Available online at www.sciencedirect.com
ScienceDirect
Gene 400 (2007) 52–59
GENE
www.elsevier.com/locate/gene

DRpp20 and DRpp40: Two protein subunits involved in *Dictyostelium discoideum* ribonuclease P holoenzyme assembly

Dimitra Kalavrizioti, Anastassios Vourekas, Denis Drainas *

Department of Biochemistry, School of Medicine, University of Patras, 1 Asklipiou st., Patras 26504, Greece

Received 20 March 2007; received in revised form 4 May 2007; accepted 25 May 2007
Available online 7 June 2007
Received by A. Rynditch

Abstract

Ribonuclease P is an essential enzyme that matures the 5′ ends of all primary tRNA transcripts. RNase P enzymes contain a similar in size RNA subunit which is absolutely required for catalysis. The holoenzyme from *Dictyostelium discoideum* possesses an essential for activity RNA subunit but the exact protein composition is still under investigation. Bioinformatic analysis of *D. discoideum* sequencing data returned seven ORFs homologous to previously characterized RNase P protein subunits from human. In the present study, DRpp20 and DRpp40 were cloned and characterized. These proteins apart from the noted similarity possess idiosyncratic regions. Immunobiochemical analysis presented herein indicates their direct involvement in the formation of the ribonucleoprotein complex of *D. discoideum* RNase P holoenzyme.

Keywords: RNase P; RNA processing; tRNA; Ribonucleoprotein

1. Introduction

Ribonuclease P (RNase P) is one of the few ribozymes of ancestral origin still represented in contemporary cells. RNase P is a ubiquitous and essential ribonucleoprotein that catalyzes a specific endonucleolytic cleavage of a phosphodiester bond thus removing the 5′ leading sequence from all precursor tRNA molecules. It has been found in organisms representing the three kingdoms of life – Bacteria, Archaea and Eukaryotes – as well as in the major subcellular organelles, mitochondria and chloroplasts (Frank and Pace, 1998). RNase P RNA is one of the first catalytic RNA molecules identified (Xiao et al., 2002). Under elevated ionic conditions *in vitro*, the RNA subunit from Bacteria (Guerrier-Takada et al., 1983), some Archaea (Pannucci et al., 1999) is catalytically active in the absence of the protein fraction of RNase P and together with the ribosome is the only known RNA catalysts naturally devoted to act *in trans*. Very recently, evidence have been put forward indicating catalytic activity of the RNase P RNA subunit from human and *Giardia lamblia* (Kikovska et al., 2007).

RNase P enzymes contain a similar in size RNA subunit which is absolutely required for catalysis. Contrary to the RNA, the size and number of protein subunits of the holoenzyme varies significantly, from one small subunit in Bacteria (~10% by mass) (Frank and Pace, 1998), to at least four protein subunits in Archaea (~45% by mass) (Hall and Brown, 2002; Kouzuma et al., 2003; Boomershine et al., 2003; Kawano et al., 2006; Wilson et al., 2006), and up to ten protein subunits in Eukarya (~70% by mass) (Chamberlain et al., 1998; Xiao et al., 2001). This enrichment of the protein fraction in eukaryotes, is thought to represent an evolutionary change in the holoenzyme's structure and function, which still remains not well understood.

Abbreviations: RNase P, ribonuclease P; DRpp20/40, Dictyostelium RNase P, Protein 20/40; Pre-tRNA, precursor transfer RNA; EST, expressed sequence tags; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside; IMAC, immobilized metal affinity chromatography; Aas, amino acids; MALDI-TOF, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

* Corresponding author. Tel.: +30 2610997746; fax: +30 2610997690.
E-mail address: drainas@med.upatras.gr (D. Drainas).

0378-1119/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2007.05.020

The only fully characterized eukaryotic protein complements concern *Saccharomyces cerevisiae* and human RNase P holoenzymes. The first is comprised of nine protein subunits (Pop1, Pop3, Pop4, Pop5, Pop6, Pop7 Pop8, Rpp1 and Rpr2) which show large variations in size (15.5–100.5 kDa) (Chamberlain et al., 1998) and are essential for yeast viability and enzymatic activity (Xiao et al., 2001). Similarly, studies on human RNase P from HeLa cells have revealed the existence of ten protein subunits (hPop1, hPop5, Rpp14, Rpp20, Rpp21, Rpp25, Rpp29, Rpp30, Rpp38 and Rpp40) ranging from 14 to 115 kDa (Jarrous, 2002). At least six of human proteins appear to be homologous to the subunits of *S. cerevisiae* RNase P (hPop1/Pop1, Rpp30/Rpp1p, Rpp29/Pop4, Rpp21/Rpr2, Rpp20/Pop7p, hPop5/Pop5). The relationships between Rpp14, Rpp25 and Rpp40 with the yeast proteins are not clear. The set of eukaryotic protein subunits contains only two acidic proteins, Pop8 (p*I* 4.6) from yeast and Rpp40 (p*I* 5.2) from human while the rest are quite basic (p*I*>9) (Walker and Engelke, 2006). The yeast Pop7, the human Rpp20 and Rpp25 have predicted sequence similarity to the Alba superfamily of proteins which appear to have originated as RNA-binding proteins that formed various ribonucleoprotein complexes (Aravind et al., 2003). The homology of Rpp20 and Pop7 proteins was further supported by Rosenblad et al. (2006). Recently it was shown that Rpp20 and Rpp25 form a heterodimer which regulates their RNA-binding activity, subcellular localization and expression. The Rpp20–Rpp25 heterodimerization is resistant to both high ionic conditions and a nonionic detergent (Welting et al., 2007). Furthermore, Rpp20 was reported to exhibit ATPase activity and to interact with the proteins SMN, Hsp27 and KIAA0065 (Li and Altman, 2001; Jiang and Altman, 2001; Hua and Zhou, 2004).

