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Identification and modeling of a phosphatase-like domain in a tRNA 2'-*O*-ribosyl phosphate transferase Rit1p

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Abbreviations: FR, fold recognition; nt, nucleotide; e-value, expectation value; CTD, C-terminal domain; PTP, protein tyrosine phosphatase

Cytoplasmic initiator tRNAs from plants and fungi are excluded from participating in translational elongation by the presence of a unique 2'-phosphoribosyl modification of purine 64, introduced posttranscriptionally by the enzyme Rit1p. Members of the Rit1p family show no obvious similarity to other proteins or domains, there is no structural information available to guide experimental analyses, and the mechanism of action of this enzyme remains a mystery. Using protein fold recognition, we identified a phosphatase-like domain in the C-terminal part of Rit1p. A comparative model of the C-terminal domain was constructed and used to predict the function of conserved residues and to propose the mechanism of action of Rit1p. The model will facilitate experimental analyses of Rit1p and its interactions with the initiator tRNA substrate.

Introduction

tRNA's role in decoding the genome is critical to the accuracy and efficiency of protein synthesis. Thus, the understanding of tRNA gene expression, folding of the transcripts, posttranscriptional processing, subcellular localization and their interactions with other macromolecules is essential for the full understanding of protein translation.[1-4] A key step in the initiation of protein synthesis involves the recognition of the correct starting codon on the mRNA. The protein-synthesizing apparatus of the cell must discriminate between different AUG codons encoding the same amino acid methionine—the start codon and those in the internal coding region of the mRNA. A specialized initiator tRNA ($tRNA_i^{Met}$ in Eukaryota and $tRNA^{fmet}$ in Prokaryota and organelles) exclusively recognizes the start codon located in the ribosomal P site during the initiation of translation, whereas the elongator tRNA ($tRNA_m^{Met}$) is used to recognize internal methionine codons in the ribosomal A site.[5] In bacteria, mitochondria and chloroplasts, the Met of Met-$tRNA^{fmet}$ is formylated, which blocks the α-amino group from forming a peptide bond. On the other hand, the eukaryotic cytoplasmic Met-$tRNA_i^{Met}$ is not formylated, but possesses a number of unique sequence features not found or rare in elongator tRNAs. These features include the absence of residue 17, presence of an additional residue A20 and the replacement of T54 and pyrimidine 60 in the TΨC loop (found in virtually all elongator tRNAs) by A54 and A60. As revealed by the crystal structure of $tRNA_i^{Met}$ from *Saccharomyces cerevisiae*,[6] these features cluster together in space to form a unique substructure stabilized by an extensive and intricate network of hydrogen bonds.

Cytoplasmic $tRNAs_i^{Met}$ from fungi and plants (but not from vertebrates) carry a posttranscriptional modification that links a 5'-phosphoribosyl group by a glycosidic bond to the O_2' of ribose of purine 64 in the T stem.[7] It was shown that the demodification of A64 in yeast $tRNA_i^{Met}$ or G64 in wheat germ $tRNAs_i^{Met}$ allows these tRNAs to read not only the start AUG codons, but also the internal AUG codons.[8] Thus, this posttranscriptional modification excludes the initiator tRNA from participating in translational elongation.

Thus far, over 100 posttrascriptionally modified nucleosides have been reported in different types of RNAs from diverse organisms.[9] The largest number of modified nucleosides with the greatest structural diversity is found in tRNAs.[10] These modifications are introduced by a large number of enzymes, which may act alone or in complexes and pathways.[11-14] While many of these enzymes have been extensively characterized, very little is known about others (for details, see the collection of reviews in books, refs. 15–17). For example, the *RIT1* gene encoding the 2'-*O*-ribosyl phosphate transferase (Rit1p) was identified in yeast more than 10 y ago.[18] However, the mechanism of action

