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Original Article

Partial purification and characterization of RNase P from human peripheral lymphocytes

Anastassios Vourekas[1]*, Eleftheria Vryzaki[2]*, Chrisavgi Toumpeki[1], Vassiliki Stamatopoulou[1], Alexandra Monastirli[2], Dionysios Tsambaos[2] and Denis Drainas[1]

[1]Department of Biochemistry, School of Medicine, University of Patras, Greece;
[2]Department of Dermatology, School of Medicine, University of Patras, Greece
Correspondence: Denis Drainas, Department of Biochemistry, School of Medicine, University of Patras, Rio-Patras 26504, Greece, Tel.: +30 2610 969127, Fax: +30 2610 969167, e-mail: drainas@med.upatras.gr
**The authors had equal contribution to this report.*

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Abstract: Ribonuclease P (RNase P) is ubiquitous and essential Mg^{2+}-dependent endoribonuclease that catalyzes the 5′-maturation of transfer RNAs. RNase P and the ribosome are so far the only ribozymes known to be conserved in all kingdoms of life. Eukaryotic RNase P activity has been detected in nuclei, mitochondria and chloroplasts and demonstrates great variability in sequence and subunit composition. In the last few years we have developed methodologies and pursued projects addressing the occurrence, distribution and the potential physiological role of RNase P in human epidermal keratinocytes. In view of the vital importance of lymphocytes for an effective immune system and their successful application after transfection with RNase P-associated external guide sequences in gene therapy, we concerned ourselves with the isolation and characterization of RNase P of peripheral human lymphocytes. We developed a method described herein, that will enable the study of the possible involvement of this ribozyme in the pathogenetic mechanisms of diverse autoimmune, inflammatory and neoplastic cutaneous disorders and may facilitate the further development of RNase P-based technology for gene therapy of infectious and neoplastic dermatoses.

Key words: human peripheral lymphocytes – ribonucleoprotein – RNase P – tRNA

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Introduction

Ribonucleases comprise a family of enzymes capable of degrading RNA that are ubiquitously present in mammalian tissues. Ribonuclease P (RNase P) is a key enzyme acting early in the tRNA biogenesis pathway, which catalyzes the endonucleolytic cleavage of the 5′ leader sequence of precursor tRNAs (pre-tRNAs) and generates their 5′ mature end. Most forms of RNase P are ribonucleoproteins consisting of an essential RNA and protein subunits (1). The RNA component of the bacterial RNase P was one of the first identified catalytic RNAs and the first found to act as a multiple turnover enzyme *in vitro* (2). RNase P and the ribosome are so far the only ribozymes known to be conserved in all kingdoms of life (1,3).

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; pre-tRNA, precursor transfer RNA; RNase P, ribonuclease P; RNP, ribonucleoprotein.

Eukaryotic RNase P activity has been detected in nuclei, mitochondria and chloroplasts and demonstrates great variability in sequence and subunit composition. Nuclear RNase P has been purified or partially purified from several eukaryotes, primarily from yeast and human cells (4,5). In human, the nuclear holoenzyme consists of one essential RNA and at least 10 protein subunits. It was recently shown that RNA of human RNase P retains traces of catalytic activity, which suggests preservation of the latter throughout evolution (6).

In the last few years we have developed methodologies and pursued projects addressing the occurrence, distribution and the potential physiological role of RNase P in human epidermal keratinocytes (7,8). It is well known, that lymphocytes are vital to an effective immune system, whereas extensive evidence has granted them a cardinal role in the pathogenesis of a wide spectrum of diseases (9–11). On the other hand, lymphocytes transfected with RNase P-associated external guide sequences have been successfully used in gene therapy in order to silence specific viral and cellular carcinogenic messages (12–14). Therefore in

the present study we partially purified and characterized RNase P of peripheral human lymphocytes and developed a method described herein, that will enable the study of the possible involvement of this ribozyme in the pathogenetic mechanisms of diverse autoimmune, inflammatory and neoplastic cutaneous disorders.

Methods

Enzyme purification

Peripheral human lymphocytes were obtained using the Hypaque-Ficoll technique. Five millilitres of venous blood obtained from healthy subjects were resuspended in 2 ml cold buffer A [10 mM KCl, 0.1 mM ethylene glycol tetra-acetic acid (EGTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Hepes pH 7.9] and left to swell for 30 min. Non idet P-40 (0.6% v/v) was then added and the suspension was homogenized in a motor-driven glass homogenizer. The homogenate was dialyzed for 3 h in buffer B (50 mM potassium phosphate, pH 7.0, 1 mM DTT, 10% by volume glycerol, 0.2 mM PMSF) and was subsequently loaded onto a phosphocellulose (P-11) column (3 ml), which had been equilibrated with buffer B. The column was washed with the same buffer until the A_{280} dropped almost to zero. RNase P was then eluted with a 9-ml linear gradient of 100–500 mM NH_4Cl in buffer B. Activity was eluted with a peak at approximately 290 mM. The active fractions were immediately stored at −70°C.

