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INVESTIGATIVE REPORT

Isolation of Ribonuclease P Activity From Human Epidermis and its Regulation by Retinoids *In vitro*

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Ribonuclease P (RNase P) is a key enzyme in tRNA biogenesis that catalyses the endonucleolytic cleavage of tRNA precursors and generates their mature 5' ends. The activity of this ribozyme has never been isolated from living human tissues and data about epidermal tRNA biogenesis are not available. The purpose of the present study was to isolate and purify RNase P from human epidermis and to investigate the *in vitro* effects of retinoids on its activity. Enzyme isolation and purification from homogenates of keratinocytes derived after trypsinization from dispase-separated human epidermis were carried out using phosphocellulose chromatography. The optimal activity of the enzyme was found at 100 mM NH_4Cl and 5 mM $MgCl_2$ at pH 7.5 and 37°C. All-*trans* retinoic acid and acitretin revealed a dose-dependent inhibitory effect on RNase P activity. The isolation of RNase P activity from human epidermis, reported here for the first time, will enable the investigation of the possible involvement of this ribozyme in the regulation of epidermal differentiation and proliferation and the evaluation of its significance for the pathogenesis and gene therapy of various cutaneous disorders. *Key words: RNase P; skin; tRNA; acitretin; all-trans-retinoic acid.*

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Ribonucleases comprise a family of enzymes, including ribozymes, which are able to degrade RNA. They are ubiquitously present in mammalian tissues, including human skin. Some of the ribonucleases from human epidermis exhibit only hydrolytic activity (1), whereas others are thought to possess properties of essential importance for keratinocyte adhesion and desquamation (2), terminal differentiation (3), elimination of RNA processing reactions by-products or incorrectly synthesized or damaged RNAs (4) and for the innate mature defence system of epidermis (5).

Ribonuclease P (RNase P) is a key enzyme that acts early in the tRNA biogenesis pathway, catalysing the endonucleolytic cleavage of the leader sequence of precursor tRNAs (pre-tRNAs) and generating the mature 5' end of tRNAs. Most forms of RNase P are ribonucleoproteins consisting of an essential RNA and one or several protein subunits (6). Bacterial RNase P RNA was one of the first catalytic RNAs identified and the first found to act as a multiple turnover enzyme *in vivo* (7). RNase P and the ribosome are the only ribozymes known so far to be conserved in all kingdoms of life (6, 8).

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 vertebrates (6). Studies on human holoenzyme showed that one RNA subunit and at least ten essential proteins contribute to the total mass of RNase P (9).

To the best of our knowledge, RNase P activity has never been isolated from living human tissues and data about tRNA biogenesis in human epidermis are not available. In this paper, we report the isolation and purification of RNase P from human epidermis that will enable studies on the possible involvement of this ribozyme in the regulation of epidermal differentiation and proliferation. Moreover, the *in vitro* effects of two retinoids, one natural (retinoic acid) and one synthetic (acitretin) on the RNase P activity are reported.

MATERIAL AND METHODS

Separation of epidermis and dermis

Skin specimens were obtained from patients who underwent abdominoplasty for morbid obesity. The loosely adherent subcutaneous tissue was scraped away and skin specimens cut into pieces measuring 5×5 mm were incubated in dispase (240 U/100 ml) dissolved in serum-free DMEM-$F1_2$ at 4°C for 18 h. Then the epidermis was easily peeled off the dermis in a continuous sheet that was washed with Ca^{2+}- and Mg^{2+}-free Hank's buffer saline solution (BSS) and incubated in trypsin 0.025%/EDTA 0.01% in Hepes) at 4°C for 15 min. Keratinocytes were obtained by dissociating the epidermal sheet by shaking and centrifugation (1500 rpm) at 4°C for 5 min. Viability of keratinocytes was measured by the trypan blue dye exclusion test.

Enzyme purification

Cell breakage and purification of RNase P were carried out as described previously (10). A total of 5×10^6 cells were resuspended in 4 ml ice-cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride) and left to swell for 30 min.

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Nonidet P-40 (0.6% v/v) was then added and the suspension was homogenized with 20 strokes in a motor-driven glass homogenizer. After centrifugation, nuclear pellets were resuspended in 1 ml ice-cold buffer C (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1mM dithiothreitol, 20% glycerol) and shaken vigorously at 4˚C for 30 min. Nuclear debris was discarded by centrifugation for 15 min at 14,000 rpm and the extract was stored at –20˚C.