Dictyostelium discoideum RNase P holoenzyme is a ribonucleoprotein complex, consisted of RNA and proteins essential for catalytic activity. Considering its buoyant density, *D. discoideum* RNase P appears to have the higher protein content among the characterized holoenzymes of eukaryotic origin (Frank and Pace, 1998; Stathopoulos et al., 1995). Although it has been established that this enzyme contains both essential RNA and protein components, very little is known on the exact composition of the ribonucleoprotein complex. Bioinformatics analysis of the *D. discoideum* sequencing data returned seven open reading frames homologous to previously characterized RNase P protein subunits from human. The encoded proteins (Pop1, Pop5, DRpp20, DRpp25, DRpp29, DRpp30 and DRpp40) apart from the noted similarity with their human counterparts bear regions that distinct *D. discoideum* RNase P protein subunits from all others, such as low complexity motifs. The gene of *D. discoideum* RNase P RNA subunit has been identified through phylogenetic comparative analysis by Marquez et al. (2005). Recently we reported the cloning and initial characterization of *D. discoideum* RNase P protein subunit DRpp30 giving a first insight of its structure and possible role in the holoenzyme functions (Vourekas et al., 2007). In this study we describe our experimental approach to investigate the association of the polypeptides encoded by the *drpp20* and *drpp40* ORFs with the RNase P holoenzyme.

2. Materials and methods

2.1. Growth of D. discoideum and partial RNase P purification

Growth of *D. discoideum* cells, cell homogenization, RNase P activity recovery and enzyme assays were essentially carried out as previously described (Stathopoulos et al., 1995). The purification scheme included two steps of anion exchange chromatography (DEAE cellulose, Whatman) and a final purification step by cesium sulfate density gradient centrifugation of concentrated RNase P sample.

2.2. Molecular cloning of drrp20 and drpp40

D. discoideum cDNA encoding proteins homologous to known RNase P protein subunits were identified through keyword searches (Altschul et al., 1997) of the EST data base of the cDNA sequencing project in Japan (http://dictycdb.biol.tsukuba.ac.jp/cDNAproject.html) or by BLAST searches of raw sequencing data at dictyBase (http://dictybase.org/db/cgi-bin/blast.pl) using human homologues. The genes were named according to the human homologues, and the prefix D (*D*ictyostelium) was added to indicate their distinctiveness.

The *drpp20* gene was amplified by PCR with the SSF189 clone containing the complete ORF (acquired from cDNA project in Japan) as a template using the primers FLdrpp20F (5′-CATATGAGCGATACTGAATTCGA-3′) and FLdrpp20R (5′-CTCGAGACATGTTTCTTGAACTTTTAATTGTTG-3′). *Nde* I and *Xho* I sites (underlined) were incorporated to the 5′- and 3′-ends, respectively. The sequence of *drpp20* was deposited in NCBI database (GenBank accession number DQ295794).

The clone CFG414 (ddc13l04) containing an open reading frame of 1272 bp similar to human Rpp40 was likewise identified, and acquired from the same source. The *drpp40* gene was amplified by PCR using the following primer combination: FLdrpp40F (5′-CATATGAGTATTATAAATAATGAAGTACC-3′) and FLdrpp40R (5′-CTCGAGA CAATAATAATCATAAG-TACC-3′). The sequence of *drpp40* was also deposited in NCBI database (GenBank accession number DQ295793). A part of this ORF (591–1161 nts) encoding a polypeptide named Δ*drpp40* with strong antigenic epitopes was amplified by means of PCR using *D. discoideum* genomic DNA as a template and the following set of primers subrpp40F: 5′-CATATGCAAAGATACATCATCACATTGG-3′ and subrpp40R: 5′-CTCGAGAGAGATTGGAGTATCAGCG-3′. *Nde* I and *Xho* I sites (underlined) were incorporated to the 5′- and 3′-ends, respectively.

The PCR products were directly cloned in pCRII-TOPO vector and both strands were sequenced.

2.3. Overexpression and purification of recombinant proteins

The putative ORFs were subcloned into pET29a(+) expression vector (Novagen) carrying C-terminal histidine tag. The recombinant plasmids were verified by restriction digestion and sequencing to ensure that no mutations had taken place.

pET29a-*drpp40*, pET29a-*Δdrpp40* and pET29a-*drpp20* plasmid constructs were introduced into competent *E. coli* BL21 (DE3)pLysS. Expression was induced by addition of 1 mM IPTG. Overexpressed DRpp20 is soluble in 50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl and 10 mM imidazole, in contrast to DRpp40 and ΔDRpp40 which were present in the insoluble protein fraction (inclusion bodies) and required 7 mM urea to be solubilized. The three polypeptides were purified using IMAC. The purity of the recombinant proteins was confirmed by SDS-PAGE analysis and silver staining of the gels. The molecular mass of the DRpp20 was certified by MALDI-TOF MS analysis because of its anomalous migration on SDS-PAGE.

2.4. Preparation of rabbit polyclonal antibodies

Preparations of 100 mg of highly purified polypeptides (DRpp20, ΔDRpp40) in complete Freund's adjuvant were subcutaneous injected in rabbits. Initial injection was followed by four boosts at 2 weeks intervals with 100 mg of protein in incomplete Freund's adjuvant. Sera were collected every week. The levels of anti-DRpp20 and anti-ΔDRpp40 antibodies were measured using an ELISA assay on 80-well microtiter plate coated with the recombinant polypeptides. Serum collected 44 days and 37 days after the initial immunization was richest in polyclonal anti-ΔDRpp40 and anti-DRpp20 respectively. Total rabbit IgG was acquired by Protein A sepharose chromatography.