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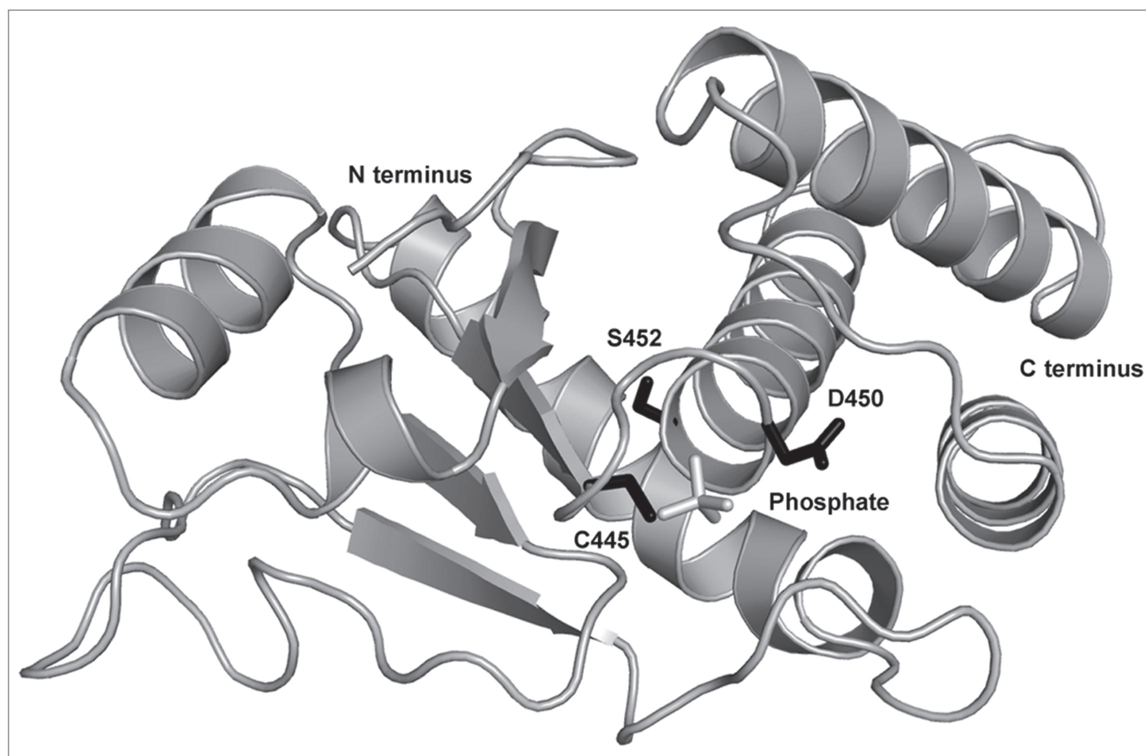

Figure 1. A model of *S. cerevisiae* Rit1 CTD. Coordinates are available from ftp://genesilico.pl/iamb/models/Rit1p/. The protein backbone is shown as a ribbon. The substrate phosphate group is shown in light gray and residues predicted to be important for catalysis of the phosphoribosyl group transfer are shown in black.

of this enzyme remains a mystery. In particular, no structural information is available for Rit1p to guide experimental analyses and determine the molecular basis of interactions with $tRNA_i^{Met}$.

Results

We submitted the sequence of *S. cerevisiae* Rit1p (NCBI gene identification number 6323939) to the GeneSilico metaserver[19] to predict the protein structure by fold-recognition methods. This analysis revealed that Rit1p is composed of two domains (roughly residues 1–340 and 341–512). The N-terminal domain (NTD) exhibited no evident similarity to any known protein structure or family. However, the C-terminal domain (CTD) of Rit1p was found to exhibit significant similarity to structures of dual-specificity phosphatases (DUSPs). Nearly all individual fold-recognition methods reported DUSP structures as the most preferred matches (e.g., HHSEARCH: 2 hcm_A, score 97.88, and 1wrm_A, score 97.28, with 100 being ideal match, and mGenThreader: 1wrm_A, score 0.001, with 0 being ideal match). Based on these primary predictions, the consensus predictor PCONS unequivocally identified the DUSP fold as the only reasonable template for modeling: the top 10 matches exhibited scores in the range of 1.94–2.65, which indicate a highly confident prediction (see Methods for explanation of scores). The similarity between Rit1p and DUSPs was originally described in a B.Sc. thesis[20] of the first author of this article, and it has been independently identified using HHsearch[21] by Arcady Mushegian.[22]

