Comparison of the microbicidal and muramidase activities of mouse lysozyme M and P

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INTRODUCTION

Lysozyme is one of the most abundant antimicrobial proteins in the airspaces of the lung. Mice express two lysozyme genes, lysozyme M and P, but only the M enzyme is detected in abundance in lung tissues. Disruption of the lysozyme M locus significantly increased bacterial burden and mortality following intratracheal infection with a Gram-negative bacterium. Unexpectedly, significant lysozyme enzyme activity (muramidase activity) was detected in the airspaces of uninfected lysozyme M−/− mice, amounting to 25% of the activity in wild-type mice. Muramidase activity in lysozyme M−/− mice was associated with increased lysozyme P mRNA and protein in lung tissue and bronchoalveolar lavage fluid respectively. The muramidase activity of recombinant lysozyme P was less than that of recombinant M lysozyme. Recombinant P lysozyme was also less effective in killing selected Gram-negative bacteria, requiring higher concentrations than lysozyme M to achieve the same level of killing. The lower antimicrobial activity of P lysozyme, coupled with incomplete compensation by P lysozyme in lysozyme M−/− mice, probably accounts for the increased susceptibility of null mice to infection. Recombinant lysozyme M and P were equally effective in killing selected Gram-positive organisms. This outcome suggests that disruption of both M and P loci would significantly increase susceptibility to airway infections, particularly those associated with colonization by Gram-positive organisms.

Key words: antibacterial protein, innate host defence, lung, lysozyme, microbicidal activity, muramidase.

EXPERIMENTAL

Generation and identification of mice

Mice deficient in lysozyme M were generated by insertion of the gene encoding EGFP (enhanced green fluorescent protein) into the lysozyme M locus [14]. Lysozyme M−/− and M+/− mice were identified by PCR amplification of a 680 bp fragment, using

Abbreviations used: BALF, bronchoalveolar lavage fluid; CFU, colony-forming units; CWFSG, cold-water-fish skin gelatin; EGFP, enhanced green fluorescent protein; RT, reverse transcriptase; SP-B, surfactant protein B.

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Lysozyme mRNA analysis

Lysozyme expression in lung tissue was assessed by RT (reverse transcriptase)-PCR using total lung RNA isolated from 5-week-old lysozyme M−/− mice and wild-type littermates. cDNA was generated by reverse transcription of 2 µg of total RNA using 1 µM oligo(dT) as primer. PCR was performed on lung cDNA using 0.1 µM of primers specific for lysozyme M (5'-GAATGGCGTGGCTACTA-3' and 5'-TCCCCAGAGCCATCACA-3'), lysozyme P (5'-GGATCCAGCTCCAGTCACC-3' and 5'-GGGACAGATCTCGTTTACGAC-3') or EGFP (5'-GCTGCCCGACAACACTACGTAG and 5'-GCTGCCCGCGTTACC) in a reaction mixture containing 1.5 mM MgCl2, 50 mM Tris/HCl (pH 7.6), 50 mM KCl, 100 µM dNTPs and 0.5 units of AmpliTaq (PerkinElmer) for 4 min at 94 °C, followed by a 30-cycle PCR amplification comprising denaturation for 10 s at 94 °C, annealing for 10 s at 60 °C and elongation for 15 s at 72 °C. Quantitative analyses of lysozyme M and P mRNAs were assessed by S1 nuclease assay [15]. S1 probes specific for lysozyme M, lysozyme P and mouse SP-B (surfactant protein B) mRNA were radiolabelled with [γ-32P]ATP and hybridized with 15 µg of total lung RNA at 56 °C overnight, followed by S1 nuclease (GIBCO BRL, Gaithersburg, MD, U.S.A.) digestion at room temperature for 1 h. Protected fragments were resolved in a 6% (w/v) polyacrylamide/8 M urea gel, dried and exposed to radiographic film at −70 °C for 4 h. SP-B served as an internal control for loading.

Analysis of protein expression in BALF

An aliquot of BALF containing 1 µg of protein was analysed by SDS/PAGE and Western blotting with a polyclonal rabbit antibody directed against human lysozyme. Relative levels of lysozyme proteins in BALF were assessed by scanning densitometry using Image-Quant software (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Spatial expression of lysozyme in the lungs

In order to assess gross lung structure and expression of lysozyme in the lungs of transgenic mice, lungs from four 5-week-old lysozyme M−/− mice, four lysozyme M+/− mice and four wild-type littermates were inflation-fixed for immunohistochemistry and light microscopy as previously described [12]. Immunostaining for lysozyme was performed using antisera directed against human lysozyme. Parallel lung sections were stained with polyclonal rabbit anti-(green fluorescent protein) antibody (Chemicon International, Temecula, CA, U.S.A.).