2.5. Western immunoblotting

Active RNase P fractions from either anion exchange chromatography or cesium sulfate gradients were analyzed on 14% polyacrylamide/SDS gel and then electro-transferred on PVDF membrane (Millipore). BSA blocked membranes were immunoblotted with a 1/1000 dilution of total IgG. As a secondary antibody was used 1/2000 dilution of anti-rabbit IgG horseradish peroxidase linked antibody. Antibody–antigen complexes were visualized using enhanced chemiluminescence. As a control for the specificity of the antisera, pre-immune serum was tested under the same conditions.

2.6. Immunoprecipitation assays

Protein A sepharose beads (Sigma) were incubated at 4 °C overnight with 30 mL pre- and post-immune sera in 1 mL IPP500 buffer (10 mM Tris–HCl pH 8.0, 500 mM NaCl, 0.1% Nonident P-40), washed with IPP500 and with IPP150 (same as IPP500, except for 150 mM NaCl). Partially purified RNase P (4 mL) was added and incubated with the beads. The mixtures were centrifuged and the supernatants were acquired and assayed for RNase P activity. The pellets were stringently washed (3 IPP150 and 3 × buffer D) and resuspended in assay buffer D (plus 10 U of RNasin and 0.5 mM PMSF) to check for RNase P activity.

2.7. Mobility shift assay

(a) A ^{32}P-labeled RNase P RNA transcript (0.5–2 pmol) (Vourekas et al., 2007) or ^{32}P-labeled pre-tRNA^{Ser} (1–4 pmol) or M1 RNA (1–4 pmol) (Milligan et al., 1987) was incubated with up to 1 mg of highly purified recombinant protein (DRpp40 and/or DRpp20) in binding buffer (5 mM $MgCl_2$, 10 mM NH_4Cl, 10 mM Tris–HCl pH 7.6, 5% glycerol, 1 mM DTT) at 25 °C. Samples were analyzed in a 4% native polyacrylamide gel and visualized by autoradiography.

(b) Purified protein (250 ng–20 mg) was incubated with 250 ng of plasmid DNA in binding buffer (20 mM MES pH 6.5, 100 mM potassium glutamate, 1 mM $MgCl_2$, 0.1 mg/ml BSA), in 10 ml total volume. After 30 min incubation at 23 °C samples were electrophoresed (in 0.7% agarose, 1 × TAE). After electrophoresis, gels were stained in EtBr and visualized under UV light.

2.8. ATPase assay

ATPase activity was assayed in 1 × ATPase buffer (50 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 1 mM DTT), 0.1 mM ATP and 0.1 mCi of [a-^{32}P] ATP at 37 °C for 30 min. After incubation, the reaction was stopped by addition of 1 ml of 0.4 M EDTA and placed on ice. An aliquot of the reaction mixture (1 ml) was spotted on a polyethyleneimine cellulose TLC plate and dried. The TLC plate was developed in 1 M NaCl and visualized by autoradiography after drying.

3. Results

3.1. Gene identification

Genomic analysis performed on *D. discoideum* publicly available sequencing data (www.csm.biol.tsukuba.ac.jp/cDNA-project.html, http://dictybase.org), resulted in the identification of two ORFs which show high degree of similarity to the Rpp20 (34% identity, 56% similarity at a length of 140 amino acids) and Rpp40 (26% identity, 45% similarity at a length of 302 amino acids) human RNase P protein subunits (Tables 1 and 2). The *D. discoideum* counterparts are named after the human homologues, with the addition of the letter 'D' for *Dictyostelium*, namely DRpp20 and DRpp40.

Initial attempts to isolate the DRpp20 and DRpp40 ORFs from a cDNA library which was made from RNA extracted from 10 h starved and 45 h migrating slug cells (strain AX4) (Vourekas et al., 2007) were unsuccessful. Two cDNA clones, the SSF189 and CFG414 containing the genes *drpp20* and *drpp40* respectively, were identified through bioinformatic

Table 1
Comparison of DRpp20 to eukaryotic RNase P protein subunits [a]

	Identity	Similarity	Length [b]	*E*-value
H. sapiens Rpp20 (U94316)	34%	56%	140	2e−10
M. musculus (BC010780)	33%	56%	140	2e−10
X. laevis (BC082214)	37%	58%	127	8e−11
D. melanogaster (NM001031907)	27%	27%	167	4e−5

[a] DRpp20 was pairwise aligned with each sequence using Blosum 62 matrix.
[b] Length (in amino acids residues) of the DRpp20 region displaying similarity.

Table 2
Comparison of DRpp40 to eukaryotic RNase P protein subunits[a]

	Identity	Similarity	Length[b]	*E*-value
H. sapiens Rpp40 (U94317)	26%	45%	302	2e−24
X. laevis (BC081021)	25%	45%	359	2e−23
M. musculus (BC024607)	25%	45%	303	8e−21
D. rerio (BC071381)	27%	48%	361	1e−21

[a] DRpp40 was pairwise aligned with each sequence using Blosum 62 matrix.
[b] Length (in amino acids residues) of the DRpp40 region displaying similarity.

analysis. These clones were kindly provided from National Institute of Genetics and University of Tsukuba in Japan.

