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Structure–function analysis of the kinase-CPD domain of yeast tRNA ligase (Trl1) and requirements for complementation of tRNA splicing by a plant Trl1 homolog

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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 domains—an N-terminal ligase component and a C-terminal 5′-kinase/2′,3′-cyclic phosphodiesterase (CPD) component—that can function in tRNA splicing *in vivo* when expressed as separate polypeptides. To understand the structural requirements for the kinase-CPD domain, we performed an alanine scan of 30 amino acids that are conserved in Trl1 homologs from other fungi. We thereby identified four residues (Arg463, His515, Thr675 and Glu741) as essential for activity *in vivo*. Structure–function relationships at these positions, and at four essential or conditionally essential residues defined previously (Asp425, Arg511, His673 and His777), were clarified by introducing conservative substitutions. Biochemical analysis showed that lethal mutations of Asp425, Arg463, Arg511 and His515 in the kinase module abolished polynucleotide kinase activity *in vitro*. We report that a recently cloned 1104 amino acid *Arabidopsis* RNA ligase functions in lieu of yeast Trl1 *in vivo* and identify essential side chains in the ligase, kinase and CPD modules of the plant enzyme. The plant ligase, like yeast Trl1 but unlike T4 RNA ligase 1, requires a 2′-PO_4 end for tRNA splicing *in vivo*.

INTRODUCTION

Yeast tRNA splicing occurs in three stages (1). First, a splicing endonuclease breaks the 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 a tRNA ligase to form a 2′-PO_4, 3′–5′ phosphodiester at the splice junction. Third, the junction 2′-PO_4 is transferred to NAD^+ to form the mature spliced tRNA, nicotinamide and ADP-ribose 1′,2′ cyclic phosphate. The yeast tRNA ligase (Trl1) catalyzes three essential reactions during the healing/sealing stage: (i) the 2′,3′ cyclic phosphate terminus is hydrolyzed to a 3′-OH, 2′-PO_4 terminus by a 2′,3′ cyclic phosphodiesterase (CPD) activity; (ii) the 5′-OH terminus is phosphorylated by a GTP-dependent polynucleotide kinase activity; and (iii) the resulting 3′-OH, 2′-PO_4 and 5′-PO_4 ends are sealed by an ATP-dependent RNA ligase activity (1–6).

Trl1 consists of an N-terminal ligase module that resembles bacteriophage T4 RNA ligase 1 (Rnl1), a central polynucleotide kinase module that resembles T4 polynucleotide kinase, and a C-terminal CPD module that resembles the 2H phosphotransferase superfamily. Initial structure–function studies showed that all three modules are essential *in vivo*, though they need not be linked in the same polypeptide (6). Complementation of a lethal *trl1*Δ mutation by the plasmid shuffle method was achieved by expressing the ligase domain Trl1-(1–388) and the kinase-CPD domain Trl1-(389–827) as unlinked polypeptides. Moreover, each of the three component catalytic activities is essential for cell growth, insofar as alanine mutations in the active sites of the ligase, kinase and CPD modules are lethal *in vivo*.

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The mechanism of the ligase component of yeast Trl1 is similar to that of T4 Rnl1, which joins broken tRNAs via three nucleotidyl transfer steps, as follows: (i) ligase reacts with ATP to form a covalent ligase-(lysyl-N)–AMP intermediate plus pyrophosphate; (ii) AMP is transferred from ligase-adenylate to the 5′-PO_4 RNA end to form an RNA-adenylate intermediate (AppRNA); and (iii) ligase catalyzes attack by an RNA 3′-OH on the RNA-adenylate to seal the two ends via a phosphodiester bond and release AMP (7–9). An important difference is that yeast tRNA ligase apparently requires the terminal 2′-PO_4 on the proximal tRNA half-molecule, whereas T4 Rnl1 does not (10). An extensive alanine scan of the N-terminal ligase domain of yeast Trl1 identified 28 individual amino acids essential for activity *in vivo* (6,11). Structure-activity relationships at these positions were then illuminated by conservative substitutions. The essential elements included: (i) putative equivalents of nucleotidyltransferase motifs I, Ia, III, IV and V found in DNA ligases, T4 RNA ligases 1 and 2, and mRNA capping enzymes (12); (ii) an N-terminal segment shared with the T4 Rnl1 subfamily only; and (iii) a constellation of conserved residues specific to fungal tRNA splicing enzymes (11).

Less is known about the mechanisms and structure-activity relationships of the kinase and CPD components. An initial alanine scan showed that mutations K404A and T405A in the P-loop (^{401}GxGKT^{405}) of the central kinase module had no effect on Trl1 function *in vivo*. The K404A and T405A mutations eliminated the ATP-dependent RNA kinase activity of Trl1 *in vitro*, but preserved GTP-dependent kinase activity. The double-alanine mutant K404A-T405A in the P-loop was lethal *in vivo* and abolished GTP-dependent kinase activity (6). These results indicated that GTP is the physiological substrate and that the Trl1 kinase has a single NTP binding site of which the P-loop is a component. Two other mutations in the central domain were lethal *in vivo* and either abolished (D425A) or severely reduced (R511A) GTP-dependent RNA kinase activity *in vitro*. Mutations of the two signature histidines of the '2H' motifs in the CPD module were either lethal (H777A) or conferred a temperature sensitive growth phenotype (H673A). Here we conduct more extensive structure–function analyses of the kinase-CPD domain of yeast Trl1, entailing alanine scanning and conservative substitutions at 34 positions, which reveal unique structural requirements, or lack thereof, for the Trl1 kinase-CPD activity *vis a vis* those of T4 polynucleotide kinase or 2H phosphoesterase family members.