Cellular and nuclear extracts were dialysed overnight in 2 l buffer B (50 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol, 10% by volume glycerol) and loaded onto a phosphocellulose (P-11) column (3 ml) equilibrated with buffer B. The column was washed with the same buffer until the A280 dropped almost to zero. RNase P was then eluted with a 9-ml linear gradient of 50 – 500 mM NH_4Cl in buffer B. Activity was eluted at 130 – 250 mM NH_4Cl. The active fractions were combined and dialysed overnight in 2 l of buffer K (5 mM $MgCl_2$, 100 mM NH_4Cl, 50 mM Tris/HCl pH 7.5) in the presence of 20% glycerol, divided into small aliquots and stored at –20˚C.

Assay for RNase P activity

Enzyme assays were carried out at 37˚C in 20 µl buffer K containing 2–5 fmol pre-tRNASer substrate (an *in vitro* labelled transcript of the *Schizosaccharomyces pombe* $tRNA^{Ser}$ gene supSI) and 1.3 µg protein from the RNase P fraction. Stock solutions of all-*trans*-retinoic acid and acitretin kindly supplied by Roche Hellas S.A. (Athens, Greece) were prepared in dimethyl sulphoxide (DMSO). Based on the results of high-pressure liquid chromatography (one single peak) and nuclear magnetic resonance (NMR) spectroscopy (400 MHz NMR Bruker instrument), both retinoids used in the present study appeared to be highly pure. Due to the high hydrophobicity of retinoids all enzyme assays were carried out in the presence of 10% DMSO, which at this concentration marginally inhibits the catalytic activity of normal human epidermal keratinocytes (NHEK) RNase P (10). 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 was quantified by Cerenkov counting of excised gel slices.

RESULTS

As shown by light microscopy, the separation of epidermis and dermis by dispase was complete, the separated epidermis retained an intact basal layer (Fig. 1) and the basement membrane remained attached to the dermis. Keratinocytes obtained by incubating the dispase-separated epidermal sheets in 0.25% trypsin solution for 15 min revealed a viability of 86%, as measured by the trypan blue dye exclusion test. The enzyme activity of epidermal keratinocytes cleaved the 110-nucleotide pre-$tRNA^{Ser}$ substrate into an 82-nucleotide mature tRNA and a 28-nucleotide 5'-sequence (Fig. 2), generating the same fragments as *Dictyostelium discoideum* RNase P (Fig. 3B, lane c). Nuclear RNase P activity eluted at 130–250 mM NH_4Cl through a phosphocellulose (P-11) column (Fig. 3) to which mitochondrial RNase P does not bind (11). The pH and temperature optima were found to be 7.5 and 37˚C, respectively. RNase P from

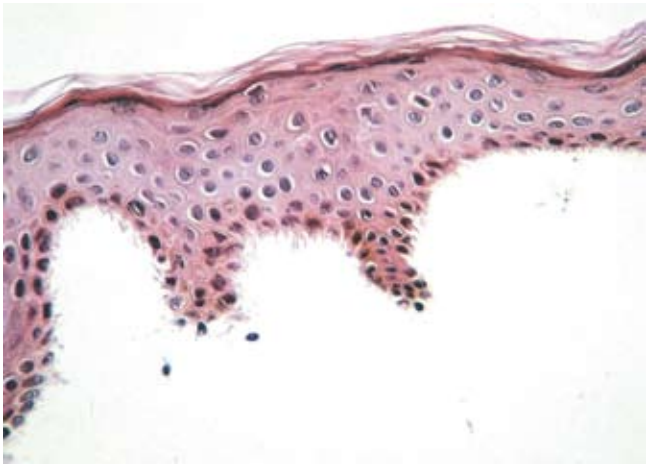

Fig. 1. Complete separation of human epidermis from dermis subsequent to incubation with dispase (haematoxylin-eosin, ×400).