3.2. Sequence analysis

The clone SSF189 contains a 684 bp open reading frame, named *drpp20* which encodes a protein (DRpp20) of 228 amino acids with a predicted molecular mass of 26.4 kDa and a theoretical isoelectric point of ~5.6. The gene *drpp20* is located on chromosome 5, at position 2393417 to 2394447 and contains two introns. Clustal W algorithm (1.83) (Milligan et al., 1987) was used to align the amino acid sequence of the four DRpp20 homologues from *Homo sapiens*, *Mus musculus*, *Xenopus laevis* and *Drosophila melanogaster* (Fig. 1). Although DRpp20 has high degree of similarity with its characterized human counterpart, as indicated by *E*-values (Table 1), the molecular mass differs significantly from Rpp20 (MW 15.6 kDa), and so does the p*I* (8.7 for the human protein versus 5.6 for DRpp20) even when the extra carboxy-terminus region is excluded (8.7 versus 4.7). DRpp20 harbors a low complexity region (aas 158 to 228), rich in threonine (aas 158 to 182) and to a lesser extent in glutamine and lysine, which confers to higher MW in comparison with its homologues, a feature also encountered and described for DRpp30 (Vourekas et al., 2007).

Likewise, the clone CFG414 contains an open reading frame of 1272 bp, named *drpp40*, encoding a polypeptide of 424 amino acids residues with calculated molecular mass of 48.2 kDa and an isoelectric point of 5.5. The gene *drpp40* is located on chromosome 3, at position 2762286 to 2763560 and does not contain introns. DRpp40 has higher molecular weight than human Rpp40 (MW 34.6 kDa, p*I* 5.4) but share almost the same p*I*. DRpp40 clusters well with putative homologues from *H. sapiens*, *M. musculus*, *X. laevis* and *Danio rerio* (Fig. 2, Table 2), apart from amino acids at positions 1 to 43. We noticed that the respective coding region (nucleotides 1 to 129) is also a part of *AbCB4* gene (ABC transporter gene) but it is translated in a different open reading frame.

Additionally, pattern search of the *D. discoideum* protein sequences using Pfam (Bateman et al., 2004) prediction tool identified Alba domain (aas 56 to 126) harbored by DRpp20. The Alba superfamily proteins appear to have originated as RNA-binding proteins which formed various ribonucleoprotein complexes, probably including RNase P. It was recruited as a chromosomal protein possibly only within the crenarchaeal lineage. It binds rRNA and mRNA *in vivo*, and may play a role

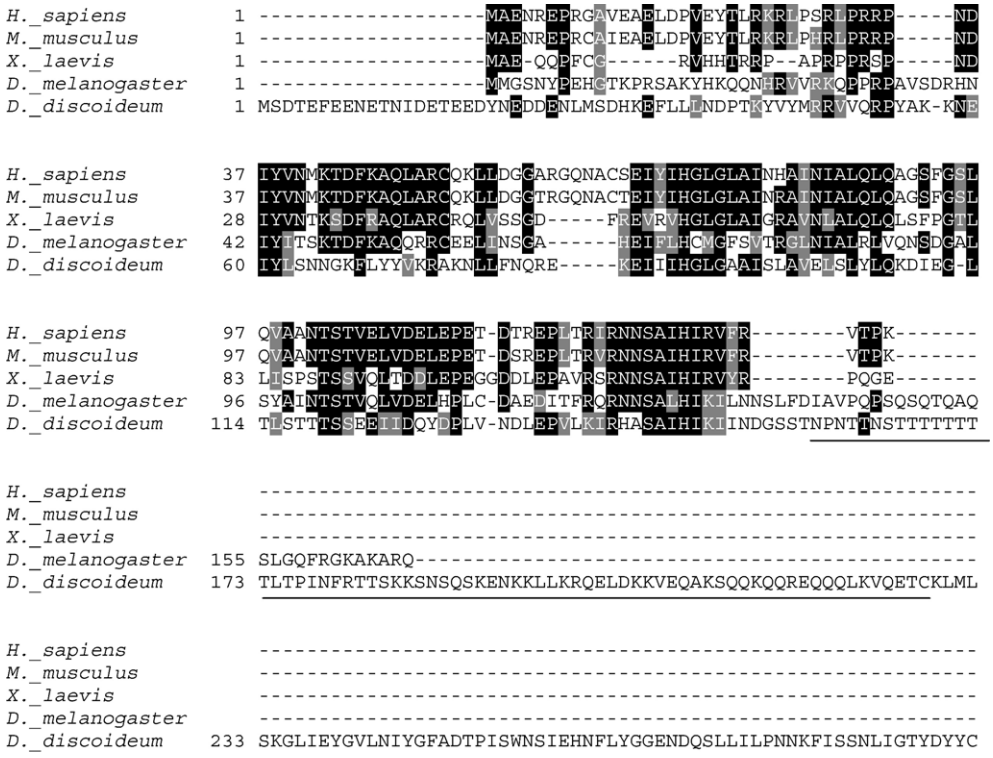

Fig. 1. Multiple sequence alignment of DRpp20 with eukaryotic potential homologues. Amino acid sequences were aligned using Clustal W algorithm (1.83) (Thompson et al., 1994). Residues exhibiting identity and similarity are highlighted black and gray respectively with a 60% threshold using BLOSUM scoring matrix. The low complexity region at the C-terminus of DRpp20 is underlined.