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. Putative homologs of yeast Trl1 are also found in the kinetoplastid protozoa *Trypanosoma* and *Leishmania* (11). Yet, Trl1-like proteins are absent from archaea and metazoans. A multifunctional Trl1-like enzyme has been purified to near homogeneity from wheat germ (13–18) and the cDNA encoding *Arabidopsis* RNA ligase was identified recently (19). Although the plant RNA ligase polypeptide displays very limited global sequence similarity to yeast Trl1, it does contain counterparts of some of the signature ligase, kinase and CPD motifs found in yeast Trl1 (19), and they are arranged in the same linear order as in yeast Trl1, i.e. ligase-kinase-CPD. Here we ask whether plant RNA ligase is a true ortholog of Trl1, by testing its ability to complement growth of a *trl1*Δ yeast strain. We report that: (i) the RNA repair activity is portable from plants to fungi; (ii) plant tRNA ligase, like Trl1, is active *in vivo* as unlinked ligase and kinase-CPD domains; and (iii) the plant ligase, like yeast Trl1 but unlike T4 Rnl1, requires a 2′-PO_4 end for tRNA splicing *in vivo*. Also, we map constituents of the ligase, kinase and CPD active sites of plant RNA ligase, thereby resolving ambiguities about what motifs are relevant and underscoring the similarities between the plant and fungal tRNA splicing enzymes.

MATERIALS AND METHODS

Yeast vectors encoding missense mutants of Trl1-(389–827)

Missense mutations and overlapping diagnostic restriction sites were introduced into the *TRL1*-(389–827) open reading frame (ORF) via the two-stage PCR overlap extension method as described previously (6). The mutated PCR products were digested with BamHI and SacII and inserted into a *CEN ADE2* plasmid to place expression of *TRL1*-(389–827) under the control of the native *TRL1* promoter (6). 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-(389–827) function by plasmid shuffle

The *trl1*Δ haploid strain YRS1 (*MAT*α *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 *TRL1*-(1–388) and a wild-type or mutated version of the *CEN ADE2 TRL1*-(389–827) plasmid (6). 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, 25 and 30°C. Lethal mutations were those that did not allow formation of FOA-resistant colonies after 10 days 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, 25, 30 and 37°C. *TRL1*-(389–827) mutants that formed 'wild-type' sized colonies at all temperatures were scored as +++. Temperature sensitive (*ts*) mutants grew at all temperatures except 37°C.

Recombinant Trl1-(389–827) proteins

DNA fragments encoding wild-type and mutated versions of the kinase-CPD domain were excised from the respective yeast *TRL1*-(389–827) plasmids with SacI and BamHI and inserted into pET28a. The resulting pET28a-His_{10}Trl1-(389–827) plasmids were transformed into *Escherichia coli* BL21(DE3). Single kanamycin-resistant colonies were inoculated into Luria–Bertani medium containing 0.06 mg/ml kanamycin. Cultures (100 ml) were incubated at 37°C until the A_{600} reached 0.5. The cultures (100 ml) were placed on ice for 30 min, adjusted to 0.3 mM isopropyl-β-D-thiogalactopyroanoside (IPTG) and 2% (v/v) ethanol.

Incubation was continued at 17°C for 16 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 (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 r.p.m. for 45 min. The soluble extracts were applied to 0.7 ml columns of Ni-NTA agarose (Qiagen) equilibrated with lysis buffer. The columns were washed with 3 ml of lysis buffer and then eluted step-wise with buffer A [50 mM Tris–HCl (pH 7.5), 0.2 M NaCl and 10% glycerol] containing 100 and 300 mM imidazole. The polypeptide compositions of the column fractions were monitored by SDS–PAGE. The recombinant Trl1-(389-827) 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 BSA as a standard.

Polynucleotide kinase assay

Reaction mixtures (10 μl) containing 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 25 μM [γ-^{32}P]GTP, 50 pmol of a synthetic 5′-OH RNA oligonucleotide (5′-AUUCCGAUAGUGACUACA) and 10 ng wild-type or mutant Trl1-(389–827) were incubated for 20 min at 37°C. The reactions were quenched by adding 10 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). The products were analyzed by electrophoresis through a 15 cm 20% polyacrylamide gel containing 7 M urea in TBE (90 mM Tris borate and 2.5 mM EDTA). The radiolabeled products were visualized by autoradiography of the gel and quantified with a phosphorimager.

Yeast vectors encoding *Arabidopsis* RNA ligase

A NotI–SalI DNA fragment containing the cDNA encoding full-length *Arabidopsis thaliana* RNA ligase (AtRNL) was excised from a pIVEX-WG vector (19) and inserted into pYX132 (*CEN TRP1*) so as to place the plant gene under the transcriptional control of a yeast *TPI1* promoter. Missense mutations were introduced into the *AtRNL* ORF via the two-stage PCR overlap extension method. The mutated PCR products were inserted into pYX132. The inserts were sequenced completely to confirm the presence of the desired mutations and to exclude the acquisition of unwanted coding changes (compared to the wild-type cDNA clone). The ability of the wild-type and mutated versions of full-length plant ligase to complement the yeast *trl1*Δ strain was tested by plasmid shuffle. Individual Trp$^+$ transformants were streaked on agar medium containing 0.75 mg/ml 5-FOA. The plates were incubated at 19, 30 and 37°C. Lethal mutations were those that did not allow formation of FOA-resistant colonies after 10 days 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 YPD medium and then tested for growth on YPD agar at 19, 25, 30, 34 and 37°C. Strains that formed 'wild-type' sized colonies at all temperatures were scored as +++. Strains that formed pinpoint colonies at 25 and 30°C (defined as + growth) and failed to grow at 34 and 37°C (ts) were scored as '+ ts'.

Domains of *Arabidopsis* RNA ligase

Deleted versions of *AtRNL* were constructed by PCR amplification using oligonucleotide primers that introduced either a start codon at amino acid 676 and an upstream flanking NcoI site or a stop codon at amino acid 676 and a downstream flanking NheI site. The *AtRNL*-(1–675) ORF was inserted into yeast *CEN TRP1* and *CEN HIS3* vectors under the control of the *TPI1* promoter. The *AtRNL*-(677–1104) ORF was inserted into a *CEN TRP1* plasmid. The plasmids encoding ligase and kinase-CPD domain fragments were tested for *trl1*Δ complementation by pairwise transformation (with each other or with plasmids encoding analogous yeast and T4 domains), followed by plasmid shuffle as described in the preceding section.

