHFATGAF-----NSLIVKES	47
Tcr	METFLDGGCFKKPWTDDGRVGVVEYAGTGKRLFCGTAF-----TSFEVKES	
Lma	MEVLKRECFWHRQATPRSTKVDTAALDG---YVARGRVTLAAVKLKDGRDVSSVAKEA	
Tbr	HQGDVSLPIAARTLLLS-ADTSEIVVRGVNKKFFDIEDVERDLMWKGDAVLQG---IW	103
Tcr	HQGDVCLKPTGARTLLLD-ADASKVMVRGVNKKFFDIEDDKTEELN-KDELWEMGS---	
Lma	SQGDVKGFLPCGARSLLIDRADGS-IVCRGINKFFDLNEVGDD---WLSDSLSWTGAYRMW	
Tbr	LQRKLAGFVVTFLFSLDGKSLGVVTKHVVEGPHVDIAYHILESMLSTEQRRLARDLPFLR	163
Tcr	LQRKAVAGFTVTLFSLDGGSGVGTTHKVVGLHVELARCVLDHLLSAEQKLLAPDLFSAE	
Lma	AVRKMAGFIVTLFSLDGVRLVMSKHALAGPHVDAARELLQV-SEMQRTRLAADLFEWG	
Tbr	AAVSCECISLKDDSHHPVPERTVFDNKLVPFSIHKRRDMREVSVPPDIMQMAKEWGFPS	223
Tcr	AAVSCECVSLKDDLHHPVLEPVEYDNKLFISVHKRNDLREIAVPIEKVQVMSKRWGLPV	
Lma	ACACCCELRRAQDYHHPVLEAQFDDQVILVIAVRQRTQLQESVCFSELTALAEKRWGLGC	
Tbr	VPCWRVKNCEELITWLGERQHWEKDPAGEA---LAEGYVVVIEDTVQRVSPESQISIFP	280
Tcr	VPSWRAGGKELQWLQKREKWLGEKDPGIP---LAEGYVVLVEIPVTRMSSSVVLEFPF	
Lma	APGVAVPNSAALRCVFAATASWNATYTLFDMCPQLAEGFVLLIEMAGSTVASGHRW----	
Tbr	TALLIRLKAKTVKYRVLRLSLRSIVSGESAHPPLFKHLKGSWTEHISGYTCLQAVTNRG	340
Tcr	TVAPLRLKAKTVKYRVLRLSLRSILTDGSTEKQLFHDVLAVWSEHIGGYKCFKQAVAQRG	
Lma	-CLPLRLKLTAKYVVLRAFRSLVCGDSRQPSVYVHQAALLGWLQLSAAE-VRRRVEEVG	
Tbr	VHQIYSEFESYINDQGNKRYRSHLSVQEAYKGLGFTKEAQTIYSAPLDVIFLCGLP	399
Tcr	VCEICSQFETYVSTHSKRLRGSHMSIQAFELNFTGEGALVKRRASLNVIMLCGIP	
Lma	VHALNDFESGLSAQQRVRHRDSQETVGDALARLQRTTALACPLGRCLPTVVVLCGLP	
Tbr	GVGKTTTLARLIGEM-GGNGC-STFRYLHLRSRDEVAKCAEHGINDSSKHKRRLRSM	457
Tcr	GAGKTTLSLALMAM-AERGD-SPFRYLILSRDQVARDVAAREGIDSSSKHKRQLRAM	
Lma	GAGKSTLAVALSRLLASQGEASPFSPVHLSRDAVHCTVAADQGITDASKHKRRLRSGAL	
Tbr	VHQVQVAFRQSILLSFLLEGPGLLIFDCAKAPSTQRWRMYLPRELASFRIYVLACAE	517
Tcr	VHALLAEVQKAVFLSLIQEGPGLLMDACNAKAEATRRVMRGLPKPKVESFRLLHVTCS	
Lma	VHQTLCNQMEVARFSAETEQSGLLLLDACNATQKSRHWRQCFPKTLHKYEVAVVECS	
Tbr	RNIIASRIARRNHSVLRGAEEAQAALYAVGKEFVAPLESEPCFCLDAAAATEDLARSV	577
Tcr	HPPELLRLAKRRHDVIRNNSAQTVLYAVKKVFLPELEDELCLVRLDTASTSAEDIARYV	
Lma	ATAMVQRAARRDGEHVLSSAAEAQAALYAVRKKFPQPSLEEVAVRWDTKCCVEEAAASL	
Tbr	LLLYEETNGPAPSCRHRAVYDHDQAERELTKHCAFTATPATSILGDHYNDSMTLARS-	633
Tcr	FSLYKGDG-----QRVATVYDVRGPEEGLKALRATIVESLLGPVGEGASTLLMS----	
Lma	RTHLTE-RAPPSSCAGCCRILTTSAIGDERRESHATLHNLNLAGSETATAALASMLSVN	
Tbr	---EVSRK-----KAVNTVAVQLL-----VKFDELYEIIISDVVKSITV	668
Tcr	---SVKRI-----KTSNAIFVRLIIS-----DELLQIASEAIMAVT-	
Lma	SAPKVARATSLQLRLDCSTEALRCAAQQLISSLDEPREPAVSFASFGRLLFHGVSA--	
Tbr	VVDLPKLSWWEKCKRGLGNRRRPGVVEKGHWHNLRLGWLDDGVEGETPPEVSVFRALE	728
Tcr	GMDCLSLGWWEKFKRALGIEIRPLCSTIEDGHTWTHNLGPDGKREKGAFSADARHAT	
Lma	---LRKISWAPSFR-VTGH-----VTRGHLKWLRLGRDPSG-PVELE--KALQ	
Tbr	DRYECQPALPHVTVFH-----GPTSAATVWSVPA-----IGSAVDVTLHSVLIDPFALC	777
Tcr	ERFECRASAPHTFAH-----DASSVESPLVPE-----AGSMANVLTSLDDRFAIC	
Lma	ERFAVVPARPHVTLLVYNAAGATSAQAISEYLSQTMHGLQEVTVKVTSLLLDRHALS	
Tbr	LGATVVLNQRQELQL-SPGCAVP-LHVTVGHTRRVKASYAGEMFDLFQQRSHNRKLEH	835
Tcr	FGATAVVGQRYEKQL-GLSETVP-LHVTVCHTERVKPSYAGEMFDLFRQWGHNNKELQH	
Lma	FGVTLLTGGQVQEQSMHGTGCTVRLHITVGTAPGVAASYAGNMFAIFDVLQAEENSLT	
Tbr	MRLGF--SVKKSRSKFFNFVQVRLA	858
Tcr	MKSGV--LVKRSRLKFFNFVQVRLA	
Lma	ERRDSFQAVNRSQRKYHNFVQVRLA	