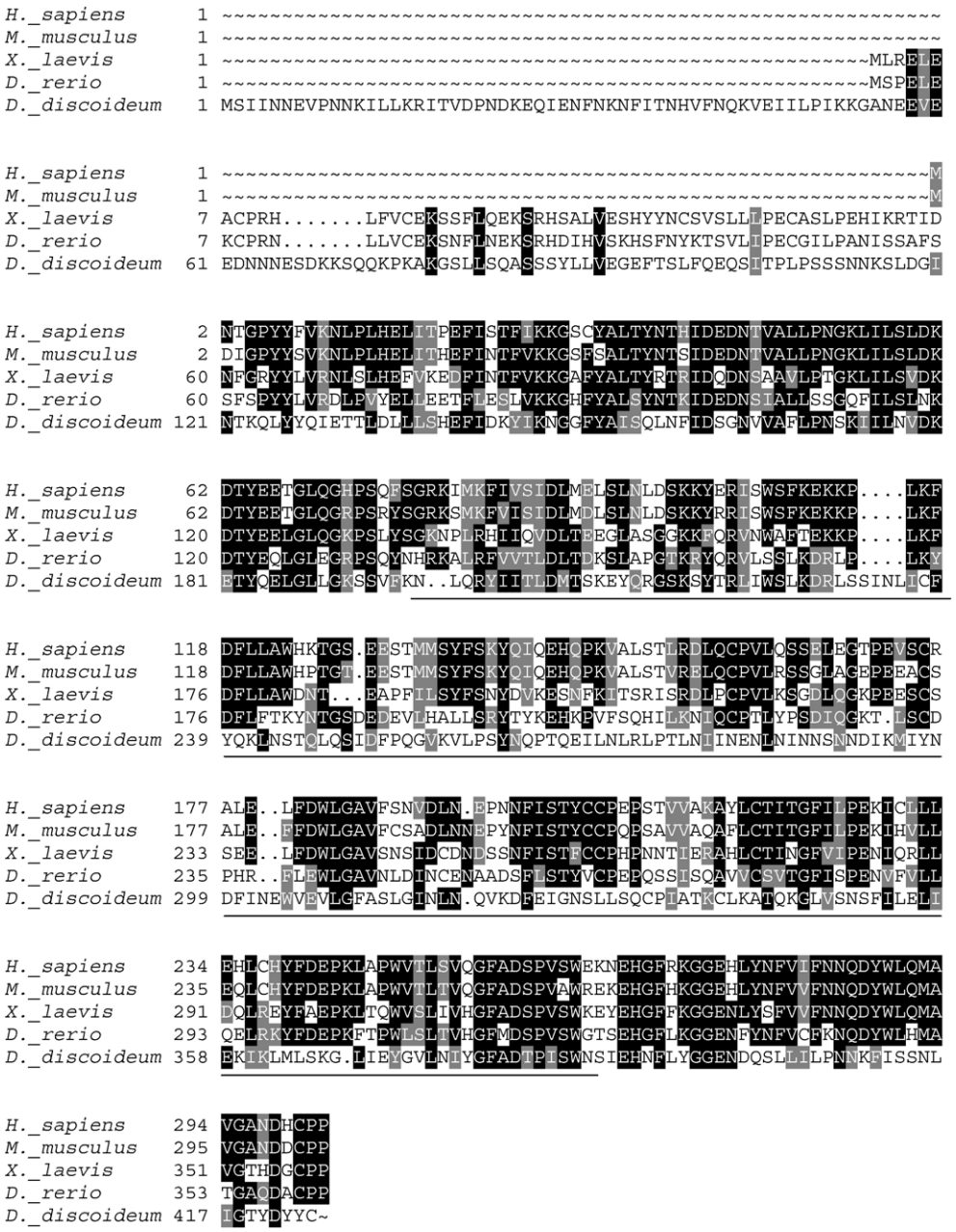

Fig. 2. Multiple sequence alignment of DRpp40 with eukaryotic potential homologues. Amino acid sequences were aligned using Clustal W algorithm (1.83) (Thompson et al., 1994). Residues exhibiting identity and similarity are highlighted black and gray respectively with a 60% threshold using BLOSUM scoring matrix. The sequence of ΔDRpp40 is underlined.

in maintaining the structural and functional stability of RNA, and perhaps, ribosomes (Aravind et al., 2003).

3.3. Overexpression and immunobiochemical analysis

In order to ascertain the proteins' functions suggested by their homology profile, we cloned and overexpressed the corresponding genes. The recombinant polypeptides were purified from the cell extract using Ni^{2+}-nitriloacetic acid agarose column. The recombinant constructs for DRpp20 and DDRpp40 were used for immunization of rabbits.

RNase P active fractions were tested for the presence of DRpp20 and DRpp40 by Western blotting using the respective purified rabbit total IgG from post-immune sera. As shown in Fig. 3, DRpp20 and DRpp40 are present only in fractions from the peak of enzymatic activity. Pre-immune serum was tested under the same conditions and showed no reactivity (data not shown). The apparent MW of the recombinant DRpp20 was higher than calculated (Fig. 3). Because of this discrepancy, the sequence and the MW of DRpp20 were certified by MALDI-TOF MS analysis.

Polyclonal antibodies raised against recombinant DRpp20 and ΔDRpp40 were also tested for their ability to precipitate

