Characterization of the 44 kDa protein co-purifying with *Tetrahymena pyriformis* tRNA 5' endoribonuclease

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Ribonuclease P (RNase P) catalyzes cleavage of the leader sequence of precursor tRNAs, generating mature 5' end of tRNAs. In eukaryotes, ribonuclease P requires both RNA and protein components for its catalytic activity. In earlier work [12], we showed that the purified RNase P from *Tetrahymena pyriformis* contains three major polypeptides with relative molecular masses about 100, 44 and 35 kDa. Here we report cloning and characterization of the gene encoding a protein with the apparent molecular weight of 44 kDa (TRP44). The detected N-terminal amino acid sequence of TRP44 was used to design the degenerated primers for molecular cloning of the gene. The calculated molecular mass and predicted pI of the product were 45.2 kDa and 6.01, respectively. Southern blot of genomic DNA demonstrated that TRP44 was present as a single-copy gene in the nuclear genome. Polyclonal antisera generated against recombinant TRP44 recognized a protein of about 44 kDa in Western blot of partially purified *Tetrahymena pyriformis* RNase P, and immunoprecipitated RNase P activity from the partially purified preparation of the enzyme, indicating that protein is associated with tRNA 5' endoribonuclease. Analysis of the deduced amino acids sequence revealed (less 30%) a homology with the translation elongation factor-1 subunit gamma (EF-1Bγ) from other eukaryotic organisms.

Key words: ribonuclease P, translation elongation factor

INTRODUCTION

Precursor tRNAs (pre-tRNAs) must undergo a complex series of processing steps during the production of a mature molecule. Ribonuclease P (RNase P) cleaves pre-tRNA molecules to produce mature 5' termini in both prokaryotes and eukaryotes. The basic understanding of the structure and function of the RNase P ribozyme has come from studies on the bacterial enzyme composed of a single RNA subunit (~ 400 nucleotides) and a single protein subunit (~ 14 kDa). The bacterial RNA subunit alone is able to recognize and cleave pre-tRNA substrates *in vitro* under high Mg^{2+} concentrations, while both RNA and protein subunits are required for the activity *in vitro* [1]. In contrast, the eukaryotic RNase P RNA subunit does not exhibit enzymatic activity *in vitro* in the absence of protein [2]. The most striking difference between the bacterial and eukaryotic nuclear RNase P is protein content, in contrast to the known RNA subunits, which are fairly well conserved in size and structure among diverse phylogenetic groups. The protein composition of eukaryotic nuclear ribonuclease P has been studied mainly in yeast and humans. In *HeLa* cells, the nuclear RNase P consists of one RNA molecule and at least ten protein subunits ranging in size from 14 to 115 kDa [2, 3]. Nine proteins have been identified as subunits of yeast nuclear RNase P ranging in size from 15 to 100.5 kDa [4]. A multisubunit ribonucleoprotein has also been described from *Aspergillus nidulans* [5]. The lack of catalytic activity for eukaryotic RNase P RNAs *in vitro* suggests a more critical role for protein subunits in eukaryotes than the protein subunits provide in the bacterial holoenzymes [2, 6]. Studies of RNase P in organelles suggest a diversity in their composition. The well-characterized mitochondrial RNase P in *Saccharomyces cerevisiae* clearly contains an RNA subunit and one 105 kDa protein subunit [7]. In contrast, *Aspergillus nidulans* mitochondrial RNase P contains 7 polypeptides ranging from 16 to 55 kDa [8]. An interesting case has been detected in spinach chloroplast: RNase P has the

properties of solely protein enzyme [9]. In this report we describe cloning of the gene encoding protein, which is associated with RNase P complex from an extremely phylogenetically diverse group of organisms – ciliate protozoa *Tetrahymena pyriformis*.