FIGURE 5. Trl1-like tRNA ligases from *Trypanosoma* and *Leishmania*. The amino acid sequence of a Trl1 homolog from *T. brucei* (Sanger Institute Tb10.6k15.2400) is aligned to related proteins encoded by *T. cruzi* (TIGR_5693) and *L. major* (Sanger Institute LM36.1.Contig1). Nucleotidyl transferase motifs I, Ia, III, IV, and V, kinase motifs GxGK(S/T) and RxxxR, and 2H motifs HVTV in the CPD domain are highlighted in shaded boxes. Individual amino acids in the protozoan proteins corresponding to known essential residues of yeast Trl1 are indicated by • above the alignment.

the cDNA encoding tRNA ligase from the plant *Arabidopsis thaliana*. Although plant RNA ligases catalyze the same set of healing and sealing reactions as does Trl1 (Gegenheimer et al.

1983; Schwartz et al. 1983; Pick and Hurwitz 1986; Pick et al. 1986), the plant tRNA ligase polypeptide has scant similarity to fungal Trl1 (Englert and Beier 2005). It will be of interest to test whether the protozoan and plant ligases can function in yeast in lieu of Trl1.

MATERIALS AND METHODS

Yeast vectors encoding missense mutants of Trl1-(1–388)

Missense mutations and overlapping diagnostic restriction sites were introduced into the *TRL1*-(1–388) open reading frame (ORF) via the two-stage PCR overlap extension method as described previously (Sawaya et al. 2003). The mutated PCR products were digested with BamHI and SacII and inserted into the isolated vector backbone of BamHI/SacII-cut *CEN TRP1* plasmid pSE358-*TRL1*(1–388) (Sawaya et al. 2003). The mutant gene thereby replaces with wild-type ORF; expression of *TRL1*-(1–388) in this vector is under the control of the native *TRL1* promoter. The inserts were sequenced completely to confirm the presence of the desired mutations and to exclude the acquisition of unwanted coding changes during PCR amplification and cloning.

