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Diminished expression of an antiviral ribonuclease in response to pneumovirus infection in vivo

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Abstract

The mouse eosinophil-associated ribonucleases (mEars) are species specific, divergent orthologs of the human antiviral RNase A ribonucleases, eosinophil-derived neurotoxin (RNase 2) and eosinophil cationic protein (RNase 3). We show here that mEar 2 is also an antiviral ribonuclease, as micromolar concentrations promote a ~sixfold reduction in the infectivity of pneumonia virus of mice (PVM) for target respiratory epithelial cells in vitro. Although initially identified as a component of eosinophilic leukocytes, mEar 2 mRNA and protein were also detected in lung tissue accompanied by enzymatically active mEar 2 in bronchoalveolar lavage fluid (BALF). At $t = 3$ days post-inoculation with PVM (strain J3666), we observed the characteristic inflammatory response accompanied by diminished expression of total mEar mRNA and protein in lung tissue and a corresponding fivefold drop in ribonuclease activity in BALF. No change in mEar expression was observed in response to infection with PVM strain 15, a replication-competent strain of PVM that does not elicit a cellular inflammatory response. However, mEar expression is not directly dependent on inflammation per se, as diminished expression of mEar mRNA and BAL ribonuclease activity were also observed in PVM-infected, inflammation-deficient, MIP-1α −/− mice. We propose that this mechanism may represent a novel virus-mediated evasion strategy, with a mechanism that is linked in some fashion to virus-specific pathogenicity.
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Keywords: Pneumovirus; Ribonuclease; Inflammation

1. Introduction

Ribonucleases, broadly defined as enzymes that catalyze the degradation of polymeric RNA, are ubiquitous among all known forms of life and participate in a wide range of biologic events in both intracellular and extracellular locales (D'Alessio and Riordan, 1997; Nicholson, 2001). While the functions of specific RNA processing ribonucleases of prokaryotes have been defined at the molecular level (Nicholson, 1997), the biology of eukaryotic, particularly mammalian ribonucleases remains less clear. Among the emerging lines of thought is the possibility that eukaryotic ribonucleases function in various ways to promote innate antiviral host defense; there is a growing body of evidence suggesting this as among the more important roles of secretory ribonucleases of plants (Bariola and Green, 1997).

The first report of antiviral activity by a mammalian ribonuclease was in 1956, in which LeClerc reported that adding "ribonuclease" (probably bovine RNase 1) to the culture medium inhibited the synthesis of hemagglutinating influenza virions (Le Clerc, 1956). More recently, several groups have characterized the antiviral activity of RNase L, an intracellular ribonuclease that is also an interferon-response gene (Silverman, 1997; Torrence, 1999). Although not from a mammalian source, several of the ribonuclease-cytotoxins isolated from oocytes of the bullfrog, *Rana pipiens*, also have activity against HIV-infected mammalian cells in vitro (Irie et al., 1998; Saxena et al., 1996).

Our work has focused on the antiviral properties of ribonucleases from the RNase A superfamily, the only enzyme family restricted to vertebrate life forms (Lander et al.,

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2001). Each ribonuclease of this divergent superfamily is secreted via an amino-terminal signal peptide and maintains invariant structural cysteines and a His–Lys–His catalytic triad (Beintema and Kleineidam, 1998). The genes encoding eight distinct human RNase A ribonucleases are all found on human chromosome 14 and fall phylogenetically into five distinct lineages (Zhang et al., 2002). The focus of our work has been the "eosinophil-associated" ribonuclease lineage, which includes the primate proteins eosinophil-derived neurotoxin (EDN/RNase 2) and eosinophil cationic protein (ECP/RNase 3). ECP is found only in eosinophilic leukocytes, while EDN is also expressed in liver, lung, and spleen (Futami et al., 1997). Interestingly, EDN and ECP are the most rapidly evolving functional coding sequences known among primates (Rosenberg et al., 1995), with clear evidence for positive (Darwinian) selection contributing to diversification of ECP (Zhang et al., 1998). We have shown that EDN and ECP reduce the infectivity of the single-stranded RNA virus pathogen, respiratory syncytial virus (RSV) for human target cells in vitro (Domachowske et al., 1998a,b); Lee-Huang et al. (1999) have demonstrated that EDN has similar efficacy against HIV.

The study of these ribonucleases, their responses, and their role in antiviral host defense in vivo presents two important challenges. One challenge relates to the evolutionary divergence of these ribonucleases. The highly divergent orthologs of EDN and ECP in rodents are species-limited clusters known as eosinophil-associated ribonucleases (Ears; Larson et al., 1996; Singhania et al., 1999; Zhang et al., 2000) that are similar to one another within each rodent species, but have at most ~50% amino acid sequence homology to their human counterparts (Fig. 1). The recent report on the complete genome sequence of *Mus musculus* documents the existence of 11 mEars in this species-specific cluster, although the authors note that this number is likely to be an underestimate (Mouse Genome Sequencing Consortium, 2002). Of the 15 mEars identified by us and by the Lees' laboratories, we find that mEars 1 and 2 are expressed in eosinophils and elsewhere (Larson et al., 1996; Cormier et al., 2001), while mEar 11 was detected in mouse lungs in response to allergic provocation (Cormier et al., 2002).

