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Mouse eosinophil associated ribonucleases: Mechanism of cytotoxic, antibacterial and antiparasitic activities

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ABSTRACT

Ribonuclease A family is a group of proteins having similar structures and catalytic mechanism but different functions. Human eosinophil granules contain two ribonucleases belonging to the RNase A family, eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN). In mouse, 15 orthologs of EDN and ECP, called mouse eosinophil associated ribonucleases (mEARs) have been reported which are expressed under different pathophysiological conditions. In this study, we have characterized mEAR2, mEAR5, mEAR7 and mEAR11, and compared them with ECP for their catalytic, cytotoxic, antibacterial and antiparasitic activities. All four mEARs had cytotoxic, antibacterial and antiparasitic activities. Generally, mEAR5 and mEAR2 were more cytotoxic than mEAR7, mEAR11 and ECP. The antimicrobial activities of mEAR7 and mEAR5 were higher than those of mEAR11 and mEAR2. The cytotoxic activity appeared to be associated with the basicity and RNase activity of mEARs, whereas no such correlation was observed for antimicrobial activities. The differential selective expression of mEARs under various pathophysiological conditions may be associated with the different biological activities of various mEARs.

1. Introduction

Eosinophils play an important role in innate immune defense. Eosinophils have been shown to have both beneficial and detrimental activities for the host [1–4]. Eosinophils respond to diverse stimuli, including non-specific tissue injury, infections, allografts, allergens and tumors. They are recruited and activated at the site of damage in lung tissue under allergy and asthma and contribute to the characteristic bronchospasm and tissue damage [4–6]. The cytoplasm of the eosinophil contains many granules in which the basic proteins are packed forming crystals [7]. The crystalloid core of the eosinophil granule is composed of major basic protein (MBP), MBP-1 and MBP-2, while its matrix contains eosinophil derived neurotoxin (EDN), eosinophil cationic protein (ECP), and eosinophil peroxidase (EPO) [8,9]. The granule proteins are released upon stimulation of eosinophils, and are responsible for many eosinophil-associated biological activities.

EDN and ECP possess ribonuclease activity and therefore are also termed as eosinophil associated ribonucleases (EARs). They manifest neurotoxic, antihelminthic and antiviral activities [10–15]. Unlike EDN, ECP also shows cytotoxic [16,17] and antibacterial activities [18,19]. ECP is comparatively a more potent helminthotoxin than EDN [14,15]. The antibacterial, cytotoxic and antiparasitic activities of ECP have been ascribed to its highly basic nature and some unique basic residues [19]. The orthologs of human EARs are found in mouse and are termed as mouse EARs (mEARs) [20,21]. Till now 15 mEARs have been identified in mouse genome and phylogenetic analysis established that human and mouse loci share an ancestral gene [22]. Further, the phylogenetic analysis of 38 functional and 23 pseudo genes of the EAR family from 5 rodent species showed a pattern of evolution by a rapid birth-and-death process and gene sorting, a pattern which is shared by the MHC, immunoglobulin and the TCR gene families [22]. It thus appears that host-defense and generation of diversity could be the primary physiological function of the rodent EARs [22]. Also, a large number of divergent EARs may have been specifically adapted to counter distinct rodent pathogens [22].

Despite the existence of 15 unique but related mEARs only transcripts encoding mEAR1 and mEAR2 are detected in significant amounts in peripheral tissues under homeostatic conditions [23]. It is suggested that the remaining mEARs might be selectively expressed under different pathophysiologic conditions [23]. While mEAR1 and mEAR2 have been shown to be predominantly expressed in the lungs of naive mice, pulmonary mEAR11 transcripts, which are absent in naive mice, accumulated as a con-

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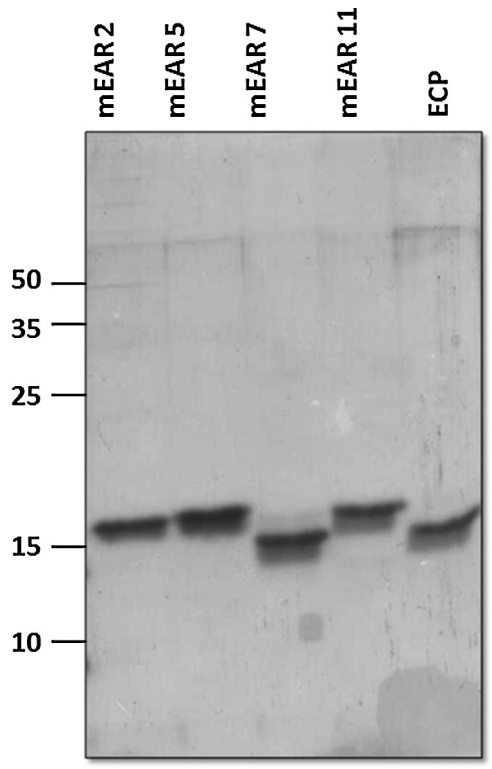

Fig. 1. Purification of mEARs. The purified proteins were analyzed by 14% SDS-PAGE under reducing condition. Molecular weight markers are shown in kDa on the left.

sequence of $T_{(H)2}$ mediated inflammation in the lung [24]. This increased expression of mEAR11 appears to mediated by $T_{(H)2}$ cytokines, as pulmonary instillation of interleukin-4 or interleukin-13 induced the accumulation of mEAR11 transcripts in naive animals [24]. mEAR6 is expressed in response to *Schistosoma mansoni* infection [25]. mEAR1 and mEAR2 have been shown to have antiviral activities against influenza A and pneumonia virus respectively [26,27].