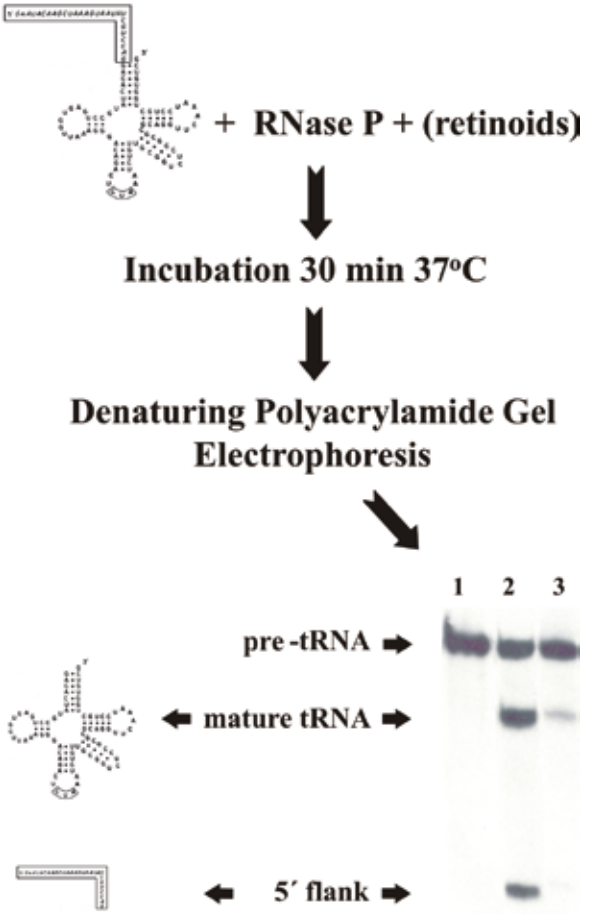

Fig. 2. Assay of ribonuclease P (RNase P) activity in the presence or absence of retinoids. *Top left:* The secondary structure of the pre-tRNASer substrate. *Bottom right:* Autoradiogram of pre-tRNASer substrate cleavage by RNase P: lane 1, pre-tRNA alone; lane 2, RNase P incubated in the absence of all-*trans*-retinoic acid; lane 3, RNase P incubated in the presence of 100 µM all-*trans*-retinoic acid.

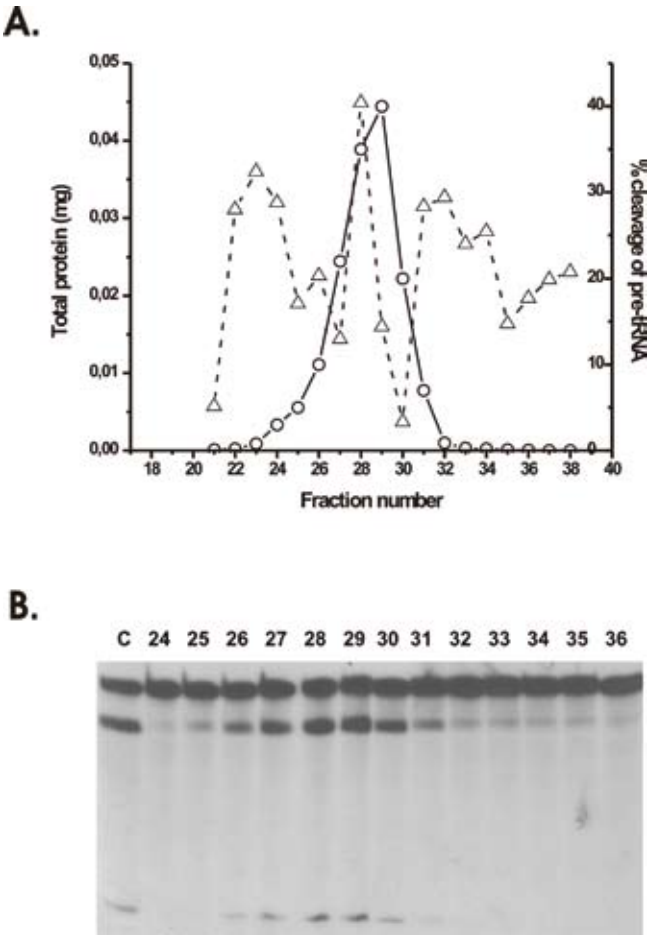

Fig. 3. (A) Epidermal ribonuclease P (RNase P) purification by phosphocellulose (P-11) chromatography; RNase P activity (○) and total protein concentration (△). (B) Autoradiogram of pre-tRNA processing activity from fractions shown in (A). The numbers above the lanes correspond to fraction numbers in the elution profile; lane c, *D. discoideum* RNase P control reaction.