A second complication relates to the complex evolution of virus pathogens. The human pneumovirus pathogen, RSV, is not a natural mouse pathogen and has little to no infectivity in rodent species. As such, RSV is not suitable for studies of innate responses to virus infection. To address this issue, we have developed a model for study utilizing the phylogenetically related pneumovirus, pneumonia virus of mice (PVM). In response to inoculation with as few as 10 plaque-forming units (pfu) of PVM (strain J3666) wild-type mice develop signs and symptoms similar to that of the most severe forms of RSV infection in humans (Domachowske et al., 2000a,b, 2002a,b), with significant morbidity and mortality accompanied by virus titers reaching 10^7 to 10^8 pfu/g lung tissue. Virions isolated from mouse lungs can infect other mice,

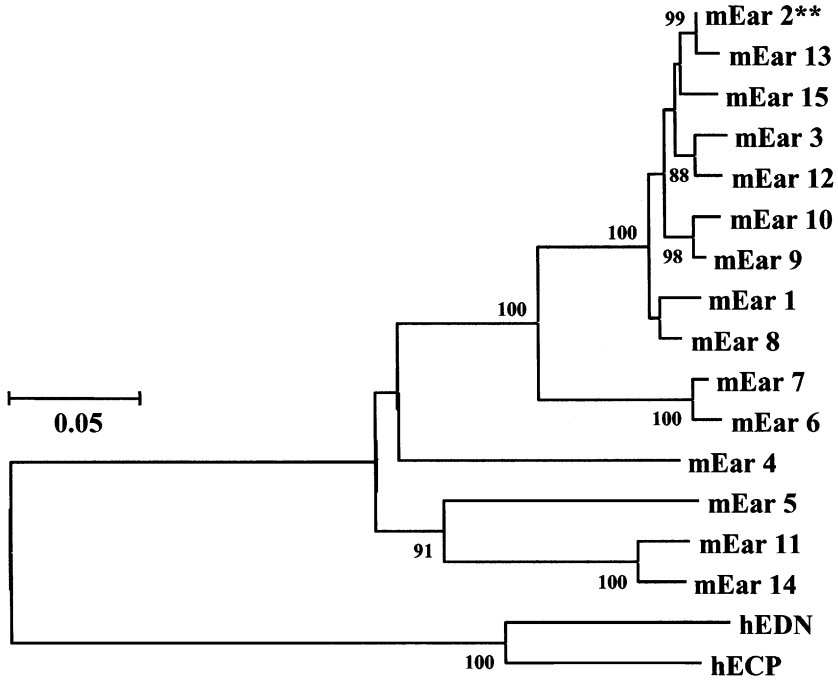

Fig. 1. Neighbor-joining phylogenetic tree. The tree demarcates distance relationships within the *M. musculus* eosinophil-associated ribonuclease (mEar) cluster as well as those between the mEars and their human orthologs, eosinophil-derived neurotoxin (EDN, RNase 2) and eosinophil cationic protein (ECP, RNase 3). Within the mEar cluster, among the closest relationships between two nucleotide sequences is mEar 2 to mEar 13 (99%) and among the most distant, mEar 2 to mEar 11 (79%). Genbank accession numbers are listed in Rosenberg and Domachowske (2001b). Tree and bootstrap replications (500; at branch points) were constructed as per MEGA Evolutionary Genetics Program (Kumar et al., 2001).

thus fulfilling the elements of Koch's postulates for a true infectious agent in this host.

Given these tools, we are now in a position to understand the biology of these ribonucleases in uninfected and PVM-infected mice, and to explore their contribution to innate host defense against RNA virus pathogens.

2. Methods

2.1. Mice, viruses, and cell lines

Six- to 10-week-old mice were used in all experiments described. Breeding pairs of MIP-1α −/− mice (Cook et al., 1995) were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained at SUNY Upstate Medical University. All procedures were reviewed and approved by the National Institutes of Health Animal Welfare Review Board (protocol LHD 8E) or the Committee on Humane Use of Animals, SUNY Upstate Medical University (CHUA #634). Virus stocks and virus titers were determined as described previously (Domachowske et al., 2000a,b). Cells of the mouse epithelial cell line LA4 were also obtained from ATCC and maintained at 32 °C, 5% CO_2 in cIMDM.

2.2. Mouse procedures

Anesthetized mice were inoculated as described above with 60–200 pfu in 80 μl IMDM. Virus titers on days indicated were corrected for protein concentration as determined by Bradford assay. Bronchoalveolar lavage fluid (BALF) was obtained from mice on days indicated by trans-tracheal cannulation and instillation of 0.8 ml phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 1 mM ethylenediaminetetraacetic acid (EDTA), repeated once for a total recovery of ∼1.5 ml BAL fluid. Cellular contents and debris were removed by centrifugation, and BAL fluids were flash-frozen as above and stored at −80 °C prior to assay. Removal and processing of lungs for RNA extraction was as described previously (Domachowske et al., 2002a). Whole lung extracts were prepared by immersing lung tissue in SDS/triton-containing buffer, blade homogenization, and centrifugation of insoluble debris.

2.3. Northern blotting, RT-PCR, and DNA sequencing

A mouse multi-tissue Northern blot was purchased from Clontech (Palo Alto, CA) and probed with the 471 bp full-length cDNA sequence of mEar 2 as described, stripped, and reprobed with beta-actin as previously described (McDevitt et al., 2001). In order to determine the distribution of cross-hybridizing mEar mRNAs represented in lung, RNA was prepared from lung tissue as described above and treated with DNase I to remove any traces of genomic contamination. DNase I-treated samples were subjected to two RT-PCR amplifications with Pfx proofreading polymerase (Invitrogen) and nested primers as follows:

outside primers: 5′-CCCTGATTTCCAGGACAACCA-3′ and 5′-GACAGGTTTCACGAGGACATG-3′

inside primers: 5′-CTAAAATGTCCCATCCAAGTGAAC-3′ and 5′-ATGGGTCCGAAGCTGCTTGAGTCT-3′

RT-PCR products were gel-purified and subcloned into the pZERO BLUNT vector (Invitrogen) and subjected to automated dideoxy sequencing. No amplification products were detected in minus RT controls.

2.4. Preparation and purification of recombinant mEar 2

Recombinant mEar 2 was prepared in *Escherichia coli* in the pFLAG-CTS expression vector (Sigma-Aldrich, St. Louis, MO) and quantity of protein determined as described previously for other RNase A superfamily ribonucleases (Rosenberg et al., 1995; Rosenberg and Dyer, 1995, 1997; Rosenberg and Domachowske, 2001a,b).