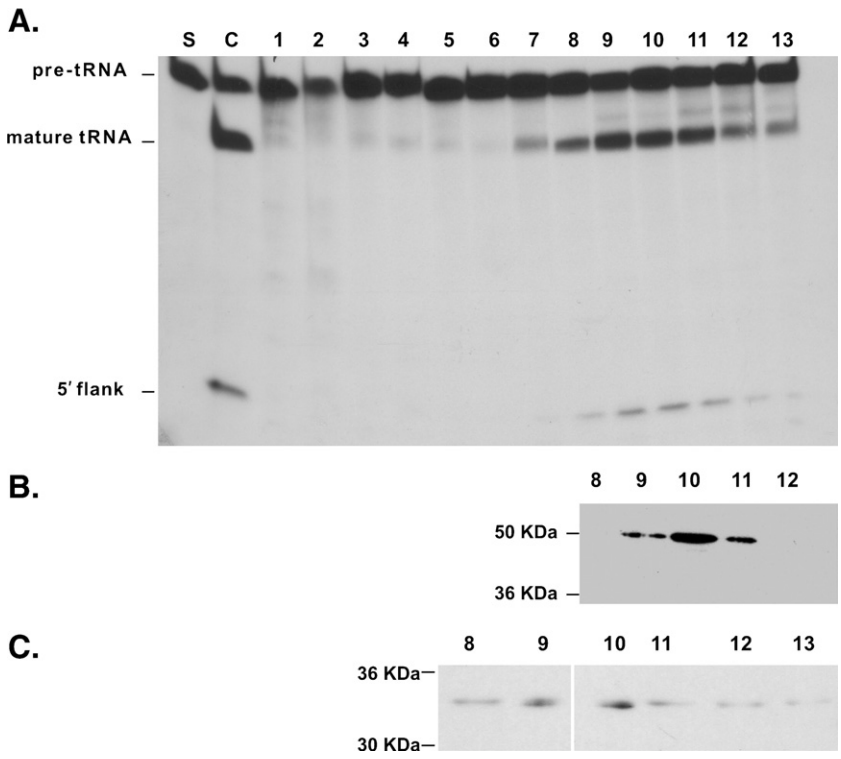

Fig. 3. DRpp20 and DRpp40 copurify with *D. discoideum* RNase P activity. (A) Fractions from the final purification step were assayed for RNase P activity using ^{32}P-labeled p*SupS1* as substrate. Reaction products were electrophoretically analyzed on a 10% polyacrylamide/8 M urea gel. S, pre-tRNA substrate alone; C, control RNase P reaction; 1–13, cesium sulfate density centrifugation fractions. RNase P cleaves the p*SupS1* (110 nucleotides), producing the mature tRNA (82 nucleotides) and the 5′ leader sequence (5′ flank, 28 nucleotides). The corresponding bands are marked on the left-hand margin. The same fractions were subjected to Western-blot analysis using anti-DRpp40 (B) or anti-DRpp20 (C) polyclonal antibodies. DRpp20 and DRpp40 were detected only in active fractions. On the left the positions of protein molecular mass markers are indicated.

catalytically active enzyme. IgG coated protein A sepharose beads were incubated with partially purified RNase P. Fractions after the immunoprecipitation procedure were tested for pre-tRNA cleavage and the reaction products were analyzed by denaturing PAGE. Anti-DRpp20 (Fig. 4A) and anti-ΔDRpp40 (Fig. 4B) antibodies bound to sepharose beads precipitate effectively active holoenzyme under stringent conditions in contrast to pre-immune sera coated beads (negative control), with anti-DRpp20 antibodies being more efficient. This finding suggests that DRpp20 and DRpp40 are associated with catalytically active RNase P complex.

3.4. Functional studies

Having established the association of DRpp20 and DRpp40 with the *D. discoideum* RNase P holoenzyme, we tested the ability of the recombinant proteins to interact with nucleic acids using the electrophoretic mobility shift assay. In a series of electrophoretic mobility shift assays using a variety of binding partners (plasmid DNA, tRNAs, RNA subunit of *D. discoideum* RNase P and M1 RNA), we did not detect any DNA or RNA-binding properties for DRpp20 and DRpp40 (data not shown), although the former contains a region that bears similarity to the Alba domain.

Human Rpp20 was shown to have ATPase activity (Li and Altman, 2001), therefore it was of interest to investigate whether DRpp20 also shows such an enzymatic property. We could not detect any ATPase activity associated with immunoprecipitated DRpp20 or the RNase P holoenzyme under the conditions tested (data not shown).

4. Discussion

4.1. DRpp20 and DRpp40 have common features and distinct characteristics with homologous proteins — implications on the structure of D. discoideum RNase P

D. discoideum RNase P protein subunits DRpp20 and DRpp40 exhibit intriguing features. The primary structures of DRpp20 and DRpp40 display significant similarity to their characterized human counterparts (Tables 1 and 2). It is highly probable that Rpp20 and Pop7 are orthologues, which together with Rpp25 form a distinct group of proteins belonging to the Alba superfamily (Aravind et al. 2003, Rosenblad et al., 2006). Although Rpp20 aligns well with Pop7, DRpp20 shows very low similarity to the yeast protein, even when the extra regions at the amino- and carboxy-terminus of the *Dictyostelium* protein (Fig. 1) were omitted from the alignment (*E*-value: 9797, data not shown), and therefore we cannot conclude on the functional relevance of these two proteins. No homologue of DRpp40 has been identified in yeast or archaeal RNase P enzymes.

On the other hand, the molecular masses of the Rpp20 and Rpp40 homologues differ significantly from their *D. discoideum* counterparts. DRpp20 harbors a region of low complexity (rich