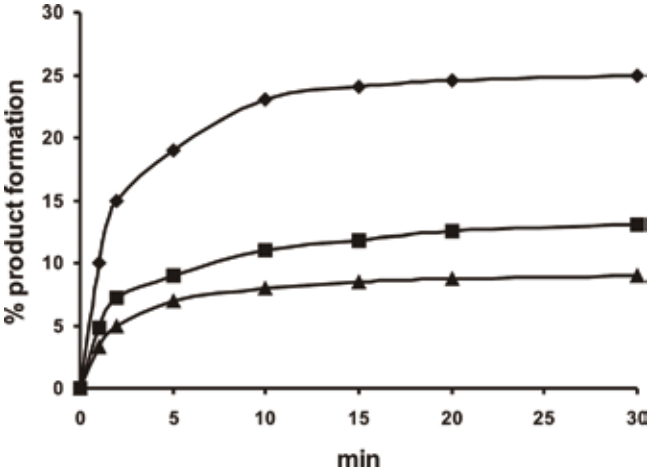

Fig. 4. Kinetics of ribonuclease P (RNase P) cleavage in the absence or presence of all-*trans*-retinoic acid or acitretin. Enzyme assays were carried out at 37°C in 20 μl buffer K in the presence of 2 fmol tRNA substrate and 1.3 μg protein of RNase P and 10% DMSO. (▲) without all-*trans*-retinoic acid or acitretin, (■) with 50 μM all-*trans*-retinoic acid, (◆) with 50 μM acitretin.

normal human epidermal keratinocytes requires both mono- and divalent cations for activity. The optimum activity was found to be at 100 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 -20°C.

In our attempt to assess the effects of retinoids on epidermal RNase activity we incubated the purified enzyme with all-*trans*-retinoic acid and acitretin. The time plots of Fig. 4 clearly show that both retinoids exert an inhibitory effect on RNase P activity. A detailed representation of the dose-response effect of all-*trans*-retinoic acid and acitretin is given in Fig. 5. The IC50 values were 55 μM (Fig. 5A) and 40 μM (Fig. 5B), respectively.

DISCUSSION

During the process of terminal differentiation, among other organelles, nuclei of epidermal keratinocytes are degraded and their DNA and RNA content is hydrolysed to oligonucleotides by nucleases (12, 13). A considerable number of ribonucleases have been isolated from human epidermis (1). Some of them are known to exhibit only hydrolytic activity, whereas others are thought to possess properties of essential importance for keratinocyte adhesion and desquamation (2), terminal differentiation (3), elimination of RNA processing reactions by-products or incorrectly synthesized or damaged RNAs (4) and for the innate mature defence system of epidermis (5).

Human RNase P has previously been isolated and partially purified from HeLa cells (6, 9) and from normal human keratinocyte monolayers (11). In this paper we describe a method by which RNase P activity can be isolated and partially purified from normal human epidermis. It was found that epidermal RNase P has: (*i*) temperature and pH optima that are very similar to those of RNase P isolated from bacterial and other eukaryotic organisms; (*ii*) absolute requirement for mono- and divalent cations; and (*iii*) a susceptibility to retinoid inhibitory action.

Retinoids are involved in gene expression, cell growth and differentiation of epithelial and non-epithelial tissues (14, 15) and exert their pleiotropic effects by interacting with retinoic acid and retinoid X receptors, which act as ligand-inducible transcription factors either affecting retinoid response elements in several genes or modifying the responses of other transcription factors (16). We have previously shown that all-*trans*-retinoic acid and acitretin exert distinct dose-dependent inhibitory effects on RNase P activity isolated from *D. discoideum* (17) and normal human epidermal keratinocytes (NHEK) monolayers (10). In the present study it was found that these retinoids exhibit similar effects on RNase P activity isolated for the first time from a living