2.5. Indirect immunofluorescence staining of PVM-infected LA4 cells

This assay is based on the overnight virus titration assay previously described (Domachowske and Bonville, 1998). Mouse epithelial LA4 cells were seeded at 3×10^4 cells per coverslip placed within a sterilized one-dram vial (Fisher Scientific, Pittsburgh, PA) in cIMDM. At 48 h post-plating (one doubling time, cells at 6×10^4 per coverslip), the culture medium was replaced with fresh medium containing various concentrations (0–3 μM) recombinant mEar 2 followed 5 min later by 3000 pfu PVM strain J3666 (MOI = 0.05). In experiments including ribonuclease inhibitor (RI; 40 U/μl; Roche Molecular Biochemicals, Indianapolis, IN), the inhibitor was added to the medium + mEar 2, which was then added to the cell monolayer after a 5-min equilibration at room temperature. The RI + mEar 2-containing medium was then added to the cell monolayer, and then the virus added after a 5-min incubation as above. After spin amplification (500 g for 60 min at 25 °C), the vials were placed at 32 °C in a 5% CO_2 incubator to permit optimum virus growth. After 72 h at 32 °C, the medium was removed, the coverslips washed four times in PBS and then fixed with cold absolute acetone (10 min), air-dried, and stored in PBS + 1% normal goat serum (NGS; Pierce Chemical Co., Indianapolis, IN) at 4 °C prior to staining. Trypan blue staining of LA4 cells treated with 3 μM mEar 2 alone or 3 M mEar 2 + 200 U RI at $t = 72$ h was 2.3 ± 0.6 and 1.8 ± 0.5%, respectively ($n = 3$ replicates). To stain, coverslips in PBS + 1% NGS were incubated for 2 h at 37 °C, followed by a 1–2-h incubation in a 1:500 dilution of rabbit polyclonal anti-virus N protein (specificity as shown previously; Domachowske

et al., 2002b) in PBS + 1% NGS also at 37 °C. Stained coverslips were washed four times with PBS, and then incubated at 37 °C for 2–16 h shielded from light in a 1:500 dilution of fluorescein-isothiocyanate (FITC)-tagged goat anti-rabbit IgG (Pierce). After a final four PBS washes, the coverslips were removed from the dram vials, mounted inverted on glass slides in mounting medium (Difco, Detroit, MI) and evaluated by fluorescence microscopy. The number of fluorescent foci (equivalent to the number of infected cells) per coverslip was determined by averaging the number of foci counted on the view-fields of the vertical and horizontal diameters and multiplying by 28.33, a conversion factor determined by the relative areas of the counted and complete coverslip fields. Each point represents the average ± standard error of triplicate samples.

2.6. Detection of mEar protein in BAL fluid

Samples of BAL fluid (1.5 ml) from uninfected mice were equilibrated overnight at 4 °C with pre-swollen heparin–Sepharose resin (Pharmacia). Resin was harvested by microfuge centrifugation, and bound proteins were eluted into SDS-containing reducing sample buffer (Invitrogen) and subjected to gel electrophoresis (14% acrylamide, Tris–glycine) and transfer to nitrocellulose. Non-specific binding to the nitrocellulose membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) prior to probing the membrane with a 1:300 dilution of polyclonal anti-mEars antiserum or 1:200 dilution of monoclonal rat anti-mEar antibody (both generous gifts of Drs. James and Nancy Lee) followed by alkaline-phosphatase conjugated goat anti-rabbit IgG or goat anti-rat IgG and developing reagents (BioRad). The loading controls were probed directly with alkaline-phosphatase conjugated goat anti-mouse IgG and followed by developing reagents.

2.7. Ribonuclease assay

Briefly, recombinant protein (2 μl, 0.36 pmol) or BAL fluid (20 or 50 μl) was added to 0.8 ml of 40 mM sodium phosphate, pH 7.4, all prepared at room temperature. The reaction was initiated with the addition of 10 μl of a stock of 4 mg/ml tRNA (Sigma-Aldrich), and reaction stopped at $t = 20$ min by the addition of ice-cold 20 mM lanthanum nitrate and 3% perchloric acid. A $t = 0$ spectrophotometric blank was prepared by adding stop solution to the buffer and ribonuclease mix prior to addition of tRNA. Acid-insoluble substrate (polymeric tRNA) was separated from acid-soluble product (ribonucleotides) by centrifugation and the amount of product per unit time (pmol/s) determined from the OD of the clarified supernatant measured at 260 nm as described (Rosenberg and Domachowske, 2001a,b). All assays were done in triplicate; the data on BAL fluid represent triplicate assays of samples from three mice.

2.8. Immunodepletion of BAL fluid samples

One microliter each of either rabbit-anti-mEars antiserum, rabbit anti-human eosinophil major basic protein antiserum (control), or PBS was added to each of three 150 μl samples of BAL fluid as described above. After 1-h equilibration at 4 °C, 2 μl of protein G agarose (Boehringer Mannheim, Indianapolis, IN) was added, and samples were equilibrated as above for another 1 h. Immunoprecipitated proteins were removed by centrifugation, and ribonuclease assays as described above were performed on the clarified BAL fluid samples.

2.9. Microarray analysis

The data shown here were taken from an extensive study of gene expression in response to pathogenic and non-pathogenic strains of PVM done at the Microarray Core Facility in Rochester, NY. Sample preparation, controls, probe, and data analysis were as described in Domachowske et al. (2002a).

2.10. Statistical and evolutionary analysis

Statistical presentation including standard deviation, standard error of the mean, one- and two-tailed *t*-tests and chi-squared analysis were as per the algorithms included in Microsoft Excel. The neighbor-joining tree and bootstrap analysis was as per MEGA (Kumar et al., 2001) available for download from the Internet at www.megasoftware.com.

3. Results

3.1. Recombinant mEar 2 has ribonucleolytic and antiviral activity in vitro

Recombinant mEar 2 is ribonucleolytically active (Fig. 2); 0.5 pmol/ml recombinant mEar 2 generated 1.8 ± 0.04 pmol acid-soluble ribonucleotides per second from acid-insoluble tRNA substrate at physiologic pH (7.4). This enzymatic activity is inhibited in a dose-dependent fashion by placental RI, a protein inhibitor that interacts tightly (of the order of 10^{-14} to 10^{-16} M) and specifically with mammalian RNase A ribonucleases (Hofteenge, 1997). Under these reaction conditions, the IC_{50} is estimated at 0.25 U RI per fmol recombinant mEar 2.