RESULTS

Mutational analysis of the kinase module of yeast Trl1

A primary structure alignment of the kinase-CPD domain of *Saccharomyces cerevisiae* Trl1 with homologous proteins from other fungal species is shown in Figure 1. Our initial mutational analysis of the kinase module highlighted: (i) the P-loop motif GxGKT^{405} (underlined in Figure 1) as a determinant of NTP binding, as evinced by the selective effects of single alanine mutations on ATP-dependent versus GTP-dependent kinase activity; (ii) the essential role of the distal arginine of the RxxxR^{511} motif (underlined in Figure 1); and (iii) the essentiality of Asp425 (shaded in Figure 1), but not Asp454 (6). These limited data suggested a relationship to the kinase domain of T4 polynucleotide kinase/phoshatase (Pnkp), the active site of which is composed of similar elements, including: (i) a classical P-loop motif (GxxGxGKS^{16}) that coordinates the beta phosphate of the NTP donor; (ii) essential side chain Arg126 (putative equivalent of Arg511 in Trl1), which also coordinates the NTP beta phosphate; and (iii) essential side chain Asp35 (putative equivalent of Asp425 in Trl1), which we proposed functions as a general acid to activate the 5′-OH for attack on the NTP γ phosphate (20–23).

To better define the active site of the yeast kinase, and search for distinctive structural components that might account for its preference for GTP as the substrate *in vitro* and *in vivo*, we performed an alanine scan of the 20 positions in the kinase module of *S.cerevisiae* Trl1 that are conserved among fungal Trl1 proteins (Figure 1; Table 1). We focused on: (i) Arg, Lys and His residues that we regarded as candidates for a direct role in catalysis of phosphoryl transfer or substrate binding; (ii) Asp and His residues as candidates for binding the requisite divalent cation cofactor(s); (iii) Gln, Asn, Ser and Thr side chains that might engage in hydrogen bonding with NTP or RNA.

The twenty *TRL1*-(389–827)-*Ala* alleles were cloned into a *CEN ADE2* plasmid so as to place their expression under the control of the native *TRL1* promoter. The plasmids were then cotransformed with a *TRL1*-(1–388) plasmid into

Figure 1. Kinase-CPD domain of fungal tRNA ligases. The amino acid sequence of the kinase-CPD domain of *S.cerevisiae* (Sce) Trl1 from residues 396–795 is aligned to the sequences of the homologous proteins of *Saccharomyces bayanus* (Sba), *Candida albicans* (Cal), *Coccidioides posadasii* (Cpo), *Magnaporthe grisea* (Mgr), *Neurospora crassa* (Ncr), *Aspergillus nidulans* (Ani) and *Schizosaccharomyces pombe* (Spo). Gaps in the alignment are denoted by dashes. The P-loop and RxxxR motifs of the kinase module and the two HxT motifs of the CPD module are underlined. Residues identified as essential by alanine scanning are indicated by 'l' and highlighted in shaded boxes. Nonessential residues are indicated by +. Position His673 at which mutations conferred a *ts* phenotype is indicated by 'Δ'.

a *S.cerevisiae trl1*Δ strain. Growth of *trl1*Δ is contingent on maintenance of a wild-type *TRL1* allele on a *CEN URA3* plasmid (6). Therefore, the *trl1*Δ 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.

Two of the *TRL1*-(389–827)-*Ala* transformants failed to give rise to 5-FOA-resistant colonies after 10 days at 18, 25 or 30°C. The two new residues defined as essential by the alanine scan were Arg463 and His515 (shaded in Figure 1). Eighteen other *TRL1*-(389–827)-*Ala* mutants supported colony formation during selection on 5-FOA at either 18, 25 or 30°C. The viable *TRL1*-(389–827)-*Ala* strains were tested for growth on rich medium (YPD agar) at 18, 25, 30 and 37°C. All of the viable strains grew at all temperatures and their colony sizes were similar to that of wild-type *TRL1*-(1–388) *TRL1*-(389–827) cells (scored as +++ growth in Table 1). We surmise that Thr406, Gln423, Asn424, Asp426, Lys430, Arg455, Asn456, Asn457, Arg461, Gln465, Lys485, Arg507, Asn513, Asn514, Gln516, Ser517, Asp549 and Asn569 are not essential for tRNA splicing activity *in vivo*. The nonessential residues are denoted by + in Figure 1.

To clarify the structure-activity relationships, we introduced conservative substitutions at the essential Arg463 and His515 positions defined presently and at the essential Asp425 and Arg511 residues identified earlier. Arginine was replaced by lysine and glutamine; histidine by asparagine and glutamine; aspartate by glutamate and asparagine. The eight conservative mutants were tested by plasmid shuffle for *trl1*Δ complementation; the results are shown in Table 1. We found that Arg463 and Arg511 were strictly essential, i.e. Lys and Gln substitutions were lethal, implying that neither positive charge

Table 1. Effect of kinase module mutations on Trl1 activity *in vivo*

Trl1(389–827) mutation	*trl1*Δ complementation
T406A	+++
Q423A	+++
N424A	+++
D426A	+++
K430A	+++
R455A	+++
N456A	+++
N457A	+++
R461A	+++
R463A	lethal
Q465A	+++
K485A	+++
R507A	+++
N513A	+++
N514A	+++
H515A	lethal
Q516A	+++
S517A	+++
D549A	+++
N569A	+++
D425E	+++
D425N	lethal
R463K	lethal
R463Q	lethal
R511K	lethal
R511Q	lethal
H515Q	lethal
H515N	lethal

nor hydrogen bonding potential at these positions sufficed for Trl1 activity *in vivo*. Our inference is that these arginines make bidentate interactions with the kinase substrates or other functional groups on the enzyme. His515 was also strictly essential, as neither Gln nor Asn (partial isosteres that can mimic the hydrogen bonding capacity of histidine Nϵ and Nδ, respectively) restored function. In contrast, glutamate was able to restore growth in lieu of Asp425, although asparagine was not (Table 1). We surmise that a carboxylate is critical at this position and that the enzyme can accommodate the extra methylene group of glutamate without severe functional interference.

Mutational effects on Trl1 kinase activity *in vitro*

Failure of *in vivo* complementation could result from loss of catalytic function of the mutated proteins or mutational effects on intracellular Trl1-(389–827) 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-(389–827) proteins was informative.