Assay for RNase P activity

Enzyme assays were carried out at 37°C in 20 μL buffer K (50 mM Tris/HCl pH 7.5, 100 mM NH_4Cl, 5 mM $MgCl_2$, 2.5 mM EGTA and 0.5 U RNasin) containing 2–5 fmol pre-$tRNA^{Ser}$ substrate (an *in vitro* labelled transcript of the *Schizosaccharomyces pombe* $tRNA^{Ser}$ gene *SupSI*) and 0.5 μg protein from the RNase P fraction in the absence or presence of the inhibitor (10 μM–1 mM neomycin). The reactions were stopped by addition of 5 μl stop dye (80% formamide, 50 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol). Reaction products were resolved on a denaturing 10% polyacrylamide/8 M urea gel and visualized by autoradiography without drying. Activity quantification was performed by phosphorimaging in a Fuji FLA-3000 using the AIDA software package.

Results

Nuclear RNase P activity from peripheral human lymphocytes eluted at 150–350 mM NH_4Cl through a phosphocellulose (P-11) column (Fig. 1) to which mitochondrial RNase P does not bind (15). Comparison of RNase P activities from peripheral human lymphocytes and *Dictyostelium*

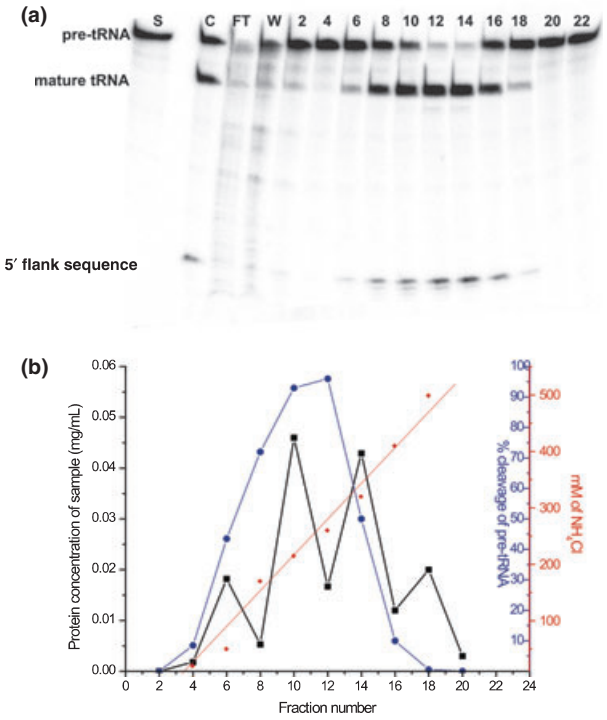

Figure 1. Phosphocellulose (P-11) chromatography. (a) Autoradiogram of the cleavage of *SupS1* precursor by RNase P. S: substrate alone, C: *D. discoideum* RNase P control reaction, FT: column flowthrough fraction, W: wash fraction, 2–20: elution fractions. Activity elutes at 240–350 mM NH_4Cl (fractions 6–14). In the left hand margin the bands corresponding to the precursor and mature tRNA molecules, as well as the 5′ leader sequence are indicated. (b) A typical phosphocellulose (P-11) elution profile. (—■—) Total protein concentration (mg/ml). (—♦—) NH_4Cl concentration (mM). (—•—) % cleavage of pre-tRNA.

discoideum using the SupS1 precursor as the substrate showed that both enzymes produce products of the same size (Fig. 1a, lane C compared to lanes 4–16). pH and temperature optima were found to be 7.5 and 37°C, respectively, as reported for nuclear RNase P from normal human epidermal kerationocytes, as well. RNase P from human peripheral lymphocytes requires both monovalent and divalent cations for activity, whose optimum was found to be at 70 mM NH_4Cl and 5 mM $MgCl_2$, when reactions were carried out at pH 7.5 and 37°C. The enzyme activity was stable for few weeks when stored in assay buffer at −70°C. Figure 2 shows the kinetic characteristics of the enzyme (data series ♦). The initial velocity was calculated by the initial slope of the time plots. The apparent K_m and the apparent V_{max} of human lymphocytes RNase P, using the SupS1 precursor as substrate, were calculated from the double reciprocal plot (Fig. 2) and were 244 nM and 0.42 pmol/min, respectively.