The antiviral activity of recombinant mEar 2 was determined by a quantitative immunofluorescence assay (Domachowske and Bonville, 1998; see Section 2). A field of PVM-infected, immunofluorescence-stained cells is shown in Fig. 3A, with the corresponding phase contrast field shown in Fig. 3B. The antiviral activity of purified recombinant mEar 2, determined as reduction in the number of immunofluorescent foci per monolayer, is shown in Fig. 3C. The antiviral activity is dose-dependent over the

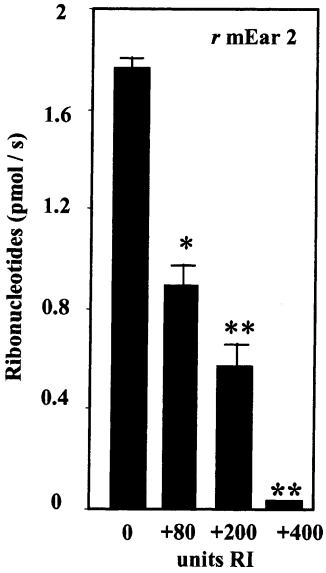

Fig. 2. Ribonuclease activity of recombinant mEar 2. Acid-soluble ribonucleotides generated per time (pmol/s) from acid-insoluble polyribonucleotide substrate by 0.5 pmol/ml recombinant mEar 2 (±S.E.) alone (−) and in the presence of 80, 200, or 400 U placental ribonuclease inhibitor (RI), respectively. Statistically significant differences, $^{*}P < 0.0005$, $^{**}P < 0.0001$ when compared to the no-inhibitor (−) control.

range tested (0–3 μM) and statistically significant differences were observed in comparisons against both the 0 μM mEar 2 control as well as against the controls that include 200 U RI; at the 3 μM concentration, we observed a three- to sixfold reduction in the number of fluorescent foci when compared to either of the relevant controls.

3.2. Detection of mEar 2 in uninfected mouse lung tissue

Although the mEars were originally isolated from mouse eosinophils (Larson et al., 1996), a significant quantity of mRNA encoding these proteins was detected in lung tissue (Fig. 4A). The mouse multi-tissue Northern blot shown was probed with the 471 nucleotide coding sequence of mEar 2 under conditions that permit cross-hybridization of mEars 1, 2, 3, 8, 9, 10, 12, 13, and 15 (all within 97–99% nucleotide sequence similarity). We also detected a substantial quantity of mEar mRNA in spleen tissue, determined by RT-PCR and sequence analysis to be a mixture of mEar 1 (7%) and mEar 2 (93%). In order to determine which of the nine mEars of this cross-hybridizing subcluster are expressed in lung tissue, we performed RT-PCR on purified mouse lung RNA using primers designed to amplify any or all of the cross-hybridizing mEars (see Section 2). We sequenced >100 independent amplicons from amplification reactions performed on three independent samples of mouse (Balb/c) lung mRNA; all (100%) encoded mEar 2. Identical results (100% mEar 2) were determined from lungs of C57BL/6J mice (~80 amplicons). These results differ somewhat from that reported by Cormier et al. (2002), who

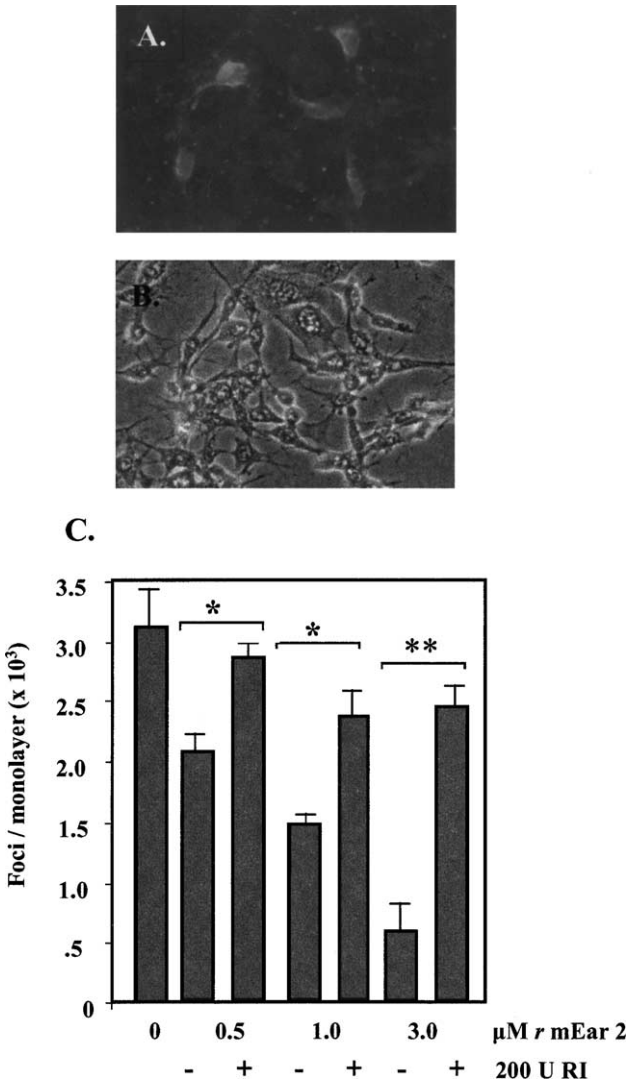

Fig. 3. The antiviral activity of mEar 2 was determined by a modification of the standard quantitative immunofluorescence assay (Domachowske and Bonville, 1998). (A) Indirect immunofluorescence staining of mouse epithelial LA4 cells infected with PVM strain J3666, and stained with polyclonal anti-viral N protein. (B) Corresponding phase contrast image. Both images were photographed at initial magnification of 40×. (C) Antiviral activity of mEar 2, fluorescent cells (foci) per monolayer ± S.E. The observed decreases were significant at $^{*}P < 0.05$ or $^{**}P < 0.005$ when compared to either each respective +200 U RI control or to the 0 μM mEar 2 control.

reported instead a mixture of mEar 1 (13%) and mEar 2 (87%) in lung tissue of C57BL/6J mice at baseline, which in their case represented mice that had been treated as controls with intraperitoneal and nebulized saline. It is possible that the C57BL/6J mouse has undergone some drift, as the mice were obtained from separate breeding facilities (Jackson Laboratories versus in-house NIH breeding). Perhaps even a relatively mild physiologic perturbation, such as nebulized saline, results in altered expression of these ribonucleases. At this point, the reason behind this discrepancy remains unclear.