Wild-type Trl1-(389–827) and ten missense mutants were produced in *E.coli* as His_{10}-tagged fusions and purified from soluble bacterial extracts by Ni-agarose chromatography (Figure 2A). The proteins were tested for kinase activity *in vitro*. The assay entails transfer of ^{32}P-label from [γ-^{32}P]GTP to an 18mer 5′-OH RNA oligonucleotide (6). Eight of the mutations that were lethal *in vivo*—D425N, R463A, R463K, R463Q, R511K, R511Q, H515A and H515Q—were found to reduce kinase activity *in vitro* to

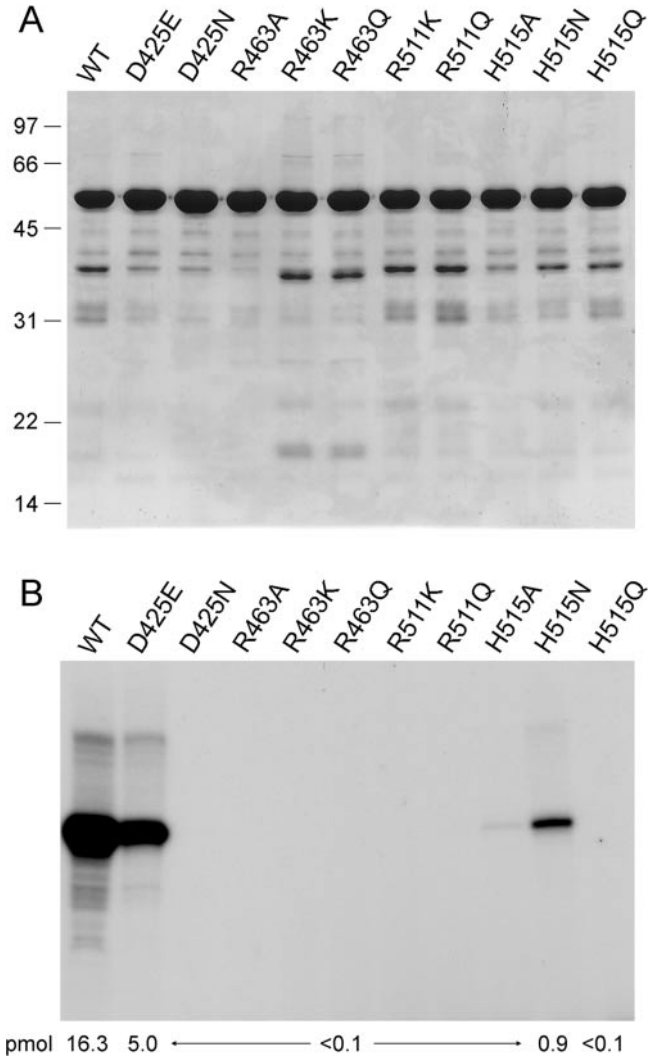

Figure 2. Mutational effects on polynucleotide kinase activity *in vitro*. (**A**) Aliquots (8 μg) of recombinant wild-type and mutated Trl1-(389–827) proteins were analyzed by SDS–PAGE. The Coomassie blue-stained gel is shown. The positions and sizes (kDa) of marker polypeptides are indicated on the left. (**B**) Kinase reaction mixtures contained [γ-^{32}P]GTP, 18mer 5′-OH RNA substrate, and the indicated Trl1-(389–827) proteins. The products were resolved by PAGE and the radiolabeled 18mer RNA detected by autoradiography. The extent of product formation is indicated below the lanes.

<1% of the wild-type level (Figure 2B). The lethal H515N mutation reduced the yield of labeled RNA product *in vitro* to 5% of the wild-type level. The D425E mutation, which restored activity *in vivo*, also revived kinase activity *in vitro* to ∼31% of the wild-type level (Figure 2B). Thus, there was good agreement between mutational effects on growth and RNA kinase activity *in vitro*. Previous studies showed that the lethal R511A mutation reduced GTP-dependent kinase activity *in vitro* to ∼14% of the wild-type level (6). Together, these data suggest that a threshold level of 5′ end-healing activity is required for yeast growth.

Mutational analysis of the CPD module of yeast Trl1

The CPD module of Trl1 contains the two signature HxT motifs of the 2H phosphoesterase family (underlined in Figure 1) (24). Alanine mutations of the histidines of the

Trl1 CPD domain were either lethal (H777; indicated by 'l' in Figure 1) or conferred a ts growth phenotype (H673; denoted by 'Δ' in Figure 1) (6). Here we sought to identify additional essential residues by alanine scanning of 10 conserved positions in the fungal CPD domain (Table 2). The ten *TRL1*-(389–827)-*Ala* plasmids were cotransformed with a *TRL1*-(1–388) plasmid into the *S.cerevisiae trl1*Δ strain and then tested for complementation by plasmid shuffle. Two of the CPD mutations were lethal, thereby defining Thr675 and Glu741 as essential. Eight other CPD module mutants were viable under 5-FOA selection and grew as well as wild-type *TRL1*-(1–388) *TRL1*-(389–827) cells on YPD agar, as gauged by colony size (+++ growth in Table 2). Thus, Gln669, His678, Arg683, Lys742, Asn773, Thr779, Ser792 and Asn793 are not essential for tRNA splicing *in vivo*. The nonessential residues in the CPD module are indicated by + in Figure 1.

Structure-activity relationships were probed by introducing conservative substitutions at the essential Thr675, Glu741 and His777 positions of the CPD module and at His673 in the proximal HxT motif, where an alanine change resulted in ts growth. Threonine was replaced by serine and valine; histidine by asparagine and glutamine; glutamate by aspartate and glutamine. Eight conservative mutants were tested by plasmid shuffle for *trl1*Δ complementation (Table 2). Asn and Gln substitutions for His777 were lethal, implying that hydrogen bonding potential did not suffice for CPD activity *in vivo*. Serine was fully functional in lieu of Thr675, but valine was not, indicating that the hydroxyl group was critical at this position. Introducing either aspartate or glutamine in place of Glu741 restored CPD activity, signifying that a carboxylate is not critical and an amide suffices, presumably via its ability to engage in hydrogen bonding. Note that all of the fungal tRNA ligases shown in Figure 1 naturally have an aspartate at the position corresponding to Glu741 of the *S.cerevisiae* enzyme. The conditional growth phenotype observed for the H673A mutant was not ameliorated by conservative replacement of His673 with Asn or Gln (Table 2).