Alveolar macrophages from 5-week-old lysozyme M−/− mice and wild-type littermates were pelleted from BALF (200 g for 10 min), resuspended in 1 ml of PBS and cytospun on to glass slides at 400 rev./min for 4 min. Cells were fixed in 4% (w/v) paraformaldehyde in PBS for 1 h at room temperature. Following rinsing in PBS, cells were permeabilized with 0.2% saponin/1% CWFSG (cold-water-fish skin gelatin)/1% BSA in PBS for 20 min and rinsed twice in PBS. The slides were incubated with a 1:1000 dilution of normal rabbit serum or a polyclonal rabbit anti-human lysozyme antibody in 3% BSA/1% CWFSG/10% normal goat antibody in PBS for 2 h, rinsed twice in 1% CWFSG in PBS and subsequently reacted with biotin-labelled goat anti-rabbit secondary antibody followed by colour development with diaminobenzidine. Slides were counterstained with 1% Nuclear Fast Red in 5% aluminium sulphate solution.
**Localiz ation of lysozyme by electron microscopic immuno cytochemistry**

Lung tissues from wild-type and lysozyme M−/− mice were fixed with 4% (w/v) paraformaldehyde and 0.1% (w/v) glutaraldehyde in 0.2 M Hepes, pH 7.3, at 4 °C for 1 h, and cryoprotected with poly(vinylpyrrolidone) and sucrose. Cryo-immunogold labelling was performed on ultrathin frozen sections as described previously [16] using a polyclonal antibody directed against human lysozyme. Control experiments were performed using normal rabbit serum at the same dilution. Immunoreactivity was visualized by 10 nM Protein A-gold probes.

**Lysozyme enzyme activity assay in BALF**

To assess lysozyme enzyme activity in BALF, an aliquot containing 1 μg of protein was incubated with 1 ml of killed Micrococcus lysodeikticus suspended in 0.4 M phosphate buffer, pH 6.7, at a D450 of 1 at 37 °C. The changes in absorbance during 30 min of incubation were plotted against time using a recording spectrophotometer. In order to determine the proportion of bacteriolytic activity attributable to lysozyme, an aliquot of BALF (containing 1 μg of protein) from lysozyme M−/−, lysozyme M+/+ and wild-type littermate mice was immunoprecipitated with anti-lysozyme antibody at 4 °C for 4 h with gentle agitation. Lysozyme–antibody complexes were precipitated with Protein G–Sepharose (Pierce, Rockford, IL, U.S.A.) and removed by centrifugation at 1000 µg for 1 min. Lysozyme enzyme activity in supernatant was assayed as described above. Purified chicken lysozyme was used to generate a standard curve (1 unit of enzyme activity gives a change in D450 of 0.001).

**Production of recombinant lysozyme M and P**

Lysozyme M and P were amplified from type II cell cDNA using specific primers with sequences encoding polyhistidine tag added to the 5’ end of the downstream primers. After verification of the appropriate DNA sequence, fragments were cloned into pVL1393 (Pharmlingen, San Diego, CA, U.S.A.). Recombinant baculovirus was generated by homologous recombination in Spodoptera frugiperda cells. Fresh monolayers of 105 Trichoplusia ni cells (Invitrogen, Grand Island, NY, U.S.A.) were infected with plaque-purified recombinant virus at a MOI (multiplicity of infection) of 2 and cultured in serum-free medium for 72 h. Recombinant lysozyme M and P were purified from the medium by Ni2+ -nitrilotriacetate chromatography and characterized by Western blotting and silver staining. The muramidase activities of lysozyme M and P were compared with native and His6-tagged recombinant human lysozyme as described above.

**Assessment of lysozyme M and P bactericidal activity in vitro**

Ps. aeruginosa, Escherichia coli, K. pneumoniae, Candida albicans, Staphylococcus aureus and group B streptococci were grown to exponential phase (for 14–16 h) at 37 °C with continuous shaking, harvested from the broth by centrifugation at 200 g for 10 min and washed and resuspended in 10 mM sterile potassium phosphate buffer (pH 7.2) + 10% medium. The concentration of the inoculum was verified by quantitative culture. One hundred CFU (colony-forming units) of each microbe was incubated for 6 h at 37 °C with increasing amounts of purified recombinant lysozyme M or P (0–150 pmol) or buffer alone in triplicate. Quantitative cultures were performed and viable pathogen counts were determined.