MATERIALS AND METHODS

Adaptor PCR isolation of S4 protein gene. Genomic DNA samples were digested with *Bam*HI or *Bgl*II, phenol/chloroform extracted and ethanol precipitated. Adaptors were annealing by mixing 2 μg of oligonucleotide Bam (5'-GATCGGTACGCAGTC) with 2 μg Uni (5'-CTCGTAGACTGCGTACC), heating for 5 min at 85 °C and slowly cooling to room temperature. Digested genomic DNA was then ligated to adaptor by overnight incubation at 16 °C. The ligation mixture was used for amplification. PCR products were ligated into pUC57/T plasmid and sequenced [10].

Recombinant protein expression. His-tagged recombinant TRP44 protein form was obtained by subcloning 700 bp TRP44 gene *Hind*III/*Sal*I fragment into *Hind*III/*Sal*I digested pQE32 expression vector. The recombinant protein was overexpressed in *E. coli* and purified from crude lysates on a nickel-charged, His-bind resin chelating affinity chromatography column (Amersham Pharmacia Biotech) according to the manufacturer's instruction. This recombinant protein was used to raise rabbit polyclonal antibodies. Sera of immunized rabbits were obtained from the Institute of Immunology. The specifity of antibodies in the sera was tested by Western blot analysis using recombinant protein.

Immunoprecipitation. Polyclonal rabbit antibodies (40 μl) were mixed with 10 mg protein A Sepharose CL-4B (Amersham Pharmacia Biotech) in 200 μl NET-2 buffer (50 mM Tris, 150 mM NaCl, 0.05% Nonidet P-40). Nutating beads made coupling overnight at 4 °C, followed by five washes of beads with NET-2 buffer. Preparation of by DEAE-Sepharose chromatography purified RNase P (80 μl) were added to the beads in final volume of 250 μl and shaked overnight at 4 °C. Beads were washed four times with NET-2. RNase P was suspended in RNase P assay buffer [11].

Assay for RNase P activity. To assay RNase P activity, an internally ^{32}P-labeled human pre-tRNA^{Tyr} substrate was transcribed *in vitro* as described previously [12]. Pre-tRNA contains 5'-end extension of 14 nucleotides in comparison with mature tRNA. The reaction mixture contained 20 mM Tris-HCl, pH 8.0, 60 mM NaCl, 8 mM $MgCl_2$, 1 mM dithiothreitol and 0.1–1.0 pmol of ^{32}P pre-tRNA. The reaction was initiated by addition of 2 μl of enzyme preparation to the reaction mixture (final volume10 μl) and incubated at 30 °C for 30 min. Reaction products were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

RESULTS AND DISCUSSION

In earlier work [12], we showed that a highly purified fraction of the tRNA 5' endoribonuclease from *Tetrahymena pyriformis* contains three major polypeptides with relative molecular masses about 100, 44 and 35 kDa. In order to characterize the proteins, these polypeptides were separated on 10% polyacrylamide/SDS gel, transferred on to a PVDF membrane and subjected to N-terminal amino acid sequence analysis. The obtained amino acid sequences DYQLYAPTG-MFRANMILTVAelt(e/s)si(r/v) correspond to N-termini of the protein with the apparent molecular weight of about 44 kDa. The degenerate oligonucleotides were designed and used in various combinations for the polymerase chain reaction from *Tetrahymena pyriformis* cDNA or from genomic DNA. PCR products were used to screen *Tetrahymena* genomic libraries. A full-length open reading frame sequence was obtained for the TRP44 polypeptide from subcloned 1.3 kb *T. pyriformis* genomic DNA fragment. The identified open reading frame consists of 1,197 nucleotides and encodes a protein of 399 amino acids with a calculated molecular mass of 45.2 kDa and a predicted pI = 6.01 (Figure, A). Southern blot analysis of genomic DNA indicates that TRP44 gene is present as a single-copy gene in *Tetrahymena* nuclear genome (Figure, C).