Our mutational data attest to the fact that the two HxT motifs of the CPD module are not functionally equivalent. Specifically, Thr675 in the proximal HxT motif of Trl1 is essential, whereas Thr779 in the distal HxT motif is not; H777 in the downstream HxT motif is unconditionally essential, while loss of the histidine in the proximal HxT element confers a ts growth phenotype.

Table 2. Effect of CPD module mutations on Trl1 activity *in vivo*

Trl1(389–827) mutation	*trl1*Δ complementation
Q669A	+++
T675A	lethal
H678A	+++
R683A	+++
E741A	lethal
K742A	+++
N773A	+++
T779A	+++
S792A	+++
N793A	+++
H673N	ts
H673Q	ts
T675S	+++
T675V	lethal
E741D	+++
E741Q	+++
H777N	lethal
H777Q	lethal

Plant RNA ligase can function as a tRNA splicing enzyme in yeast

Englert and Beier (19) reported the purification to near homogeneity of a 125 kDa RNA ligase from wheat germ that catalyzed auto-adenylylation and sealing of a precleaved synthetic intron-containing tRNA substrate. Peptide sequencing of the purified wheat enzyme enabled the isolation of an *Arabidopsis* cDNA encoding the plant RNA ligase. Recombinant His-tagged *Arabidopsis* RNA ligase prepared by *in vitro* translation and affinity chromatography was shown to seal tRNA halves to form a product with a 2′-PO_4 at the splice junction (19). Notwithstanding the identical reaction outcomes for yeast Trl1 and the plant RNA ligase, there is as yet no genetic evidence that the *Arabidopsis* enzyme is responsible for tRNA splicing *in planta*. We showed previously that tRNA repair systems are portable in yeast, e.g. bacteriophage T4 Rnl1 and Pnkp can provide the healing and sealing functions normally performed by Trl1 and thereby complement growth of the *S.cerevisiae trl1*Δ mutant (10). Here, we exploit yeast as a surrogate model system for genetic analysis of the plant RNA ligase. The key finding was that expression in yeast of the cDNA encoding the full-length 1104 amino acid plant RNA ligase polypeptide under the control of a constitutive yeast promoter on a single-copy plasmid sufficed to complement growth of the yeast *trl1*Δ strain (Table 3). Thus, the plant RNA ligase is a true ortholog of yeast Trl1.

Mapping essential constituents of the RNA ligase module of plant tRNA ligase

Despite their biochemical similarities and functional overlap as tRNA splicing enzymes, the yeast and plant RNA ligases have, at first glance, remarkably little primary structure similarity (19). Whereas the C-terminal segment of plant ligase

Table 3. Mutational effects on plant tRNA ligase activity

AtRNL allele	*trl1*Δ complementation
Wild-type	+++
K152A	lethal
E218A	lethal
E243A	+++
E326A	lethal
K436A	+++
K438A	+++
D529A	+++
K541A	lethal
K543A	lethal
D564A	+++
K586A	+++
K588A	+++
K700A	+++
S701A	lethal
D726A	lethal
R804A	+ *ts*
H999A	+ *ts*
T1001A	lethal
H1060A	lethal
T1062A	+++

includes two HxT motifs and is therefore recognizable as homologous to the fungal CPD domain, the putative plant kinase module in the central portion of the protein is quite divergent from that of yeast Trl1, and the plant N-terminal domain is hardly recognizable as a polynucleotide ligase, except for the presence of a candidate adenylylation site (motif I; ^{152}KHSG) (19).

Further scrutiny of the sequence of the *Arabidopsis* polypeptide in light of the now extensive structure–function analysis of yeast Trl1 affords some clues to the organization of the plant ligase. Focusing on the N-terminal domain reveals three potential candidates for a counterpart of nucleotidyl transferase motif IV (^{326}EGLVA, ^{529}DGLAD, and ^{564}DGAAA), three candidates for a counterpart of motif V (^{434}LMKEK, ^{539}MIKMK, and ^{584}DGKQK), and two candidates for a counterpart of motif III (^{213}MCISMELV and ^{238}VTAVTELG). To begin to sort out whether the plant ligase actually has an active site composed of the 'standard' motifs (12) and to sort out which of the candidate plant motifs are 'real' (i.e. important for function) versus 'impostors', we conducted an alanine scan of the underlined residues and tested the Ala alleles in yeast for *trl1*Δ complementation. The results of this analysis are shown in Table 3.

The first salient finding was that the Lys152 side chain in putative nucleotidyltransferase motif I is essential for plant tRNA ligase activity, consistent with the prediction that this lysine is the nucleophile in the auto-adenylylation reaction of the RNA ligase module. The KxSG motif I of plant tRNA ligase diverges at the serine position from the KxNG motif I of fungal tRNA ligases (11) and from the canonical KxDG signature of DNA ligases, mRNA capping enzymes and T4 Rnl1 (12). In the case of *S.cerevisiae* Trl1, the Asn of the KxNG motif is nonessential for tRNA splicing activity *in vivo* (6).

Nucleotidyltransferase motif IV typically consists of an essential Asp or Glu followed by a glycine and three aliphatic residues. Motif V (typically ϕϕKϕK, where ϕ is an aliphatic side chain) contains two essential lysines. Motifs IV and V are located close together in the primary structure; they are separated by 10–20 amino acids in DNA ligases, RNA capping enzymes and fungal tRNA ligases. Only one of the three candidates for a plant motif IV contained an essential carboxylate side chain, that being ^{326}EGLVA (shaded black in Figure 3). Because the Asp529 and Asp564 side chains in the other motif IV candidates (^{529}DGLAD, and ^{564}DGAAA) were nonessential, we judged these motifs to be impostors (shaded gray in Figure 3). These results were puzzling, insofar as the real motif IV has no candidate motif V located at the expected distance downstream, whereas both of the 'false' motifs IV actually do have a KxK sequence situated immediately downstream (Figure 3). Mutating the alternating lysines in the motif V candidates highlighted ^{539}MIKMK as the real motif V, containing essential Lys541 and Lys543 constituents, and relegated the two other peptides (^{434}LMKEK and ^{584}DGKQK) to impostor status. Thus, the separation between motifs IV and V in the protein primary structure (208 amino acids) is exceptionally long in plant RNA ligase.