**RESULTS**

Lysozyme M−/−, lysozyme M+/− and wild-type offspring were identified by PCR amplification of a 680-bp fragment of EGFP and a 220-bp fragment of exon 1 of lysozyme M (Figure 1A). Lysozyme M mRNA, assessed by RT-PCR, was not detected in lung tissue of lysozyme M−/− mice (Figure 1B), whereas EGFP mRNA was readily detected, consistent with a null genotype (Figure 1D). Lysozyme P mRNA was also detected in lung tissues (Figure 1C) and type II alveolar epithelial cells (results not shown). S1 nuclease assays using total lung RNA isolated from each of the genotypes demonstrated that lysozyme P mRNA was increased approx. 4-fold in lysozyme M−/− mice and 12-fold in lysozyme M+/− mice (Figure 2); in contrast, there was no difference in SP-B mRNA levels among the three genotypes. As expected, lysozyme M mRNA was detected in wild-type mice and at lower levels in lysozyme M+/− mice, but not in lysozyme M−/− mice (Figure 2).
Figure 3 Concentration of lysozyme protein in BALF

(A) BALF was obtained from 5-week-old lysozyme M−/− mice and wild-type littermates. Total BALF protein (1 µg) from wild-type mice and serial dilutions of BALF (0.125–1 µg) from lysozyme M−/− mice were resolved on SDS/PAGE in order to assess the relative abundance of total lysozyme protein in BALF. (B) The relative abundance of lysozyme M and P in BALF from lysozyme M−/− and wild-type littermate mice was compared by resolving 1 µg (wild-type) or 0.5 µg (lysozyme M−/−) of BALF protein by SDS/PAGE and Western blotting using an antibody against human lysozyme.

Spatial distribution of lysozyme in the lungs was assessed by immunohistochemistry on paraffin-embedded lung sections from 5-week-old lysozyme M−/− mice (B, D) and wild-type (WT) littermates (A, C) using an antibody against EGFP (A, B) or human lysozyme (C, D). Arrows indicate alveolar type II epithelial cells. Scale bar, 50 µm. The inset shows cytospun alveolar macrophages from lysozyme M−/− mice immunostained for lysozyme.

Figure 4 Cellular expression of lysozyme

To determine if elevated lysozyme P mRNA levels were accompanied by an increase in the corresponding protein, lungs of mice from each genotype were lavaged and the BALF was analysed by Western blotting. Immunoreactive lysozyme protein was elevated 4-fold in lysozyme M−/− mice relative to wild-type littermates (Figure 3A). Because of the unanticipated increase in lysozyme protein in the BALF of lysozyme M−/− mice, the levels of lysozyme P were assessed in wild-type and lysozyme M−/− mice by SDS/PAGE and Western blotting. Lysozyme P migrated more slowly than lysozyme M and was barely detectable in BALF from wild-type mice. In contrast, lysozyme P was dramatically elevated in BALF from lysozyme M−/− mice (Figure 3B). Taken together, these results indicate that the synthesis and secretion of lysozyme P are increased in the absence of lysozyme M.

The cellular sources of lysozyme in lung tissue were identified by immunohistochemistry on tissue sections from lysozyme M−/− mice. EGFP (expressed from the lys locus) was detected only in alveolar type II epithelial cells and alveolar macrophages (Figure 4B), suggesting that lysozyme M was synthesized by both cell types. Similarly, incubation of tissue sections from lysozyme M−/− mice with antibody directed against human lysozyme detected lysozyme P in type II cells and macrophages (Figure 4D). Immunogold labelling of ultrathin cryosections of lung tissue from lysozyme M−/− mice detected lysozyme P in multivesicular bodies and lamellar bodies of type II epithelial cells (Figure 5) as well as macrophages (results not shown). This pattern of subcellular localization is similar to that previously reported for lysozyme in rat type II cells [17], and suggests that both lysozyme M and P are packaged and co-secreted with pulmonary surfactant.