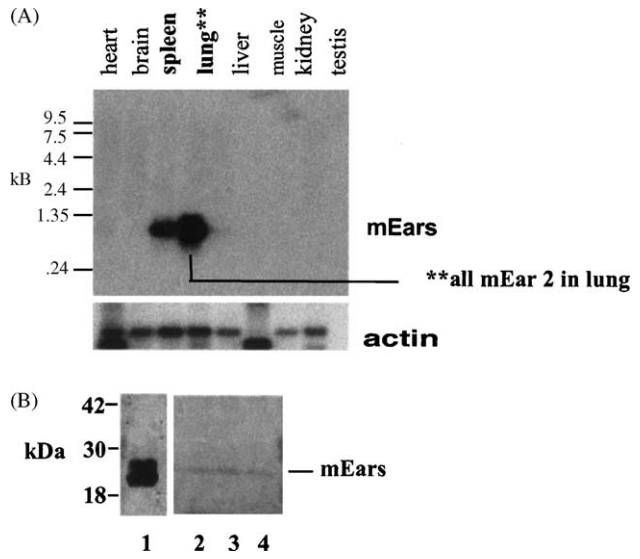

Fig. 4. (A) Mouse multi-tissue Northern blot. The blot shown was probed with the 471 bp cDNA encoding mEar 2 under conditions that result in cross-hybridization of mEars 1, 2, 3, 8, 9, 10, 12, 13, and 15. Hybridization with a beta-actin cDNA probe to document relative loading is shown below. To determine the precise distribution of mEars in lung tissue, >100 amplicons derived from three independent samples of mouse lung RNA were sequenced; all (100%) encoded mEar 2. (B) Western blot of bronchoalveolar lavage (BAL) fluid samples. Samples probed with anti-mEar polyclonal antiserum. Lane 1, recombinant mEar 2 prepared in Sf9 cells (McDevitt et al., 2001); lanes 2–4, BAL fluid concentrates from three mice.

The Western blot in Fig. 4B includes three independent samples of concentrated mouse BAL fluid probed with a rabbit-polyclonal antibody prepared against purified eosinophil-derived mEars (a generous gift of Drs. James and Nancy Lee). Mouse Ears detected in BAL fluid migrate as a single band with an apparent molecular mass of ~22 kDa.

The ribonuclease activity of three (unconcentrated) BAL fluid samples is shown in Fig. 5A, with 20 μl BAL fluid in a final volume of 0.8 ml generating acid-soluble ribonucleotides at a rate of 2–3 pmol/s. This activity is inhibited in a dose-dependent fashion by placental RI (IC_{50}~2 U RI/μl BAL fluid). The immunodepletion experiment in Fig. 5B indicates that virtually all (99%) of the ribonuclease activity detected in BAL fluid can be attributed to mEars.

3.3. Differential expression of mRNA encoding mEar 2 in response to pneumovirus infection

As part of a more extensive evaluation of gene expression in mouse lungs in response to pneumovirus infection (Domachowske et al., 2002a), duplicate microarray analysis of two independent isolates of RNA from sham-infected, PVM (pathogenic) strain J3666-infected, and PVM (non-pathogenic) strain 15-infected mice on day 3 post-inoculation yielded a reproducible and internally consistent twofold decrease in total (cross-hybridizing) mEar expression in response to infection with PVM strain J3666,

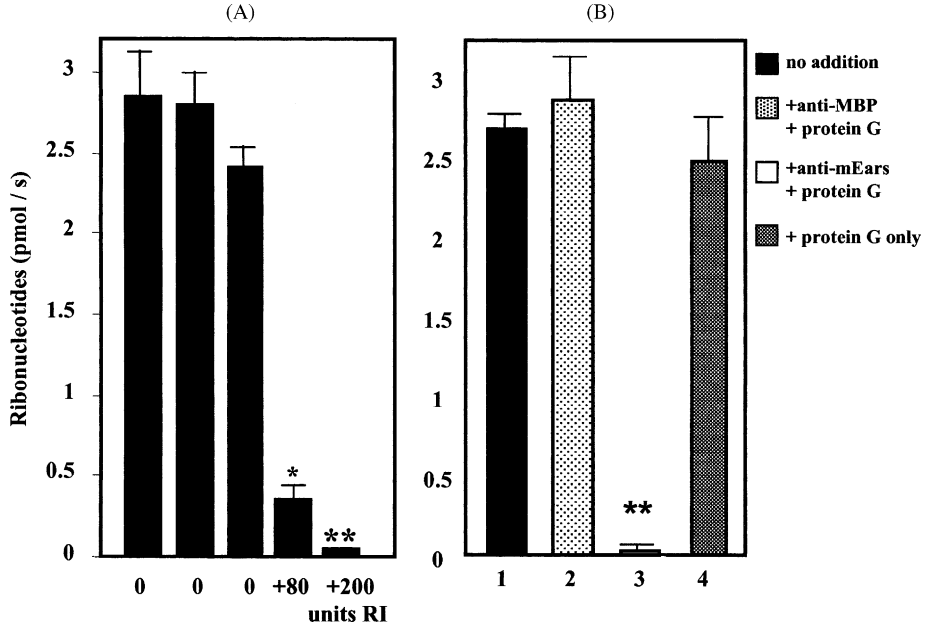

Fig. 5. (A) Ribonuclease activity in BAL fluid. Acid-soluble ribonucleotides generated per time (pmol/s) from acid-insoluble polyribonucleotide substrate by the catalytic activity present in 20 μl unconcentrated BAL fluid (±S.E.). Activity from BAL fluids from three independent samples alone (0) or with the addition of 80 or 200 U placental ribonuclease inhibitor (RI); statistically significant differences, $^{*}P < 0.005$, $^{**}P < 0.0005$, respectively, when compared to activities observed in any of the three samples without RI. (B) Immmunodepletion of BAL fluid with anti-mEars polyclonal antiserum. Lane 1, no addition; lane 2, + 1 μl rabbit anti-human MBP antiserum (control) + 2 μl protein G agarose; lane 3, + 1 μl rabbit anti-mEars antiserum + 2 μl protein G agarose; lane 4, + protein G agarose only. Statistically significant differences, $^{**}P < 0.0005$ when compared to any of the three control bars (lane 1, 2, or 4).