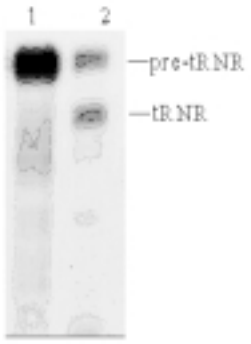
We next performed immunochemical analysis of the isolated TRP44 protein. Rabbit polyclonal antibodies were generated against *E. coli*-expressed recombinant polypeptide representing 233 amino acids a long the N-terminal part of TRP44. Immunoblotting of the purified tRNA 5' endoribonuclease fractions revealed a strong cross-reactivity of the anti-TRP44 antibody with ~ 44 kDa protein band, demonstrating that the identified protein co-purifies with RNase P complex (data not shown).

In an effort to provide independent evidences that isolated TRP44 protein is physically associated with RNase P complex, we tested whether RNase P activity can be co-immunoprecipitated with an anti-TRP44 antibody. *Tetrahymena pyriformis* RNase P was partially purified by liquid chromatography on DEAE-Sepharose resin as described elsewhere [12]. The fractions containing the enzyme activity were subjected to immunoprecipitation with anti-TRP44 antibody and the bound IP fraction was analyzed by its ability to process the 5' sequence of the tRNA precursor.

A

MDYQLYAPTGNFRANMILTVAELTGVKVELVHTEYSTIKTPEFLQKNPLGKVPVLVTPEVPSSNLTPSLDTSPEPLVSSTVETS
TNLPSSTNSWDMCPHRIIAALTTTLYAIFGFKPADKEVLKAAKTETMTVLRIFNERLTKNKFLAGEHLTIADIQLATFLNLAFR
VTFSGEQKKPFAKLVEYFVSIAILPEFTKFQGRPHFTTSEYQTVAVPEAKDNKKKEAVKTKDAPKKDAPKKEAPKAKKEEEEEE
EAPTGPAKWNLYDYKTLYANAKDKEEAITDLVANYNPAEMCIYHLKYQKYDGDGKVLYQFNNMKNNFVQRCDPARKKAFGTYSI
YGDEPNLEIAGVWLFLGAEIPKEMNENPAFEYHDLKKLDITKPEDLQLVREYWTRTVEDESVA

B



C

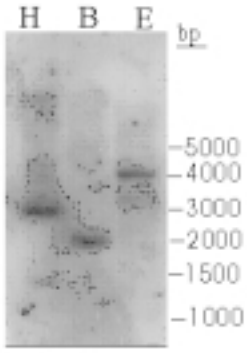


Figure. **A**. Amino acid sequence of *Tetrahymena pyriformis* TRP44 protein. **B.** TRP44 associates with catalytically active RNase P were tested for RNase activity in the processing of precursor $tRNA^{Tyr}$. Lane1 – pre tRNA, lane 2 – immunoprecipitated fraction was used for assay of RNase P activity. **C**. Southern blot analysis of the TRP44 gene organization. *T. pyriformis* genomic DNA was digested with BglII (B), EcoRI (E) and HindIII (H)

Imunoprecipitation assays showed that the anti-TRP44 antibody effectively pulls down tRNA 5' endoribonuclease activity, indicating that TRP44 protein interacts with the catalytically active RNase P complex (Figure, B).

BLAST searches using the determined amino acid sequence of TRP44 protein showed no significant homology to the other known protein subunits of the eukaryotic RNase P. However, *T. pyriformis* TRP44 protein displayed a low similarity to the γ-subunit of the translation elongation factor 1B (eEF-1Bγ) from different organisms. The highest similarity was found between TRP44 and *Arabidopsis thaliana* eEF-1Bγ (30% of identical amino acids). TRP44 protein also contains regions similar to eEF-1Bγ and glutathione S-transferase conserved domains, suggesting that the identified protein may be a γ-subunit of a translation elongation factor 1B. The gene encoding *Tetrahymena* eEF-1Bγ has not been reported before. If TRP44 is a translation factor, how could it be involved in tRNA processing? The eukaryotic polypeptide elongation factor EF-1, which consists of four different subunits, is not only a major translational factor, but also one of the most important multifunctional proteins. Recently, eukaryotic elongation factors have been shown to be concerned or likely to be concerned in various important cellular processes, among them in nuclear processes such as RNA synthesis, tRNA processing and transport coordination [15]. Moreover, recent observations that transcription and translation are coupled within nuclei of mammalian cells [16] are consistent with the role of EF-1 within the nucleus.