Although, mEARs have a very high degree of similarity among themselves, they have differences in their physicochemical properties. In this study, we have functionally characterized four mEARs, namely mEAR2, mEAR5, mEAR7 and mEAR11 to analyze structure-function relationship in these ribonucleases. The biological activities of mEARs have been compared with that of ECP. All four mEARs were found to have antimicrobial and cytotoxic activities, however there were quantitative differences.

2. Materials and methods

2.1. Cloning of DNAs encoding mEARs

Mouse bone marrow cells, taken from the femur bone were washed thrice with RPMI 1640 and used as the source of RNA. RNA was isolated from cells using RNeasy kit (Qiagen, GmBH) following the manufacturer's protocol, and used for synthesizing the first strand of cDNA. Briefly, 1 μg of RNA in 10 μl diethylpyrocarbonate (DEPC)-treated water was mixed with 4 μl of dNTP mix (2.5 mM each) and 2 μl of random hexamers (0.4 μg/μl). The mixture was heated at 70 °C for 5 min followed by instantaneous transfer to ice. To this mixture, 2.0 μl of 10× reaction buffer, 1.0 μl of M-MuLV reverse transcriptase (200 U/μl) and 1.0 μl of RNase inhibitor was added, and incubated at 42 °C for 60 min. The activity of reverse transcriptase was quenched by incubation at 95 °C for 5 min. The reaction mixture was diluted to 50 μl with DEPC-treated water.

Based on the nucleotide sequences of mouse EARs from NCBI, primers were designed and DNA encoding four mEARs, mEAR2, mEAR5, mEAR7 and mEAR11 were amplified by PCR. The sequences of various PCR primers used are as follows.

mEAR2: 5′ATTTAATATGGATCCCTGGGACAAACCCCTTCCCAG3′
5′AATTTAATTAAGCTTAAAATGTCCCATCCAAGTGAAC3′
mEAR5: 5′ATTTAATATGGATCCCAGCGACCAACCCCTTCCCAG3′
5′AATTTAATTAAGCTTAAAATAGCCGATCCAAGTGAACCG3′
mEAR7: 5′ATTTAATATGGATCCCAGCGACCAACCGCATCCCAG3′
5′AATTTAATTAAGCTTAAAATGTCCCATCCAAGTGAAC3′
mEAR11: 5′ATTTAATATGGATCCCAGCCATTGACCCCCTCCCCG3′
5′AATTTAATTAAGCTTAAAATATCCCATCCAAGTGAACTG3′

A 35 cycle PCR was performed in 100 μl containing 10 mM Tris-HCl buffer, pH 8.3, 0.2 μg cDNA, 50 mM KCl, 1.5 mM $MgCl_2$, 250 μM of each dNTP, 0.2 μM each of the sense and antisense primers and 2.5 U of Taq polymerase. Each PCR cycle consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C and 1.5 min of extension at 72 °C. The amplified products were run on 1% agarose gels and purified using QiaQuick gel extraction kit according to manufacturer's instructions (Qiagen, Hilden, Germany). The purified PCR product was digested with BamHI and HindIII and ligated into pQE-30 vector (Qiagen) digested with the same enzymes. The resulting constructs were verified by DNA sequencing.

2.2. Expression and purification of recombinant mEARs

mEARs were expressed in *E. coli* strain BL21 Codon Plus (λDE3)-RIL. The host cells transformed with pQE-mEAR constructs were cultured in LB broth supplemented with 100 μg/ml ampicillin. For the expression of recombinant proteins, 0.5 mM isopropyl-β-D-thiogalactopyaronide (IPTG) was added when culture A_{600} reached 1.5; the cultures were further incubated for 6 h. The cells were harvested by spinning the culture at 4000 rpm for 15 min at 4 °C. All proteins were found to accumulate in the cell in the form of inclusion bodies. The inclusion bodies were isolated and the recombinant proteins were purified after denaturation and renaturion in vitro as described by Buchner et al. [28]. Briefly, the inclusion bodies were dissolved in 6 M guanidine hydrochloride, reduced with dithioerythritol, and renatured by diluting the protein 100-fold in a refolding buffer containing L-arginine-HCl and oxidized glutathione, and incubating at 4 °C for 48 h. The renatured proteins were dialysed against 20 mM MES buffer, pH 6.0 containing 100 mM urea, and purified to homogeneity using cation exchange and gel filtration chromatography using SP Sepharose and Superdex 75 columns respectively. The proteins were analyzed for their purity by SDS-PAGE.

2.3. Assay of ribonuclease activity of mEARs

The RNase activity of various mEARs was assayed on yeast tRNA following the method of Bond [29]. Serial dilutions of each protein were incubated with 40 μg of yeast tRNA in 10 mM Tris-Cl, pH 7.5 at 37 °C for an hour. The reaction was stopped by the addition of 5% (v/v) perchloric acid and 0.25% (w/v) uranyl acetate. The undigested large molecular weight RNA was precipitated on ice for 30 min and removed by centrifugation at 15000 × g for 10 min. The acid soluble product, present in the supernatant, was quantified by measuring the absorbance at 260 nm. The activity was expressed as $\Delta Absorbance_{260}$/minute/mg protein.

2.4. Cytotoxicity assay

The cytoxicity of mEARs and ECP was analyzed on A431, a human epidermoid carcinoma cell line; Hela, a human adenocarcinoma cell line; Jurkat, a human T-cell leukemia cell line; J774A.1, a mouse monocyte-macrophage cell line, and U373, a