In our attempt to assess the effects of aminoglycosides on RNase P activity from human peripheral lymphocytes we incubated P-11 purified enzyme with neomycin B.

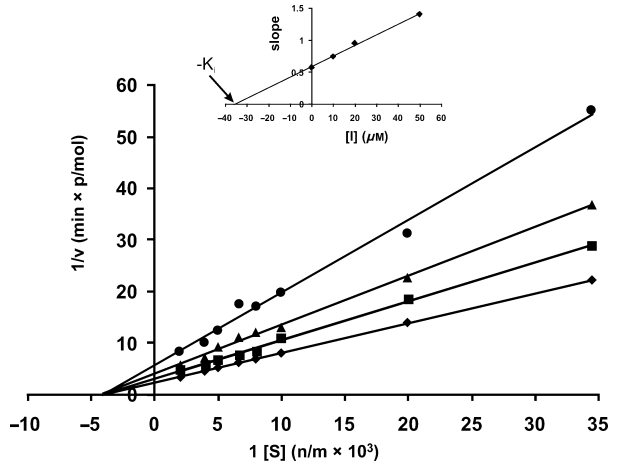

Figure 2. Double-reciprocal plot ($1/v$ versus $1/$[pre-tRNA]) for RNase P reaction in the absence and presence of various concentrations of neomycin B; (♦) 0 μM of neomycin B (control); (■) 10 μM; (▲) 20 μM; (●) 50 μM. Top panel: Slope replot of double–reciprocal plots versus [inhibitor]. The slope is linear, characteristic for non–competitive inhibition. The intercept of the [I]-axis is equal to K_i. All data presented are averaged values of three individual experiments.

A detailed representation of the dose response effect of neomycin is given in Fig. 3. The IC_{50} value was 74 μM for neomycin B (Fig. 3b). Increasing Mg^{2+} from 3 to 20 mM, at a constant neomycin concentration (50 μM), resulted in a considerable recovery of the activity (about 40%) (Fig. 3c). The mode of neomycin-induced inhibition of RNase P activity isolated from human peripheral lymphocytes was further investigated by detailed kinetic analysis in the presence of 5 mM Mg^{2+}. Figure 2 shows double reciprocal plots with increasing concentration of neomycin, a finding indicating that the inhibition is of non-competitive type. The slopes of the lines in Fig. 2 were plotted against the concentration of neomycin (results shown in the top panel of Fig. 2). The linearity of this plot is indicative for simple non-competitive inhibition and permits the graphical determination of $K_i = 37$ μM from the negative intercept of the line with the *I*-axis.

Discussion

RNase P isolated for the first time in the present study from human peripheral lymphocytes, revealed temperature and pH optima very similar to those of RNase P isolated from bacterial and other eukaryotic organisms and an absolute requirement for monovalent and divalent cations. However, its activity elutes from a cation exchange column in a substantially higher NH_4Cl concentration (peaks at 285 mM NH_4Cl), as compared to that of RNase P isolated from human epidermal keratinocytes (peaks at 190 mM NH_4Cl) (8). This difference in chromatographic behaviour suggests that lymphocyte and keratinocyte RNase P have