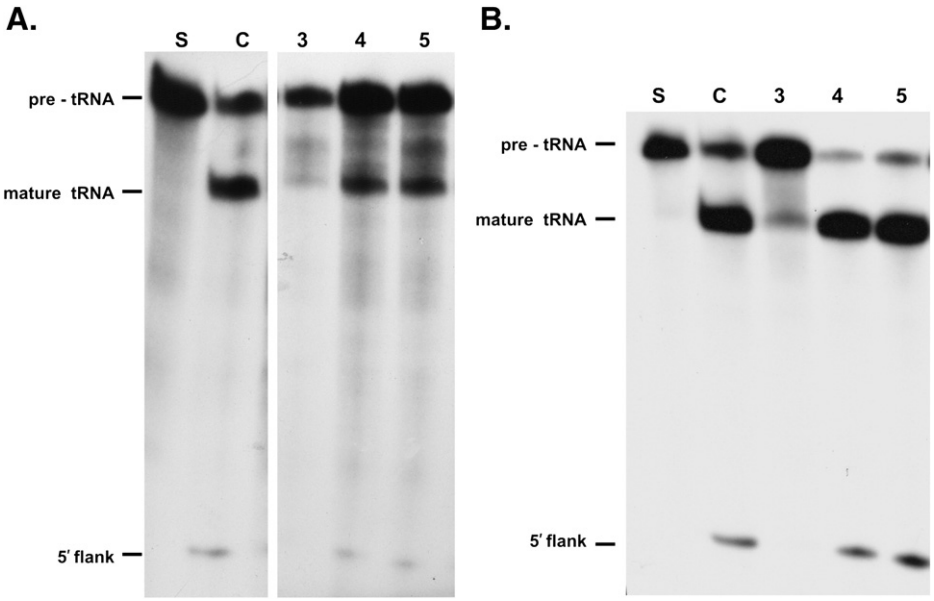

Fig. 4. Immunoprecipitation of RNase P activity with polyclonal antibodies against DRpp20-His_6 (A) and ΔDRpp40-His_6 (B). Rabbit antisera were used to immunoprecipitate RNase P activity from a partially purified *D. discoideum* RNase P preparation. Protein A Sepharose beads coated with pre-immune serum (lanes 3) or serum after immunization with DRpp20-His_6 (A: lanes 4 and 5: 37 days post-immune serum) or DRpp40-His_6 (B: lanes 4 and 5: 44 days post-immune serum) were mixed with partially purified RNase P, washed stringently and assayed for RNase P activity. S, substrate alone; C, control RNase P reaction.

in threonine residues) which confers to the higher MW in comparison with the human homologue (Table 1). Such regions have not been encountered so far in proteins of this kind in other organisms but are abundant in *D. discoideum* proteome. Among the identified protein subunits of *D. discoideum* RNase P, DRpp30 (Vourekas et al., 2007) and DRpp25 also carry regions rich in specific amino acids most notably threonine. It remains to be proven if these features contribute to higher structure conformations and possibly the function of DRpp proteins (Eichinger et al., 2005).

D. discoideum RNase P has the lowest buoyant density among eukaryotic RNase P enzymes studied so far, but interestingly, consists of fewer protein subunits than human and yeast RNase P enzymes. However, DRpp20 and DRpp40 (this study), DRpp30 (Vourekas et al., 2007) as well as Pop5, DRpp25, DRpp29 (currently under investigation) have higher MW than the homologues, thus contributing to the proteinaceous character of their macromolecular complex.

4.2. Functional association of identified proteins with RNase P holoenzyme

DRpp20 and DRpp40 are functionally associated with the RNase P ribonucleoprotein catalytic complex. Using anti-DRpp20 and anti-ΔDRpp40 antibodies we ascertained the concurrence of DRpp20 and DRpp40 with purified RNase P activity after standard purification schemes. Moreover, the nature of this association permits the precipitation of RNase P activity through antigen–antibody interaction using the same antibodies. The polyclonal anti-DRpp20 antibodies are much more efficient in the immunoprecipitation of RNase P activity. In the experimental conditions used most of the RNase P was bound on the IgG coated protein A sepharose beads. In comparison, polyclonal antibodies raised against the ΔDRpp40 which is a part of DRpp40 have lower efficiency in the same experiment but give better results in the detection of their partner on the Western blots (Fig. 4). In our view, this is due to the use of the native full length DRpp20 polypeptide for the immunization of the rabbits.

4.3. Possible roles of DRpp20 and DRpp40 — direction of future studies

RNA–protein interactions between the protein subunits, the RNA moiety and/or the RNA substrate are expected in the holoenzyme complex, and therefore the ability of DRpp40 and DRpp20 to bind to RNA molecules was investigated. Although no such ability was revealed, we cannot rule out the possibility that DRpp20 in complex with its protein partners exhibits a potential to bind RNA molecules, due to the Alba domain. It is also possible that DRpp40 and DRpp20 contribute to the formation of *D. discoideum* active RNase P complex only through protein–protein interactions.

Additionally, despite the similarity between DRpp20 and its human counterpart, no ATPase activity was detected in immunoprecipitated preparations of DRpp20 and RNase P holoenzyme. The limited data concerning this activity at this point hampers our efforts to study it further in *D. discoideum* RNase P.

From an evolutionary point of view, both Rpp20 and Rpp40 proteins were recruited on the RNase P holoenzyme after the emergence of the eukaryotes, and could serve for lineage specific interactions of RNase P with other nuclear molecular mechanisms, such as transcription by RNA polymerase III

(Reiner et al., 2006). This work shows that these proteins are integral parts of the RNase P from the lower eukaryote *D. discoideum* and their roles in RNase P function as well as their possible involvement in coordination of tRNA maturation and RNA gene transcription provide the basis for future studies.

Acknowledgments

We thank Dr. Constantinos Stathopoulos for critical discussions and helpful comments on the manuscript, and Dr Ioannis K. Zarkadis for sharing experience on cloning procedures. We thank the members of the Japan cDNA project of *Dictyostelium discoideum* (*Dictyostelium* cDNA project in Japan with support by Japan Society for the Promotion of Science and Ministry of Education, Science, Sports and Culture of Japan) for providing us with the cDNA clones SSF189 and CFG414. The *D. discoideum* AX4 genomic DNA and cDNA lambda ZAPII library was a kind gift from Dr. Dan Fuller (University of California, San Diego, USA). We thank the European Social Fund (ESF), Operational Program for Educational and Vocational Training II (EPEAEK II), and particularly the Program HERAKLITOS, for funding the above work.