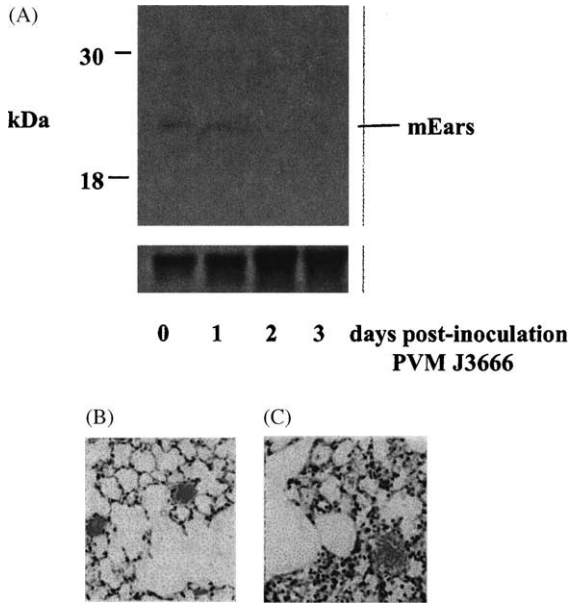

Fig. 6. (A) Western blot of lung tissue homogenates. Samples from uninfected (day 0) and PVM strain J3666-infected (days 1–3) mice probed with rat anti-mEars (MT-32.1) monoclonal antibody (cross-reacts with all mEars tested); loading control (below) probed with goat anti-mouse IgG. (B and C) Microscopic pathology. H&E-stained lung tissue sections from day 0 uninfected (B) and day 3 infected (C) mice, demonstrating the profound inflammatory response to the J3666 strain of this virus.

a reduction that falls in range with similar alterations in expression of genes encoding crucial host defense proteins including interferon-β (+2.4-fold), eotaxin (+2.8-fold), catalase (−2.1-fold), and the TNF-RE binding protein (−2.8-fold). Infection with the non-pathogenic PVM strain 15 resulted in no change in mEar expression. The decrease in mRNA expression in response to strain J3666 infection is reflected in a parallel overall net decrease in mEar protein detected in lung tissue as shown by Western blot in Fig. 6A. Fig. 6B and C is representative histology of lung tissue from sham-infected and PVM strain J3666-infected mice, respectively, at day 3 post-inoculation with 200 pfu of virus, demonstrating the profound inflammatory response to this strain (Domachowske et al., 2000b). Lung RNA from uninfected and strain J3666-infected (2 and 3 days post-inoculation) mice were subjected to RT-PCR amplification with cross-hybridizing primers (see above and Section 2); the results (Table 1) indicate that not only is there an overall net decline in mEar expression (Fig. 6A), but a change in the distribution, with mEar 2 reduced from 100% on day 0 to 57% of the total mEar cDNAs detected on day 3 ($^{*}P < 0.0001$ by chi-square). Since mEar 1 mRNA contributes to the signal detected in the microarray analysis, the overall decline in mEar 2 mRNA expression compared with the uninfected mouse samples likely to be somewhat larger than twofold, calculated at $2 \times 100/43$, or 4.7-fold.

3.4. Ribonuclease activity and pneumovirus infection

The reduction in mEar mRNA and protein expression in response to strain J3666 infection is paralleled by a prominent and significant reduction in BAL fluid ribonuclease activity (Fig. 7A). Prior to inoculation, 50 μl BAL fluid under standard reaction conditions generated 7.8 ± 0.9 pmol ribonucleotides/s, a rate that declined rapidly by four- to fivefold, to 1.7 ± 0.07 pmol ribonucleotide/s by day 3 post-inoculation. This decline persisted through day 4 ($^{*}P < 0.005$) correlating with virus titers reaching $\sim 10^6$ and 10^7 pfu/g lung tissue on days 3 and 4, respectively. Consistent with the results from microarray analysis, we observed no reduction in ribonuclease activity in BAL fluid of mice infected with the non-pathogenic PVM strain 15, despite indistinguishable rates of virus replication in lung tissue (Fig. 7B; Domachowske et al., 2002a). In our previous study, we demonstrated that there were very few of the characteristic antiviral inflammatory responses to PVM strain 15 infection, including no production of antiviral interferon-β, no local production of the proinflammatory CC-chemokine MIP-1α, and no cellular inflammatory response (Fig. 7C and D).

3.5. Differential mRNA expression of mEar 2 in inflammation-deficient mice

In previous work, we identified the CC-chemokine MIP-1α as the master-switch promoting the antiviral inflammatory response, as mice devoid of either MIP-1α or its receptor CCR1 cannot recruit inflammatory cells to the lung in response to PVM strain J3666 infection (Fig. 8A and B; Domachowske et al., 2000b). In order to determine the nature of the stimulus—whether differential expression of mEars is a response to virus replication per se or to the MIP-1α-mediated proinflammatory sequelae, we evaluated microarray data performed as described above comparing

Table 1
Sequences of cDNAs isolated from lung RNA of uninfected (day 0) and PVM J3666-infected (days 2 and 3) mice

Day	# cDNAs sequenced	# mEar 2	mEar 2 (%)	# mEar 1	mEar 1 (%)
0	27	27	100	0	0
2	34	33	97	1	3
3	16	9*	57	7*	43

The cDNAs were isolated by RT-PCR from RNA from lungs combined from two to three mice per point using primers specific for all possible known Ears (see Fig. 1) as described in Section 2.