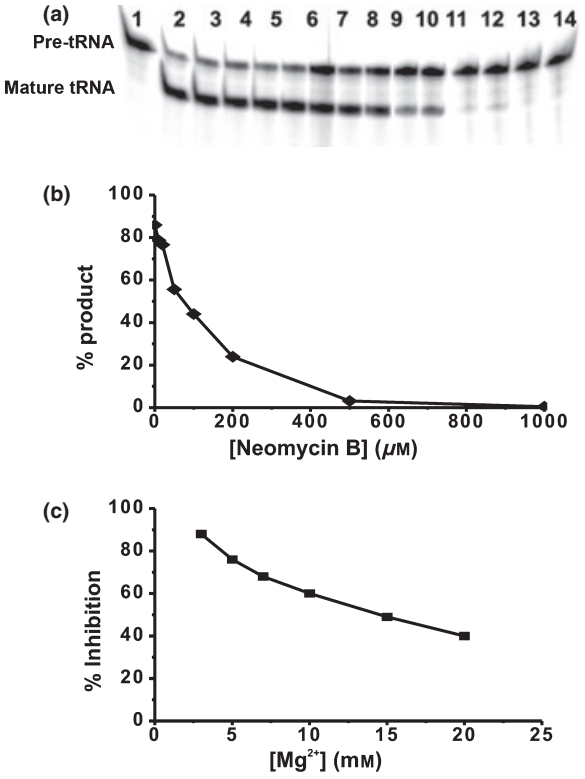

Figure 3. Dose–response effect of neomycin B in the pre-tRNA maturation by RNase P from human peripheral lymphocytes. (a) Autoradiography of the cleavage of the SupS1 precursor by RNase P in the presence of neomycin B. lane 1: SupS1 alone; lanes 2–3: control reaction; lanes 4–15: reaction in the presence of various concentrations of neomycin B (10 μM–1 mM). Reactions are in duplicate for each concentration point. In the left hand margin the bands corresponding to the precursor and mature tRNA molecules are indicated. (b) RNase P activity as a function of increasing concentration of neomycin B. (c) Inhibition of the RNase P reaction in the presence of increasing concentrations of Mg^{2+}. All data presented are averaged values of three individual experiments.

different physicochemical properties, which may reflect a possible different protein composition of the enzymatic complex. Nevertheless, the kinetic analysis suggests that the two enzymes have the same affinity for the SupS1 substrate. The kinetic constant K_m (245 nM) of RNase P activity isolated from lymphocytes for the tRNA maturation reaction is comparable to that of RNase P isolated from normal human epidermal keratinocytes ($K_m = 233$ nM) (7). The difference in V_{max} values (0.42 pmol/min for the RNase P from human peripheral lymphocytes, compared to 2.4 pmol/min for the normal human epidermal keratinocytes enzyme) may be due to different enzyme concentration of the two RNase P preparations (HPL and NHEK) used for the kinetic analysis. We cannot exclude the possibility that the differences in the V_{max} values reflect differences in the protein content of the two enzymes, which may also explain the differential chromatographic

behaviour. If the latter hypothesis is true, we could speculate that the tRNA maturating complex of RNase P in various tissues is formed by a core consisting of less than 10 protein subunits and the RNA subunit, and that the presence of other RNase P proteins (such as eukaryotic specific Rpp40 and Rpp14) may serve additional purposes.

Recent preliminary studies have shown that there are differences in the expression levels of several protein subunits (Rpp38, Rpp30, Rpp21, Rpp20 and Rpp14) of RNase P between various human normal tissues, and between human tumors and normal tissue, as well (16). The characterization of RNase P activity from various tissues is the first step for the identification of possible tissue specific RNase P enzymatic complexes that may differ in their protein content. Currently there is limited or no information for either the contents of RNase P ribonucleoprotein particles in various mammalian tissues or their regulation.

Since susceptibility to various protein inhibitors such as aminoglycosides is a common feature of eukaryotic RNase P enzymes, we tested whether neomycin, a representative of this group, is capable of *in vitro* affecting RNase P activity from peripheral human lymphocytes. The IC_{50} value obtained for neomycin B was similar to those previously obtained with RNase P from *D. discoideum* (17) and human epidermal keratinocytes (18). The inhibition caused by neomycin was sensitive to Mg^{2+} concentration. When the concentration of Mg^{2+} was increased from 3 to 20 mM, a 40% recovery of the activity was observed. The fact that upon increase of Mg^{2+} concentration the enzyme activity was partially restored clearly implies the occurrence of a competition of the aminoglycoside molecule with the metal atoms for the same sites on the enzyme. Our findings are in agreement with previously reported data on aminoglycoside inhibition of *D. discoideum* (17) and human epidermal keratinocytes RNase P (18), which suggest that these antibiotics inhibit ribozyme cleavage by replacing magnesium ions with protonated amino groups. Detailed kinetic analysis showed that neomycin behaves as a classical non-competitive inhibitor and allowed the accurate evaluation of the potency of this aminoglycoside on the basis of K_i value. It is important to note that despite any structural differences found between RNase P enzymes isolated from various eukaryotic sources, their response to protein inhibitors is similar. Thus, it seems that modulators of RNase P enzymatic activity still represent precious tools for the study of its properties.

The method for isolation and characterization of RNase P activity from human peripheral lymphocytes described in this paper will enable the study of the possible involvement of this ribozyme in the pathogenetic mechanisms of diverse autoimmune, inflammatory and neoplastic cutaneous disorders and may facilitate the further development of RNase P-based technology for gene therapy of infectious and neoplastic diseases.

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