DUSPs are a heterogeneous group of protein phosphatases that can dephosphorylate both phosphotyrosine and phosphoserine or phosphothreonine residues, also within the same substrate. They have been implicated as major modulators of signaling pathways critical for cell growth and differentiation (reviewed in refs. 23–25). They are often composed of multiple domains, with the conserved catalytic domains fused to other domains with various folds and functions.[23] There are also known cases of DUSP-related proteins, such as MK-STYX, that lack the conserved residues in the active site and appear enzymatically inactive. While the evolutionary relationship and the resulting structural similarity between the CTD of Rit1p and DUSPs appears evident beyond any reasonable doubt, the question arises whether Rit1p possesses the DUSP-like active site and may exhibit a similar activity.

Based on the alignments from the metaserver, we have built a comparative model of the CTD (**Fig. 1**) using the "FRankenstein's monster" modeling approach.[26] Details of the modeling procedure and an explanation of the model assessment scores are presented in the Materials and Methods section. The best-scoring structure was evaluated as potentially "extremely good" by the PROQ method (predicted LGscore: 4.317), and the MetaMQAP method[27] predicted that the model's root mean square deviation to the (currently unknown) true structure is around 3.4 Å. These values indicate that our structural prediction is likely to be correct and support the prediction that Rit1 has a common evolutionary ancestor with phosphatases. The

Figure 2. Multiple alignment of representative sequences from the Rit1 family and the known dual specificity phosphatase structure (2 hcm) identified by the fold-recognition analysis as the best template for modeling of the CTD. Secondary structures predicted for Rit1 and observed in the template crystal structures are indicated as arrows and tubes (strands and helices, respectively). Conserved motifs are indicated. Residues predicted to be functionally important and shown in **Figure 1** are indicated above the alignment by "*."

model can, therefore, be used to make functional inferences at the level of individual residues (however, not with atomic precision) in connection with the analysis of sequence conservation in the Rit1p family.

A search of the non-redundant database at the NCBI revealed that Rit1 homologs are present in green plants, fungi and *Giardia lamblia*, and that all members of this family possess both domains. Analysis of the multiple sequence alignment (**Fig. 2**) reveals that

the NTD is more conserved and contains several sequence motifs rich in invariant or nearly invariant residues, while the CTD contains two conserved motifs. One of the conserved motifs (hhhxCxxGxDhS, where h indicates a hydrophobic residue and x a non-conserved residue) in the CTD corresponds to the active site motif of dual-specificity phosphatases. Interestingly, most (but not all) Rit1 family members possess a conserved Cys residue (C445 in *S. cerevisiae* Rit1p) that corresponds to the catalytic Cys residue of DUSPs, which is used to form a cysteinyl-phosphate intermediate in the phosphate group transfer reaction.[28] A catalytic Asp residue that fulfills a role of general acid in the reaction performed by DUSPs has no homologous counterpart in the Rit1 family. However, a spatially equivalent position in the putative phosphate-binding pocket is occupied by a conserved Asp residue (D450 in *S. cerevisiae* Rit1p). This spatial equivalence, which became apparent only upon inspection of the model and was not obvious from the sequence alignment, suggests that the CTD of Rit1 proteins possesses a putative active site similar to those of DUSPs.