* $P < 0.0001$ vs. day 0 (modified chi-square).

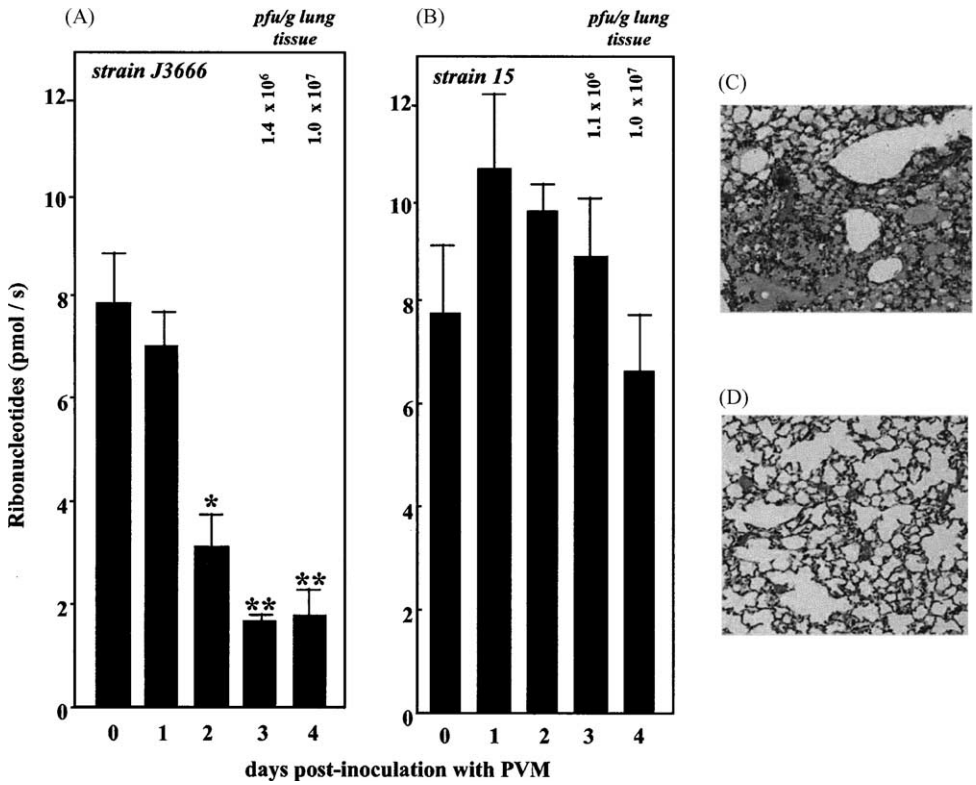

Fig. 7. (A and B) Ribonuclease activity in BAL fluid of infected mice. Acid-soluble ribonucleotides generated per time (pmol/s) from acid-insoluble polyribonucleotide substrate by the ribonucleolytic activity present in 50 μl unconcentrated BAL fluid (±S.E.) obtained from uninfected (day 0) and either PVM strain J3666-infected (A), or PVM strain 15-infected mice (B) on days 1–4 post-inoculation. The infectivity of each virus, as determined by pfu/g lung tissue are as shown, and are analogous to those reported previously (Domachowske et al., 2002a) documenting the indistinguishable rates of replication of these two strains. The observed decreases were determined to be statistically significant at $^{*}P < 0.05$ and $^{**}P < 0.005$ when compared to the activity measured at day 0. (C and D) Microscopic pathology. H&E-stained lung tissue sections from PVM strain J3666-infected (C) and PVM strain 15-infected (D) mice on day 5 post-inoculation, demonstrating the differential inflammatory responses to these two strains of virus.

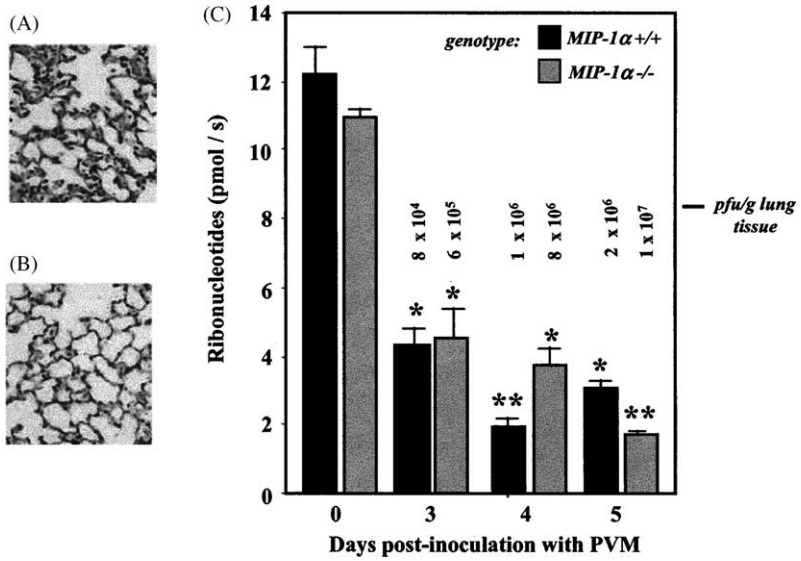

Fig. 8. (A and B) Microscopic pathology. H&E-stained lung tissue sections from day 3 PVM strain J3666-infected wild-type (A) and MIP-1α −/− inflammation-deficient (B) mice, demonstrating the differential inflammatory responses in these two strains of mice. (C) Ribonuclease activity in BAL. Acid-soluble ribonucleotides generated per time (pmol/s) from acid-insoluble polyribonucleotide substrate by the ribonucleolytic activity present in 60 μl BAL fluid (±S.E., three mice per time point) obtained from mice prior to inoculation (day 0) and on days 3–5 after inoculation. The infectivity of the virus in both wild-type and MIP-1α −/− mice, as determined by pfu/g lung tissue is as shown. The observed decreases were determined to be significant at $^{*}P < 0.005$ and $^{**}P < 0.0005$ when compared to the activity measured at day 0 for each mouse strain.