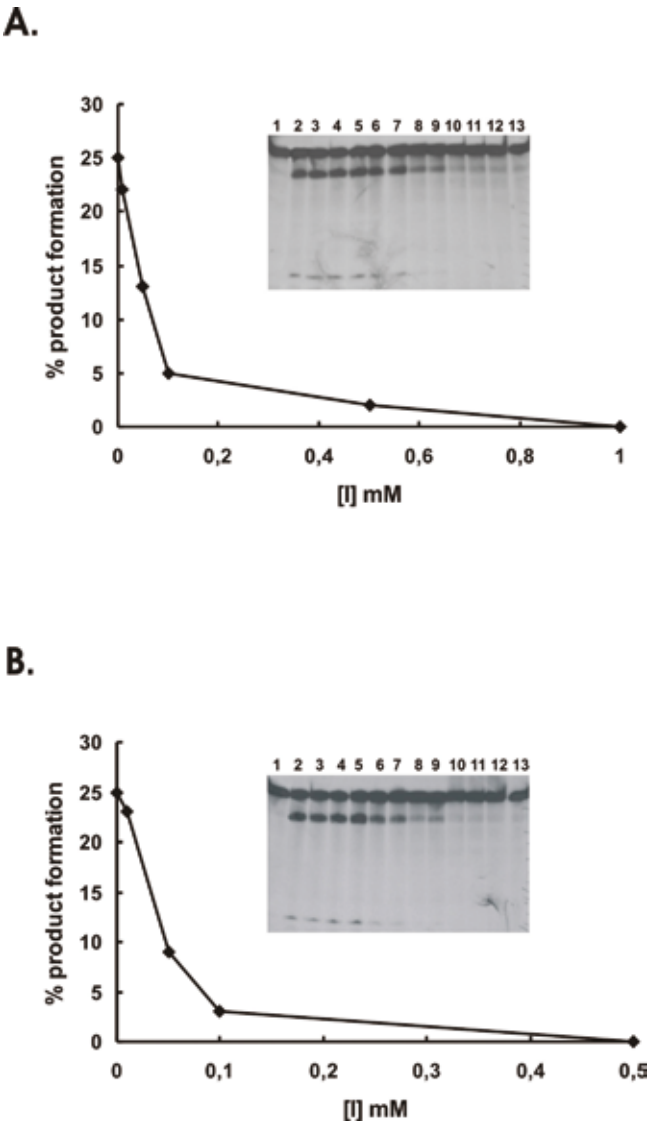

Fig. 5. Dose-response effect of all-*trans*-retinoic acid (top) or acitretin (bottom) on RNase P activity. The diagram shows the RNase P activity as a function of increasing concentrations of retinoids. Insert: autoradiogram of the cleavage of pre-tRNA by RNase P in the presence of retinoids, lane 1, pre-tRNA alone, lanes 2, 3, control (RNase P in the absence of all-*trans*-retinoic acid), lanes 4 – 5, 6 – 7, 8 – 9, 10 – 11, 12 – 13 incubated RNase P in the presence of 0.01, 0.05, 0.1, 0.5, 1.0 mM of either all-*trans*-retinoic acid, or acitretin.

human tissue, i.e. from normal human epidermis. The IC50 values obtained for all-*trans*-retinoic acid and acitretin are similar to those previously obtained with RNase P from *D. discoideum* (80 and 40 μM, respectively) (17) and from NHEK monolayers (60 and 45 μM, respectively) (10). The susceptibility exhibited by the epidermal RNase P toward synthetic retinoid action seems to represent a further feature shared by various eukaryotic RNase P enzymes.

The results of the present study clearly show that the tested retinoids, apart from their well-known suppressive action on protein and DNA synthesis and their regulatory effects on transcription (18), are also capable of affecting tRNA biogenesis by directly inhibiting epidermal RNase P activity. Furthermore, these data provide additional support for the ability of retinoids also to exert their biological effects through mechanisms in which their binding to the corresponding nuclear receptors is not involved. It remains, however, to be elucidated whether these inhibitory effects of retinoids on epidermal RNase P activity are of importance for the mechanisms of their therapeutic action.

The isolation of RNase P activity from human epidermis will enable the investigation of the possible involvement of this ribozyme in the regulation of epidermal differentiation and proliferation and the evaluation of its significance for the pathogenesis of various cutaneous disorders. Furthermore, since RNase P can be used as an effective gene-targeting agent for therapeutic application (19, 20), the results of the present study may facilitate the development of RNase P-based technology for gene therapy of infectious and neoplastic dermatoses.

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