Nucleotidyltransferase motif III was identified initially as ϕϕϕDGEϕϕ, although it was soon recognized that the upstream aspartate was not strictly conserved, being replaced by an aliphatic or aromatic side chain in many members of the covalent nucleotidyltransferase superfamily (25). It is now clear that the glutamate is the defining component of motif III; the glutamate contacts the ribose sugar of the NTP substrate and is essential for enzyme activity. The findings that Glu218 is essential and Glu243 is not point to the ^{213}MCISMELV peptide as a likely motif III equivalent in plant RNA ligase and eliminate the ^{238}VTAVTELG peptide from contention.

Essential constituents of the CPD and kinase modules of plant tRNA ligase

The ^{999}HxT and ^{1060}HxT motifs of the C-terminal CPD module of plant RNA ligase were subjected to alanine scanning. Tests of function of the mutants in yeast by plasmid shuffle showed that: (i) His1060 in the second HxT motif was unconditionally essential, whereas loss of His999 in the first motif conferred a severe ts phenotype and (ii) Thr1001 in the first HxT motif was essential, whereas Thr1062 in the second motif was not (Table 3). These mutational effects, which echo the findings for alanine substitutions at the equivalent positions of yeast Trl1, underscore the nonequivalence of the two signature motifs of the CPD domain.

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MDAPFESGDSSATVVAEAVNNQFGGLSLKESNTNAPVLPSQTTSNHRVQNLVWKPKSYGTVSGSSSATEVGKTSA  75
VSQIGSSGDTKVGLNLSKIFGGNLLEKFSVDKSTYCHAQIRATFYPKFENEKTDQEIRTRMIEMVSKGLATLEVS  150
LKHSGSLFMYAGHKGGAYAKNSFGNIYTAVGVFVLSRMFREAWGTKAPKKEAEFNDFLEKNRMCISMELVTAILG  225
DHGQRPLDDYVVVTAVTELGNGKPQFYSTSEIISFCRKWRLPTNHVWLFSTRKSVTSFFAAFDALCEEGIATSVC  300
RALDEVADISVPASKDHVKVQGEILEGLVARIVSSQSSRDMENVLRDHPPPPCDGANLDLGLSLREICAAHRSNE  375
KQQMRALLRSVGPSFCPSDVEWFGDESHPKSADKSVITKFLQSQPADYSTSKLQEMVRLMKEKRLPAAFKCYHNF  450
HRAEDISPDNLFYKLVVHVHSDSGFRRYHKEMRHMPSLWPLYRGFFVDINLFKSNKGRDLMALKSIDNASENDGR  525
GEKDGLADDDANLMIKMKFLTYKLRTFLIRNGLSILFKDGAAAYKTYYLRQMKIWGTSDGKQKELCKMLDEWAAY  600
IRRKCGNDQLSSSTYLSEAEPFLEQYAKRSPKNHILIGSAGNLVRTEDFLAIVDGDLDEEGDLVKKQGVTPATPE  675
PAVKEAVQKDEGLIVFFPGIPGSAKSALCKELLNAPGGFGDDRPVHTLMGDLVKGKYWPKVADERRKKPQSIMLA  750
DKNAPNEDVWRQIEDMCRRTRASAVPIVADSEGTDTNPYSLDALAVFMFRVLQRVNHPGKLDKESSNAGYVLLMF  825
YHLYEGKNRNEFESELIERFGSLIKMPLLKSDRTPLPDPVKSVLEEGIDLFNLHSRRHGRLESTKGTYAAEWTKW  900
EKQLRDTLVANSEYLSSIQVPFESMVHQVRGELKTIAKGDYKPPSSEKRKHGSIVFAAINLPATQVHSLLEKLAA  975
ANPTMRSFLEGKKKSIQEKLERSHVTLAHKRSHGVATVASYSQHLNREVPVELTELIYNDKMAALTAHVGSVDGE  1050
TVVSKNEWPHVTLWTAEGVTAKEANTLPQLYLEGKASRLVIDPPVSISGPLEFF*                       1104
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Figure 3. *Arabidopsis* RNA ligase. The amino acid sequence of *A.thaliana* RNA ligase is shown. Candidate motifs subjected to mutational analysis are shaded. Peptide motifs containing an essential amino acid side chain are shaded black. Nonessential 'impostor' motifs in the N-terminal ligase domain are shaded gray.

The central portion of the plant ligase polypeptide contains a candidate P-loop motif GIPGSAKS[701] (Figure 3). Alanine scanning showed that Lys700 was nonessential, whereas the loss of the Ser701 hydroxyl was lethal (Table 3). The dispensability of the P-loop lysine is a consistent finding in yeast and plant ligases, but the plant enzyme is apparently more reliant on the vicinal hydroxyamino acid of the P-loop. Located ~100 amino acids downstream of the plant P-loop is a sequence RVLQR[804] that we considered a potential homolog of the RVIKR[511] motif conserved in the fungal Trl1 proteins, which includes the Arg511 residue essential for polynucleotide kinase activity. The corresponding R804A mutation of plant RNA ligase elicited a severe growth defect in yeast; *R804A* cells formed pinpoint colonies on YPD agar at 19, 25 or 30°C and failed to grow at 34 or 37°C. We also mutated Asp726 in the plant RNA ligase and found it to be essential for activity in yeast (Table 3). Asp726 is located 25 amino acids downstream of the P-loop and is a plausible equivalent of the Asp425 side chain that we found to be essential for the kinase function of yeast Trl1.

Delineation of separable sealing and healing domains of plant tRNA ligase

The results of the alanine scan of plant RNA ligase show that tRNA splicing activity *in vivo* depends on all three catalytic modules. Moreover, the delineation of essential motifs provides valuable internal landmarks for comparing the plant ligase to other RNA repair enzymes. Because previous studies of yeast Trl1 showed that its component domains were autonomous and functional in *trans*, we asked whether this is also the case for the plant enzyme. We chose a plausible domain boundary between motif V of the ligase module and the P-loop of the kinase module and cloned the ORFs encoding the N-terminal ligase module (1–675) and the C-terminal kinase-CPD domain (677–1104) into yeast centromeric plasmids, where their expression is driven by a constitutive yeast *TPI1* promoter. The instructive finding was that complementation of the *trl1*Δ strain could be achieved by cotransformation with *CEN HIS3 AtRNL*-(1–675) and *CEN TRP1 AtRNL*-(677–1104) plasmids (Table 4). The *AtRNL*-(1–675) *AtRNL*-(677–1104) strain grew as well as a wild-type *TRL1* strain on YPD agar at 25, 30, 34 and 37°C (scored as +++ growth in Table). Control experiments showed that neither the *AtRNL*-(1–675) nor the *CEN TRP1 AtRNL*-(677–1104) plasmid alone supported growth on *trl1*Δ cells under 5-FOA selection (scored as — in Table 4). Thus, the plant RNA ligase consists of separable ligase and kinase-CPD domains.