We next determined if elevated levels of lysozyme protein in BALF of lysozyme M−/− mice were accompanied by an increase in lysozyme enzyme (muramidase) activity in the airways. Relative to wild-type littermates, muramidase activity in BALF was
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Figure 5  Subcellular localization of lysozyme P

Ultrathin cryosections of lung tissues from 5-week-old lysozyme M−/− mice were labelled with an antibody directed against human lysozyme. MVB, multivesicular body; LB, lamellar body; MIT, mitochondria. Scale bar, 200 nm.

decreased by 28% in lysozyme M+/− mice and 58% in lysozyme M−/− mice (Figure 6). In order to determine the contribution of lysozyme to muramidase activity in BALF, lysozyme protein was removed from each BALF sample by immunoprecipitation before assay. Following lysozyme immunoprecipitation residual muramidase activity in BALF was similar for all three genotypes (Figure 6). These results indicate that there was substantial muramidase activity in the airways of lysozyme M−/− mice and that some of this activity was due to secretion of the P form of lysozyme.

Significantly decreased muramidase activity in lysozyme M−/− mice in the face of apparently increased lysozyme protein suggested that the enzymic properties of lysozyme P were different from the M isoform. To address this issue, recombinant lysozyme was purified from the medium of SF9 insect cells infected with baculoviruses encoding lysozyme M or P (Figure 7). The electrophoretic mobility of recombinant M and P lysozyme was different (Figure 7A), confirming the finding that native lysozyme P migrated more slowly than the M form by SDS/PAGE. Interestingly, lysozyme P was approx. 7-fold more immunoreactive than lysozyme M in Western blots (Figure 7B), indicating that the concentration of the P isoform in BALF from lysozyme M−/− mice was overestimated (Figure 3). At equimolar concentrations, recombinant lysozyme P required roughly twice as much time as recombinant lysozyme M to achieve 50% lysis of substrate (Figure 8A). Dose–response curves generated for the recombinant proteins indicated that muramidase activity was approx. 3-fold greater for the M form (results not shown). The muramidase activity of recombinant lysozyme M was identical with that of recombinant human lysozyme and native human lysozyme, suggesting that the C-terminal, His6 tag (used to purify the recombinant protein) did not affect the enzymic properties of lysozyme (Figure 8A). Similarly, the ability of recombinant lysozyme M to kill K. pneumoniae was not significantly different from recombinant human lysozyme or native human lysozyme, supporting

Figure 6  Muramidase activity in BALF

BALF was obtained from 5–6-week-old lysozyme M+/−, lysozyme M−/− and wild-type mice. Muramidase activity was assessed in 1 µg of BALF protein by incubating in a suspension of M. lysodeikikus as described in the Experimental section (open bars). In order to determine the relative contribution of lysozyme to muramidase activity in BALF, lysozyme was removed by immunoprecipitation before muramidase assay (filled bars). *P < 0.01 relative to pre-precipitation sample; **P < 0.01 for lysozyme M−/− mice compared with wild-type mice before immunoprecipitation.

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The hypothesis that the His6 tag did not alter the bactericidal properties of lysozyme (Figure 8B). The antimicrobial activity of purified, recombinant lysozyme M and P was tested against Gram-negative bacteria (P. aeruginosa, E. coli, and K. pneumoniae) and Gram-positive organisms (S. aureus and group B streptococci) and C. albicans. One hundred CFU of each microbe was incubated with increasing amounts of recombinant protein, and the number of viable pathogens was counted after 6 h of incubation (Figure 9). Lysozyme M and P were equally effective against C. albicans requiring less than 40 pmol to kill 50% of the pathogen. There was also no significant difference in the ability of the two isoforms to kill group B streptococci and S. aureus, although more enzyme was required to kill these Gram-positive bacteria. Lysozyme M was significantly more effective than lysozyme P in killing E. coli, Ps.

**DISCUSSION**

The present study was initially undertaken to test the hypothesis that lysozyme deficiency predisposes mice to colonization of the airspaces by a variety of pathogens. Although mice express two lysozyme genes, the relative amount of P lysozyme mRNA in lung tissue was previously reported to be very small [9]. Consistent with this report, only trace amounts of P lysozyme protein were detected in BALF from wild-type mice (Figure 3). These findings confirmed that M lysozyme is the major form of lysozyme in lung tissue and predicted that disruption of the locus encoding this enzyme would eliminate virtually all lysozyme protein and enzyme activity in the airspaces. Unexpectedly, robust muramidase activity was detected in BALF from lysozyme M−/− mice amounting to approx. 25% of the activity detected in wild-type mice (after correction for non-lysozyme-related muramidase activity). Lysozyme-related muramidase activity in lysozyme M−/− mice was shown to be due to elevated P lysozyme protein in the airspaces.