Test of Trl1-(1–388) function by plasmid shuffle

The *trl1Δ* haploid strain YRS1 (*MATα ura3-1 ade2-1 trp1-1 his3-11,15 leu2-3,11 can1-100 trl1::kanMX p360-TRL1*) was cotransformed with a *CEN TRP1* plasmid bearing a wild-type or mutated version of *TRL1*-(1–388) and a *CEN ADE2 TRL1*-(389–827) plasmid (Sawaya et al. 2003). Transformants were selected on medium lacking tryptophan and adenine. Two individual colonies were transferred to fresh selective medium. The isolates were then streaked on agar medium containing 0.75 mg/mL 5-FOA. The plates were incubated at 18°C, 25°C, and 30°C. Lethal mutations were those that did not allow formation of FOA-resistant colonies after 7 d at any of the temperatures tested. Other mutated alleles supported FOA-resistant colony formation at one or more of the growth temperatures. At least two individual colonies from each streak were picked from the FOA plate, transferred to yeast extract/peptone/dextrose (YPD) medium and then tested for growth on YPD agar at 18°C, 25°C, 30°C and 37°C. *TRL1*-(1–388) mutants that formed “wild-type” sized colonies at all temperatures were scored as +++. Temperature sensitive (*ts*) mutants grew at all temperatures except 37°C. Cold-sensitive (*cs*) mutants grew at all temperatures except 18°C.

Plasmids for expression of Trl1-(1–388) in bacteria

The T7-based expression vector pET28a-His₁₀ was constructed by replacing the MluI–NdeI fragment of pET28a (encoding a His₆ tag) with the MluI–NdeI fragment of pET16b (encoding a His₁₀ tag). The ORF encoding the ligase domain Trl1-(1–388) was excised from pUC-Trl1-(1–388) with BamHI and SacI and inserted into pET28a-His₁₀ to yield pET28a-His₁₀Trl1-(1–388). DNA fragments encoding mutated versions of the ligase domain mutants were excised from the respective yeast p358-*TRL1*-(1–388) plasmids with BamHI and SacII and inserted into the isolated vector

backbone of BamHI/SacII-cut pET28a-His₁₀Trl1-(1–388) in lieu of the wild-type ORF.

Recombinant Trl1-(1–388) proteins

pET28a-His₁₀Trl1-(1–388) plasmids were transformed into *E. coli* BL21(DE3). Single kanamycin-resistant colonies were inoculated into Luria-Bertani medium containing 0.06 mg/mL kanamycin. One-hundred-milliliter cultures were incubated at 37°C until the *A*₆₀₀ reached 0.5. The cultures were placed on ice for 30 min, adjusted to 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 2% (v/v) ethanol. Incubation was continued at 17°C for 15 h with constant shaking. Cells were harvested by centrifugation and the pellets stored at –80°C. All subsequent procedures were performed at 4°C. Thawed bacterial pellets were resuspended in 7 mL of lysis buffer (50 mM Tris-HCl at pH 7.5, 1.2 M NaCl, 15 mM imidazole, 10% glycerol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride) containing 1 mg/mL lysozyme and 0.2% Triton X-100. The lysates were sonicated to reduce viscosity. Insoluble material was removed by centrifugation in a Sorvall SS34 rotor at 14,000 rpm for 45 min. The soluble extracts were applied to 0.75-mL columns of Ni-NTA agarose (Qiagen) equilibrated with lysis buffer. The columns were washed with 5 mL of lysis buffer and then eluted step-wise with buffer A (50 mM Tris-HCl at pH 7.5, 0.2 M NaCl, 10% glycerol) containing 100 and 300 mM imidazole. The polypeptide compositions of the column fractions were monitored by SDS-polyacrylamide gel electrophoresis (PAGE). The recombinant Trl1-(1–388) proteins were retained on the column and recovered in the 300 mM imidazole eluates. Peak fractions were pooled and stored at –80°C. Protein concentrations were determined using the BioRad dye-binding assay with bovine serum albumin as a standard.

Velocity sedimentation

Aliquots (40 μg) of His₁₀-Trl1-(1–388) or His₁₀-Trl1-(389–827) were mixed with catalase (30 μg), BSA (30 μg), and cytochrome c (30 μg). The protein mixture was applied to a 4.8-mL 15–30% glycerol gradient containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 2.5 mM DTT, and 0.1% Triton X-100. The gradient was centrifuged in a Beckman SW50 rotor at 50,000 rpm for 16 h at 4°C. Fractions (~0.2 mL) were collected from the bottom of the tubes.

ACKNOWLEDGMENTS

This research was supported by NIH grant GM42498. S.S. is an American Cancer Society Research Professor.

Received January 27, 2005; accepted March 2, 2005.

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