We proceeded to test whether the separate sealing and healing domains of plant RNA ligase could function *in vivo* when paired with a heterologous RNA healing or sealing enzyme.

Table 4. Separable Sealing and Healing Domains of Plant RNA Ligase

Sealing domain	Healing domain	*trl1*Δ complementation
AtRNL-(1–675)	AtRNL-(677–1104)	+++
AtRNL-(1–675)	None	—
none	AtRNL-(677–1104)	—
AtRNL-(1–675)	Trl1-(389–827)	+++
Trl1-(1–388)	AtRNL-(677–1104)	+++
AtRNL-(1–675)	T4 Pnkp	—

These experiments showed that: (i) the plant ligase domain was functional *in vivo* when paired with the yeast kinase-CPD domain and (ii) the plant kinase-CPD domain was active when paired with the yeast ligase domain. Given that there is scant primary structure similarity between the plant and yeast proteins, the findings that the plant and yeast domains are functionally interchangeable as separate polypeptides suggests that a hypothetical physical interaction between the plant ligase and plant kinase-CPD domains is not essential for tRNA splicing activity *in vivo*.

Previous studies showed that the yeast tRNA splicing system can be replaced *in vivo* by coexpression of bacteriophage T4 Rnl1 and Pnkp (10). However, whereas the yeast ligase domain and T4 Rnl1 are functionally interchangeable as isolated sealing modules, the respective end-healing enzymes are not. T4 Pnkp could substitute for the yeast kinase and CPD modules in tandem with T4 Rnl1, but was inactive when paired with the yeast ligase module. Here we find that the plant ligase module is also unable to function in tRNA splicing when paired with T4 Pnkp as the source of the end-healing activities. Because the kinase modules of the phage Pnkp and plant kinase-CPD enzymes generate identical products (a 5′-PO_4 RNA end), we ascribe the fact that the plant ligase functions only in tandem with plant or fungal kinase-CPD to the distinctive 3′-OH, 2′-PO_4 end configuration generated by yeast CPD versus the 3′-OH, 2′-OH end produced by Pnkp. In other words, the *Arabidopsis* tRNA ligase requires the 2′-PO_4 terminus to seal tRNAs *in vivo*. This inference is consistent with the requirements for a 2′-PO_4 for RNA joining *in vitro* by wheat germ RNA ligase (14,16,18).

DISCUSSION

The present study adds to our knowledge of tRNA splicing mechanisms by: (i) identifying the essential constituents of the kinase-CPD domain of fungal tRNA ligase; (ii) illuminating structure-activity relationships at the putative kinase and CPD active sites; (iii) demonstrating that an analogous RNA ligase from plants, AtRNL, is a bona fide tRNA splicing enzyme capable of performing all requisite tRNA healing and sealing steps in a *trl1*Δ yeast strain; and (iv) mapping the essential constituents of the ligase, kinase and CPD modules of AtRNL, thereby revealing the conservation of active site motifs between plant and fungal tRNA ligases, notwithstanding their very low level of global primary structure similarity. Below we discuss and interpret the functional data, drawing on the atomic structures of enzymes with biochemical activities similar to those of the tRNA ligase domains.

The kinase domain

The key landmarks of the fungal kinase domain are the [404]Lys-Thr[405] dipeptide of the P-loop, plus Asp425, Arg463, Arg511 and His515. These essential residues are strictly conserved among fungal tRNA ligases (Figure 1). Yet conservation *per se* is clearly not predictive of essentiality, insofar as nineteen other conserved residues subjected to alanine scanning were found to be nonessential for Trl1 activity *in vivo*. We can speculate about the roles of some of the essential components of the fungal kinase by reference to the crystal structures and extensive mutational analyses of the kinase domain of

T4 Pnkp (20–23). The GSGKS[16] P-loop element of T4 Pnkp connects a beta strand to an alpha helix and forms and 'oxyanion hole' for binding of the beta phosphate of the NTP substrate. The binding occurs via multiple contacts of the phosphate oxygens to the backbone amides of the loop, plus contacts to the lysine and serine side chains. It is reasonable to think that the same NTP contacts are made by the P-loops of the yeast and plant kinases. Yet, structure-activity relations differ for the T4, yeast and plant P-loops. For example, T4 kinase activity is ablated by single alanine mutations at Lys15 or Ser16 of the P-loop, whereas the equivalent Lys404 and Thr405 side chains appear to be functionally redundant in yeast Trl1, requiring a double K404A-T405 mutation to abolish activity *in vivo* and *in vitro* (6). However, in the plant enzyme, where the P-loop sequence GxxGSAKS deviates from the consensus, the P-loop lysine is dispensable, but the serine is essential for activity *in vivo*.

The RxxxR motif located ~100 amino acids downstream of the P-loop is located within an alpha helix and loop that form a 'lid' over the NTP binding pocket. The distal arginine of this motif (Arg126 in T4 Pnkp) contacts the alpha and beta phosphates of the nucleotide substrate. Mutation of this arginine side chain ablates the kinase activity of T4 Pnkp, as do mutations of the equivalent Arg511 residue in yeast Trl1. An alanine mutation of the likely equivalent of the plant enzyme, Arg804, results in a severe growth defect. Thus, we speculate that the fungal and plant kinases probably have a lid structure similar to that of T4 kinase. The proximal arginine side chain of the RxxxR motif of T4 Pnkp (Arg122) forms a π-cation stack on the purine base of the NTP substrate. Replacing Arg122 with alanine had no effect on T4 kinase activity; nor did the equivalent mutation of Arg507 in yeast Trl1 affect its activity *in vivo*. A noteworthy finding here is that the essential His515 residue of yeast Trl1 is located just downstream of the RxxxR motif, in what would be the loop component of the lid of T4 Pnkp. The putative loop sequence immediately following the RxxxR motif in yeast Trl1 (GNNHQSIK) is conserved among fungal tRNA ligases (Figure 1). The equivalent segment of T4 Pnkp (GTKAVPID) has little similarity to that of the fungal proteins. It is conceivable that this motif, which includes an essential histidine, is a determinant of the specificity of the yeast polynucleotide kinase for GTP as the phosphate donor. The corresponding sequence of the plant kinase (VNHPGKLD) includes an Asn-His dipeptide that could be related to the yeast element. At present, the NTP donor specificity of the *Arabidopsis* kinase remains uncharted.