Table 2
Sequences of cDNAs isolated from lung RNA of uninfected (day 0) and PVM J3666-infected (day 2) MIP-1α −/− mice

Day	# cDNAs sequenced	# mEar 2	mEar 2 (%)	# mEar 1	mEar 1 (%)
0	7	7	100	0	0
2	9	0*	0	8*,a	89

The cDNAs were isolated by RT-PCR from RNA from lungs combined from two to three mice per point using primers specific for all possible known Ears as described in Section 2.

* $P < 0.0001$ vs. day 0 (modified chi-square).

[a] One non-mEar 1 sequence identified.

the responses of wild-type mice (sham-infected and PVM J3666-infected) to sham-infected and PVM J3666-infected MIP-1α −/− mice. The results obtained were indistinguishable from those determined for wild-type mice, with a ∼twofold down-regulation of mEar mRNA expression in infected MIP-1α −/− mice, together with the change in distribution of amplified cDNAs (Table 2). Similarly, a three- to fivefold reduction in ribonuclease activity in response to PVM J3666 infection was observed in BAL fluids from both MIP-1α −/− and MIP-1α +/+ mice (Fig. 8C), indicating that the diminished expression of mEar is a direct response to virus infection, and not dependent on the MIP-α-mediated inflammatory response pathway.

4. Discussion

In this manuscript, we explore the biology of the Ears and their relationship to the pathogenesis of infection with the pneumovirus, PVM in vivo. While the initial report on the mouse genome (Mouse Genome Sequencing Consortium, 2002) predicted 11 mEars in this species-limited cluster, we have found only one, mEar 2, to be expressed constitutively in mouse lung tissue. We demonstrate here that recombinant mEar 2, similar to its human orthologs, EDN (RNase 2) and ECP (RNase 3), is ribonucleolytically active and has antiviral activity against an evolutionarily relevant pneumovirus pathogen, PVM and both activities are inhibited by placental RI. It is interesting to note that mEar 2 and its human ortholog, EDN (RNase 2), seem to have developed antiviral activity independently; we base this statement on findings from our previous study (Zhang and Rosenberg, 2002) in which we documented the relatively weak antiviral activity of an inferred ancestral protein of RNase 2. Although the mechanism of this antiviral activity remains unclear, there is evidence to suggest a specific, saturable interaction between the ribonuclease and an as-yet-unspecified molecule or molecules on the target cell surface (Rosenberg and Domachowske, 2001b).

Using both polyclonal and monoclonal antibodies, we detected native mEar 2 in mouse lung tissue and BAL fluid, and ribonuclease activity that was also inhibited by RI. Infection of wild-type mice with the pathogenic PVM strain J3666 results in a twofold decrease in mRNA encoding the cross-hybridizing mEars accompanied by a concomitant decrease in mEar protein in lung tissue and a four- to fivefold drop in BAL fluid ribonuclease activity, shown to be directly attributable to the activity of mEars. The reductions in mRNA and BAL fluid ribonuclease activity were not observed in response to infection with the non-pathogenic PVM strain 15, despite indistinguishable rates of virus replication. As PVM strain 15 does not induce local synthesis of the crucial chemokine MIP-1α, and thus induces no cellular inflammatory response, the role of the inflammatory response itself in inducing differential mEar expression required some consideration. Results analogous to the wild type, including a decrease in mEar mRNA expression in lung tissue accompanied by a decrease in detectable ribonuclease activity in BAL fluid, were observed in response to pathogenic PVM strain J3666 in MIP-1α −/− mice that are unable to mount an inflammatory response in this setting (Domachowske et al., 2000b), suggesting that decreased transcription and synthesis of mEars is a direct response to virus replication rather than secondary to antiviral inflammation per se.

We propose that the virus-mediated down-regulation of mEars may represent a virus-mediated strategy to evade host protective responses. Both DNA and RNA viruses employ a large number and variety of mechanisms to subvert innate antiviral host defense (Brander and Walker, 2000; Tindle, 2002; Abendroth and Arvin, 2001; Shisler and Moss, 2001; Piguet and Trono, 2001; Chensue, 2001; Rosenkilde et al., 2001; Alcami and Koszinowski, 2000; Mahalingam et al., 2002), many of which involve evasion of interferon and interferon-regulated responses (Levy and Garcia-Sastre, 2001; Grandvaux et al., 2002). There is comparatively little known about such strategies among the pneumoviruses (Gotoh et al., 2001). Of the three specific reports, Schlender et al. (2000) have described the role of bovine RSV proteins NS1 and NS2 in antagonizing signaling by interferons α and β in vitro, Teng and coworkers (1999, 2000) have reported on the attenuation of human RSV in vitro and in the chimpanzee model upon deletion of the NS1 and M2-2 proteins, and Tripp et al. (2001) have shown potential chemokine-mimicry of the RSV G protein.

One of the most interesting differences observed here is the fact that decreases in steady-state levels of mEar mRNA and the resulting decline in synthesis and secretion were observed only in response to the pathogenic PVM J3666, which was originally isolated from mice and has

been passed only through mice. The non-pathogenic strain 15 has gone through multiple passages in tissue culture, and while still infectious (see Fig. 6), virus replication in vivo does not result in the signs and symptoms typical of severe respiratory virus infection (Domachowske et al., 2002a). Among the known differences between the two strains, Randhawa et al. (1995) have shown that the F (fusion) proteins differ by only four amino acids, while the G (attachment) proteins differ substantially due to distinct translational start sites. In addition to differences in mEar expression, a comparative microarray analysis of lung tissue mRNAs of strain J3666 and strain 15-infected versus normal controls mice also demonstrated differential expression of interferon-β, interferon-response genes, and several proinflammatory chemokines (Domachowske et al., 2002a). At this point, we can only speculate on a potential relationship between the interferon responses and antiviral ribonuclease expression.

The relationship between virus infection, virus pathogenicity, and the regulation of antiviral ribonuclease expression is most intriguing and awaits further evaluation from a number of perspectives, specifically in mEar gene-deleted mice. Similarly intriguing is the possibility of using these ribonucleases as therapeutic agents for the treatment of pneumovirus infection.

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