Discussion

Our prediction of a DUSP domain in Rit1p with a partially conserved DUSP-like active site suggests that this domain may be directly involved in the phosphotransferase activity of Rit1p, based on the same mechanism used by DUSPs. However, the structure of the remaining N-terminal part of Rit1p remains unknown. The structurally uncharacterized NTD is actually more conserved than the DUSP-like CTD. Therefore, it cannot be excluded that the transfer of the phosphoribosyl group is catalyzed by the conserved NTD, while the CTD performs some secondary function. There are known cases of inactivation of the enzymatic function of a protein and utilization of the enzyme-like domain for non-enzymatic roles. Among RNA modification enzymes, relevant examples are the Gcd10p subunit of tRNA:m1A58 MTase Trm6p,[29,30] Sen15 and Sen54 subunits of the tRNA splicing endonuclease[31] or the N-terminal domain of rRNA MTase RsmC.[32] In these proteins, "degenerated" enzyme-like domains are typically used for RNA binding. The partial conservation of the DUSP-like active site suggests that the CTD of Rit1p may be involved in interactions with the phosphate group, but this interaction could be limited to binding of the phosphate backbone of the tRNA substrate. Alternatively, the NTD and the CTD may fulfill distinct enzymatic functions; a number of RNA modification enzymes that catalyze more than one reaction have been characterized, exemplified by the trifunctional capping enzyme in the mimivirus, which is composed of a metal-dependent RNA triphosphatase module fused to a guanylyltransferase and methyltransferase domains.[33] Such can be the case with Rit1p one of its domains could be involved in an activity that yet remains to be discovered.

The ultimate test for our predictions may be provided by experimental analysis, which is out of scope of this article. The multiple sequence alignment, which was used as the basis for our modeling of the CTD, suggests a number of conserved residues in both domains that may be targeted by site-directed mutagenesis for functional studies of Rit1p. The charged or hydrophilic character of these conserved residues suggests involvement in recognition and binding of the tRNA substrate or in other aspects of catalysis. Potential targets for further analyses include invariant residues in the NTD (e.g., S48, D52, N72, R74, H97, R107, N109, D136, T141, D280, D281 and E283) and highly conserved C445, D450, S452 and R502 in the CTD. These experiments may provide clues as to whether Rit1p CTD is enzymatically active or plays only a supporting role by, e.g., binding the substrate RNA.

Materials and Methods

Sequence database searches and alignment. Searches of the non-redundant version of current sequence database (nr) were performed using PSI-BLAST,[34] with the e-value threshold of 1e-3. The multiple sequence alignment of Rit1p and proteins identified in the database was calculated using MUSCLE[35] with default parameters and refined by hand to ensure that no unwarranted gaps had been introduced within α-helices and β-strands.

Protein fold prediction. Protein structure predictions were performed via the GeneSilico metaserver gateway[19] for secondary structure prediction and fold recognition. Structural predictions were performed both for the full-length Rit1p sequence and for the individual domains. It is important to indicate that different FR servers use completely different scoring systems with different scales (e.g., Z-scores, e-values, percent values, etc.). Moreover, the meaning of scores changes over time and may not be the same as reported in original publications describing the methods, as servers are modified and databases grow continuously. The comparable reliability thresholds for a number of servers are estimated, e.g., by the Livebench benchmark[36,37] conducted by Leszek Rychlewski and coworkers. The GeneSilico metaserver uses the PCONS[38] consensus method to compare different predictions with each other and to assign its overall score. According to Livebench-8 analysis, a PCONS score of 0.67 corresponds to the average score for their 8th incorrect predictions, which amount to 5% of incorrect predictions for all targets in the Livebench test set.[39] In general, scores > 1 indicate confident predictions with low chance of false positives.

Protein structure modeling. Homology modeling of the catalytic core was performed using the "FRankenstein's monster" approach (see ref. 26 and 40 for a detailed description). Briefly, preliminary models were built with MODELLER[41] based on the sequence alignment between Rit1p and the template structure 2 hcm obtained from various fold-recognition servers queried via the GeneSilico metaserver. The preliminary models were scored by MetaMQAP to predict their accuracy at the level of individual residues,[27] and a hybrid model was generated by merging fragments with consensus alignment with those non-consensus fragments that exhibited the best MetaMQAP scores. Additional evaluations of protein structure quality were performed with PROQ.[42] The hybrid model was refined with the REFINER method, which uses a reduced representation of the protein chain and a statistical potential of mean force to describe intramolecular interactions.[43] REFINER is a real-space version of a lattice-based algorithm CABS[44] that we had earlier successfully combined with

the "FRankenstein's monster" method in CASP6.[45] The folding was performed with restraints on predicted secondary structure. Models generated during the simulation had their full-atom representation rebuilt and were scored using PROQ and MetaMQAP.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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