The CPD domain

Atomic structures are available for several members of the 2H phosphotransferase family, including human and rat brain 2′,3′ cyclic nucleotide phosphodiesterase (26,27), archaeal 2′–5′ RNA ligase (28) and *Arabidopsis* ADP-ribose 1′,2′ CPD (29,30). These structures, and especially the structures of the plant CPD with sulfate or cyclic vanadate inhibitor bound and human CPD with phosphate bound, illuminated the likely catalytic roles of the HxT motifs and other functional groups in the active site. The 2H enzymes share a symmetrical bilobed tertiary structure with each lobe consisting of a three- or four-stranded beta sheet at the lobar interface and alpha helices on the periphery. The HxT motifs reside within beta strands lining the active site. Both histidines and both threonines make contacts to the phosphate oxygens in the ligand complexes. The proposed mechanism of the 2H enzymes invokes acid-base catalysis by the histidines of the HxT motifs. The histidine of the distal HxT motif is posited to act as a general base to activate a water for attack on the phosphorus center. The histidine of the proximal HxT motif is proposed to act as a general acid to donate a proton to the ribose O leaving group. The two threonines are supposed to stabilize the transition state via hydrogen bond donation to the phosphate oxygens.

Mutational analyses of rat brain, zebrafish and yeast 2H family enzymes underscore that both histidines are essential for catalysis (26,31–33). Yet, we find that only the distal histidine is strictly essential for the *in vivo* CPD function of both the yeast and plant tRNA ligases. Mutation of the proximal histidine of the yeast and plant proteins results in a conditional growth defect. Mutational analysis of brain CPD showed that both threonines are essential for catalysis of the 2′3′ CPD reaction (26). The threonine of the distal HxT motif was also shown to be essential for the zebrafish 2′,3′-CPD activity (31). In contrast, we find that only the proximal theonine is essential for tRNA splicing and mutation of the distal threonine had no apparent effect on the *in vivo* CPD activity of either the yeast or plant tRNA ligase. Thus, the contributions of the individual amino acids at the CPD active site are significantly different in the tRNA ligases *vis a vis* other 2H family members.

We speculate that the relaxed requirement for the proximal histidine in tRNA ligases might reflect the capacity to switch from a general acid mechanism, where the histidine donates a proton to the leaving O3′ ribose atom, to one of specific acid catalysis in which a water can occupy the active site and act as a proton donor. Alternatively, another functional group on the enzyme or the tRNA could assist in leaving group expulsion when the histidine is missing. Whatever the mechanism, the proximal histidine-less ligases can apparently sustain a threshold level of CPD activity at lower growth temperatures, but not at 37°C.

The benign effect of the distal threonine mutations in the yeast and plant tRNA ligases signifies either that: (i) the proximal threonine suffices for binding the tRNA 2′,3′ cyclic phosphate and stabilizing the transition state or (ii) there are additional constituents of the enzyme that serve this role to the extent that the distal threonine is redundant. In the same vein, Nasr and Filipowicz (33) reported that neither of the hydroxyamino acids of the Hx(S/T) motifs of yeast ADP-ribose 1′,2′ CPD was required for activity with the physiological substrate Appr>p. However, when the yeast ADP-ribose 1′,2′ CPD was assayed with 2′,3′ cyclic AMP, the proximal threonine was essential, a situation that echoes our findings for the 2′,3′ CPD activity of tRNA ligases.

The plant ligase domain

We discussed previously the structure-activity relationships at the numerous essential residues within the ligase domain of yeast Trl1 and their interpretation in light of known ligase structures (11). The remarkable aspect of the plant RNA ligase domain is how little it resembles the fungal tRNA ligase or any other RNA ligase. By sorting out the functionally relevant

putative equivalents of the classical covalent nucleotidyltransferase motifs from other irrelevant impostors, we have placed the plant ligase firmly within the covalent nucleotidyltransferase superfamily, while highlighting the divergence in motif sequence and spacing in the plant protein versus other superfamily members. In particular, the large interval between motifs IV and V is unique to the plant ligase and accounts, in good part, for the much larger size of the autonomous ligase domain of AtRNL (675 amino acids) compared to that of yeast Trl1 (388 amino acids). A search of the nonredundant GenBank database with the 208 amino acid polypeptide sequence separating motifs IV and V uncovered no similar polypeptide from a non-plant source. It is conceivable that this large insert plays a plant-specific role in tRNA splicing (or some other RNA repair process).

The recently reported crystal structure of bacteriophage T4 Rnl1 bound to the nonreactive substrate mimetic AMPCPP (34) provides the closest structurally defined homolog of the fungal tRNA ligase domain, which belongs to an Rnl1-like subfamily of viral and cellular RNA sealing enzymes (35,36). The residues defined herein as essential in the plant tRNA ligase domain—Lys152, Glu218, Glu326, Lys541 and Lys543—are the putative equivalents of residues Lys99, Glu159, Glu227, Lys240 and Lys242 that comprise the active site of T4 Rnl1. T4 Rnl1 can execute the sealing steps of tRNA splicing in yeast when it is provided with either a 2′-OH, 3′-OH or a 2′-PO_4, 3′-OH terminus on the proximal tRNA half. In contrast, the plant and yeast ligases can only seal the 2′-PO_4, 3′-OH ends generated by the CPD component of tRNA ligase. Accounting for the unique requirement of yeast and plant ligases for a 2′-PO_4 and divining the contributions of the plant-specific and yeast-specific primary structure elements will depend on success in obtaining atomic structures of the yeast and plant ligase domains. The delineation of an autonomous plant ligase domain using yeast as a surrogate genetic system is an initial step toward this goal.

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