Estimation of the precise concentration of lysozyme P in lysozyme M−/− mice is complicated by differences in the muramidase activity and immunoreactivity of lysozyme M and P. Given that the muramidase activity of P lysozyme is 2–3-fold less than the M protein, we estimate that the concentration of P lysozyme in BALF of lysozyme M−/− mice is approx. 50–75% of that in wild-type mice. This estimate agrees well with the results of Western blotting, which indicate that the concentration of P lysozyme in the BALF of null mice is approx. 60% of that in wild-type mice (after correction for increased P immunoreactivity). Thus inactivation of the locus encoding lysozyme M resulted in up-regulation of the P gene leading to partial compensation of lysozyme protein in the airspaces.
and enzyme activity. A similar finding was recently reported by Ganz et al. [11] who demonstrated compensatory expression of P lysozyme in alveolar macrophages, but not neutrophils, of lysozyme M−/− mice.

Lysozyme is an abundant protein that constitutes 6–7% of protein in BALF from rat lung [5]. The cellular sources of lysozyme in BALF are alveolar macrophages and type II epithelial cells. Lysozyme is the major protein in lamellar bodies of type II cells and is secreted with surfactant phospholipids into the alveolar airspace [3,5]. Lysozyme P was detected in type II cells (in the present study) and alveolar macrophages [11] from lysozyme M−/− mice, confirming up-regulation of lysozyme P expression in both cell types; however, the relative contributions of type II cells and macrophages to muramidase activity in BALF is not known. A non-lysozyme muramidase activity of unknown source was also detected in BALF from lysozyme M−/− mice. This activity did not change in BALF from wild-type, lysozyme M+/− or M−/− mice, indicating that expression of lysozyme P was induced selectively in response to disruption of the lysozyme M locus.

Lysozyme M and P are both synthesized as a 148-amino-acid precursor containing an 18 residue N-terminal signal peptide. The 130-amino-acid mature lysozyme peptides are 92% identical, differing at only 11 positions (Figure 10). These 11 amino acid substitutions give rise to differences in electrophoretic migration, immunoreactivity, muramidase activity and microbicidal activity between lysozyme M and P. Lysozyme M and human lysozyme were very similar with respect to each of these properties, confirming experimentally that these enzymes are orthologues. Lysozyme P is identical with human lysozyme at four of the 11 amino acids (Table 1), suggesting that the remaining seven amino acids (residues 4, 11, 24, 73, 74, 114 and 115) may account for the differences between M and P lysozyme. Each of these seven residues is predicted to be located at the surface of the molecule (Figure 10). Interestingly, two of these amino acids, residues 114 and 115, map to a helix (residues 107–115 of human lysozyme) that has potent microbicidal activity against both Gram-positive

Figure 9  Antimicrobial properties of recombinant lysozyme M and P

Group B streptococci (GBS), E. coli, P. aeruginosa, K. pneumoniae, S. aureus or C. albicans (10^3 CFU) were incubated with 0, 10, 20, 80 or 150 pmol of recombinant lysozyme M or P at 37 °C for 3 h. The number of pathogens at the end of incubation was assessed by quantitative culture. The broken lines indicate the concentration of lysozyme at which 50% of microbes were killed.

*P < 0.05.

Figure 10  Differences in amino acid sequence between mature human lysozyme, and mouse lysozyme M and P peptides

Lysozyme M and P differ at 11 of 130 positions. Residue # indicates amino acid position in the mature peptide (indicated in bold in the primary sequence). The primary sequence, predicted secondary structure and predicted solvent accessibility of individual amino acids in human lysozyme is shown beneath the Table. Darker shades indicate less solvent accessibility.
and Gram-negative organisms [18]. Since lysozyme was previously shown to have microbicidal activity independent of its enzymic (muramidase) activity [7,8], it is conceivable that amino acid substitutions at positions 114 and 115 contribute to the difference in antimicrobial activity of lysozyme M and P. It is not clear which amino acid(s) substitution is responsible for altered muramidase activity.

Lysozyme P was less effective in killing selected Gram-negative bacteria in vitro, requiring higher concentrations than lysozyme M to achieve the same level of killing. The lower antimicrobial activity of P lysozyme coupled with incomplete compensation by P lysozyme in lysozyme M−/− mice probably accounts for the significantly increased bacterial burden and mortality in null mice following intratracheal infection with K. pneumoniae [13]. Lysozyme M and P were equally effective in killing selected Gram-positive organisms in vitro. This outcome suggests that disruption of both M and P loci would significantly increase susceptibility to airway infections, particularly those associated with colonization by Gram-positive organisms. Whether or not the loss of both M and P lysozyme would lead to up-regulation of the gene(s) responsible for the non-lysozyme muramidase activity in BALF remains to be tested.

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