The protein subunits suggested play a more important role in eukaryotic RNase P than in bacterial holoenzymes. In addition to possible function in RNase P catalysis, the protein subunits may have other roles *in vivo*: the human subunit Rpp20 is suggested to have an ABC transporter-like ATPase activity [17] and Rpp14 together with the protein partner OIP2 is an 3'–5' exoribonuclease involved in 3' terminus processing of tRNA precursors [18]. A new function is attributed to Rpm2, the protein subunit of the *S. cerevisiae* mitochondrial RNase P, which has a role in the translation regulation of mitochondrially encoded subunits of cytochrome c oxidase [19]. Further investigations need to be done to understand the exact role of TRP44 protein in RNase P catalysis.

ACKNOWLEDMENT

This work was supported by Lithuanian Government Program "Molecular Basis of Biotechnology: Gene

Structure, Functions and Mechanisms of Their Regulation" (grant No. 219/2490-7).

Received 14 October 2002

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44 KDA BALTYMO, ASOCIJUOTO SU INFUZORIJŲ *TETRAHYMENA PYRIFORMIS* TRNR 5' ENDORIBONUKLEAZE, CHARAKTERIZAVIMAS

S a n t r a u k a

Charakterizuojant laboratorijoje identifikuotą ir išgrynintą infuzorijų *Tetrahymena pyriformis* ribonukleazę P – ribonukleoproteiną, atsakingą už tRNR pirmtakų 5' galinės sekos brandinimą, buvo identifikuoti trys polipeptidai (apie 35, 44, 100 kDa), kurie, manoma, sudaro fermento baltyminį subvienetą. Nustatyta išskirto 44 kDa polipeptido N-galinių amino rūgščių seka. Panaudojant degeneruotus oligonukleotidinius pradmenis, sukonstruotus remiantis šia nustatyta amino rūgščių seka, buvo amplifikuoti PGR būdu ir klonuoti keli *Tetrahymena pyriformis* genominės DNR bei kDNR fragmentai. Nustačius šių klonuotų fragmentų nukleotidinę seką, identifikuotas genas, koduojantis 399 amino rūgščių polipeptidą (TRP44). Apskaičiuota šio polipeptido molekulinė masė yra 45,2 kDa, o numatomas pI = 6,01. Atlikus genominės DNR Souther'no hibridizaciją, nustatyta kad *T. pyriformis* genome šis genas yra vienkopijinis. Atliktas imunoišsodinimas, panaudojus antikūnus prieš rekombinantinį TRP44 baltymą. Nustatyta, kad šie antikūnai išsodina iš dalinai išvalyto DEAE chromatografijos kolonėlėje fermentinio preparato katalitiškai aktyvų RNazės P fermentinį kompleksą. Tai leidžia manyti, kad klonuoto fragmento koduojamas baltymas TRP44 yra asocijuotas su katalitiškai aktyviu ribonukleazės P fermentiniu kompleksu. Atlikus klonuoto fragmento koduojamos polipeptidinės sekos kompiuterinę analizę ir palyginus ją su baltymų duomenų bazėse esančiomis sekomis, pastebėta (iki 30%) nedidelė homologija su kitų eukariotų (*Arabidopsis thaliana*, *Xenopus*, mielių, žmogaus ir kt.) transliacijos elongacijos faktoriaus-1B gama subvienetu (eEF-1Bγ). Analogijos su kitais žinomais eukariotų RNazės P komplekso baltymais nerasta.