References

Altschul, S.F., et al., 1997. Gapped BLAST and PSI BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Aravind, L., Iyer, L.M., Anantharaman, V., 2003. The two faces of Alba: the evolutionary connection between proteins participating in chromatin structure and RNA metabolism. Genome Biology 4, R64.

Bateman, A., et al., 2004. The Pfam protein families database. Nucleic Acids Res. 32, 138–141.

Boomershine, W.P., McElroy, C.A., Tsai, H.Y., Wilson, R.C., Gopalan, V., Foster, M.P., 2003. Structure of Mth11/Rpp29, an essential protein subunit of archaeal and eukaryotic RNase P. Proc. Natl. Acad. Sci. U. S. A. 26, 15398–15403.

Chamberlain, J.R., Lee, Y., Lane, W.S., Engelke, D.R., 1998. Purification and characterization of the nuclear RNase P holoenzyme complex reveals extensive subunit overlap with RNase MRP. Genes Dev. 12, 1678–1690.

Eichinger, L., et al., 2005. The genome of the social amoeba *Dictyostelium discoideum*. Nature 435, 43–57.

Frank, D.N., Pace, N.R., 1998. Ribonuclease P: unity and diversity in a tRNA processing ribozyme. Ann. Rev. Biochem. 67, 153–180.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., Altman, S., 1983. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell 35, 849–857.

Hall, T.A., Brown, J.W., 2002. Archaeal RNase P has multiple protein subunits homologous to eukaryotic nuclear RNase P proteins. RNA 8, 296–306.

Hua, Y., Zhou, J., 2004. Rpp20 interacts with SMN and is re-distributed into SMN granules in response to stress. Biochem. Biophys. Res. Commun. 314, 268–276.

Jarrous, N., 2002. Human ribonuclease P: subunits, function, and intranuclear localization. RNA 8, 1–7.

Jiang, T., Altman, S., 2001. Protein–protein interactions with subunits of human nuclear RNase P. Proc. Natl. Acad. Sci. U. S. A. 98, 920–925.

Kawano, S., Nakashima, T., Kakuta, Y., Tanaka, I., Kimura, M., 2006. Crystal structure of protein Ph1481p in complex with protein Ph1877p of archaeal RNase P from *Pyrococcus horikoshii* OT3: implication of dimer formation of the holoenzyme. J. Mol. Biol. 2, 583–591.

Kikovska, E., Svard, S.G., Kirsebom, L.A., 2007. From the cover: eukaryotic RNase P RNA mediates cleavage in the absence of protein. Proc. Natl. Acad. Sci. U. S. A. 104, 2062–2067.

Kouzuma, Y., et al., 2003. Reconstitution of archaeal ribonuclease P from RNA and four protein subunits. Biochem. Biophys. Res. Commun. 306, 666–673.

Li, Y., Altman, S., 2001. A subunit of human nuclear RNase P has ATPase activity. Proc. Natl. Acad. Sci. U. S. A. 98, 441–444.

Marquez, S.M., et al., 2005. Structural implications of novel diversity in eucaryal RNase P RNA. RNA 11, 739–751.

Milligan, J.F., Groebe, D.R., Witherell, G.W., Uhlenbeck, O.C., 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res. 15, 8783–8798.

Pannucci, J.A., Haas, E.S., Hall, T.A., Harris, J.K., Brown, J.W., 1999. RNase P RNAs from some Archaea are catalytically active. Proc. Natl. Acad. Sci. U. S. A. 96, 7803–7808.

Reiner, R., Ben-Asouli, Y., Krilovetzky, I., Jarrous, N., 2006. A role for the catalytic ribonucleoprotein RNase P in RNA polymerase III transcription. Genes Dev. 20, 1621–1635.

Rosenblad, M.A., Lopez, M.D., Piccinelli, P., Samuelsson, T., 2006. Inventory and analysis of the protein subunits of the ribonucleases P and MRP provides further evidence of homology between the yeast and human enzymes. Nucleic Acids Res. 34, 5145–5156.

Stathopoulos, C., Kalpaxis, D.L., Drainas, D., 1995. Partial purification and characterization of RNase P from *Dictyostelium discoideum*. Eur. J. Biochem. 228, 976–980.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Vourekas, A., Kalavrizioti, D., Zarkadis, I.K., Spyroulias, G.A., Stathopoulos, C., Drainas, D., 2007. A 40 kDa Rpp30/Rpp1p homologue is a protein subunit of *Dictyostelium discoideum* RNase P holoenzyme. Biochimie 89, 301–310.

Walker, S.C., Engelke, D.R., 2006. Ribonuclease P: the evolution of an ancient RNA enzyme. Crit. Rev. Biochem. Mol. Biol. 41, 77–102.

Welting, T.J., et al., 2007. Heterodimerization regulates RNase MRP/RNase P association, localization, and expression of Rpp20 and Rpp25. RNA 13, 65–75.

Wilson, R.C., Bohlen, C.J., Foster, M.P., Bell, C.E., 2006. Structure of Pfu Pop5, an archaeal RNase P protein. Proc. Natl. Acad. Sci. U. S. A. 4, 873–878.

Xiao, S., Houser-Scott, F., Engelke, D.R., 2001. Eukaryotic ribonuclease P: increased complexity to cope with the nuclear pre-tRNA pathway. J. Cell. Physiol. 187, 11–20.

Xiao, S., Scott, F., Fierke, C.A., Engelke, D.R., 2002. Eukaryotic ribonuclease P: a plurality of ribonucleoprotein enzymes. Ann. Rev. Biochem. 71, 165–189.