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

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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.

INTRODUCTION

In addition to serving as a physical barrier against microbial invasion, the respiratory epithelium secretes multiple antimicrobial peptides that enhance the elimination of micro-organisms either directly or in co-operation with professional phagocytes. Lysozyme, one of the most abundant antimicrobial proteins in the airways [1], is secreted by pulmonary epithelial cells and macrophages [2–5]. Lysozyme hydrolyses the glycosidic linkage between *N*-acetylglucosamine and *N*-acetylmuramic acid, leading to degradation of peptidoglycan in the bacterial cell wall (reviewed in [6]). The muramidase activity of lysozyme is thought to be primarily responsible for lysis of Gram-positive bacteria, which contain abundant peptidoglycan. Lysozyme is also active against some Gram-negative bacteria; however, because peptidoglycan is less abundant and less accessible in Gram-negative organisms, it is not clear if the muramidase activity and/or a non-enzymic bacteriolytic activity is responsible for this property [7,8].

The number of genes that encode lysozyme and the pattern of lysozyme expression varies considerably among species. Humans contain a single lysozyme gene, whereas two genes, lysozyme M and P, are expressed in mice [9,10]. Lysozyme M is the predicted orthologue of human lysozyme and is the predominant form in most mouse tissues with the exception of the small intestine, where lysozyme P predominates. In lung tissue, lysozyme M mRNA is much more abundant than that of lysozyme P and appears to constitute the major antimicrobial form of the enzyme in the airways [11].

Although the antimicrobial properties of lysozyme have been characterized extensively *in vitro*, relatively little is known about the importance of lysozyme in airway host defence *in vivo*. A previous study in transgenic mice demonstrated that overexpression of lysozyme in the distal respiratory epithelium enhanced killing, decreased systemic spread and improved survival following intratracheal infection with group B *Streptococcus* or *Pseudomonas aeruginosa* [12]. These findings suggested that lysozyme is an important component of innate host defence and that deficiency of lysozyme might increase susceptibility to infection by airway pathogens. Consistent with this hypothesis, bacterial burden and mortality were significantly increased following intratracheal infection of lysozyme $M^{-/-}$ mice with the Gram-negative bacterium *Klebsiella pneumoniae* [13]. Unexpectedly, significant muramidase activity was detected in BALF (bronchoalveolar lavage fluid) from uninfected lysozyme $M^{-/-}$ mice. A similar finding was recently reported by Ganz et al. [11] who showed partial compensation by P lysozyme in macrophages of lysozyme $M^{-/-}$ mice. The present study was therefore undertaken to assess the extent of P lysozyme compensation in the airspaces of lysozyme $M^{-/-}$ mice and compare the muramidase and microbicidal activities of lysozyme M and P.

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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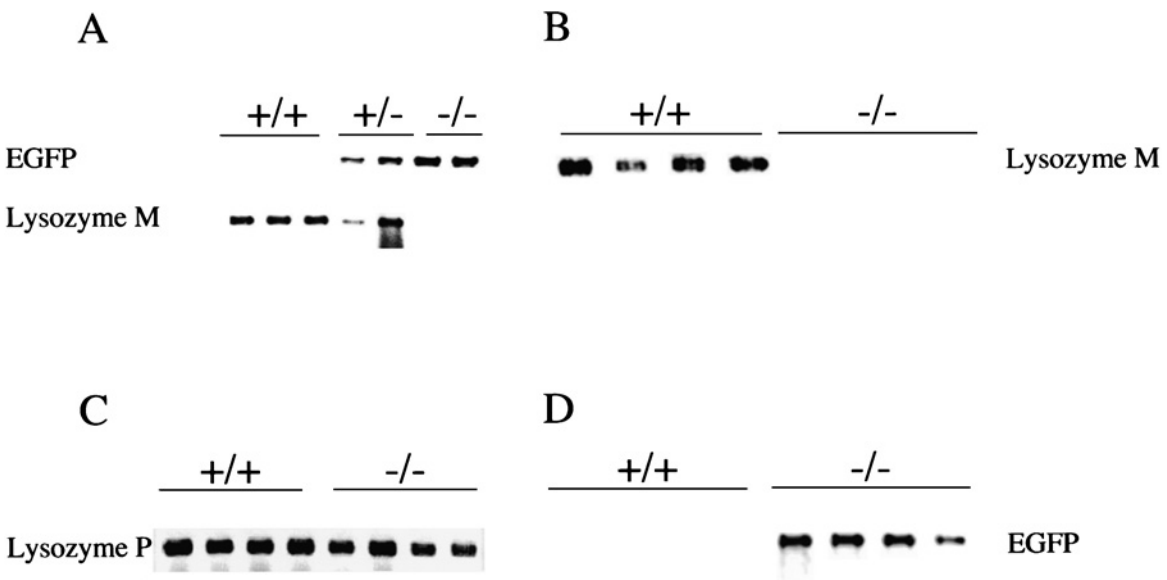

Figure 1 Identification and characterization of lysozyme $M^{-/-}$ mice

(**A**) The lysozyme M locus was disrupted by the insertion of the gene that encodes EGFP into exon I and intron I. Mice were identified by PCR amplification of intact exon 1/intron 1 (220 bp; lysozyme M) or exon 1/EGFP fragment (680 bp; EGFP). Expression of mRNA for lysozyme M (**B**), lysozyme P (**C**) or EGFP (**D**) was assessed by RT-PCR of total lung RNA.

an upstream primer to exon I (5′-AAGCTGTTGGGAAAGGAGGG-3′) and a downstream EGFP-specific primer (5′-GTCGCCGATGGGGTGTTCT-3′). The wild-type allele of lysozyme M was co-amplified to yield a 220 bp fragment using a downstream primer (5′-TCGGCCAGGCTGACTCCATA-3′) complementary to the sequence that was replaced by the targeting construct. To minimize variability arising from genetic background, all mice used in the present study were littermates from sibling matings of lysozyme $M^{+/-}$ mice. All mice were housed in pathogen-free conditions in the vivarium at Cincinnati Children's Hospital Medical Center.

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′-GAATGGCTGGCTACTA-3′ and 5′-TCCCACAGGCATTCACA-3′), lysozyme P (5′-GGTACCAGCCTCCAGTCACC-3′ and 5′-TGGGACAGATCTCGGTTTTGAC-3′) or EGFP (5′-GCTGCCCGACAACCACTACCTGAG and 5′-GCTGCGGCCGCTTTACCTGT) in a reaction mixture containing 1.5 mM $MgCl_2$, 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) were radiolabelled with [γ-^{32}P]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 antiserum 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.

Localization of lysozyme by electron microscopic immunocytochemistry

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 D_{450} of 1 at 37 °C. The changes in attenuance 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 ***g*** 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 D_{450} 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 (Pharmingen, San Diego, CA, U.S.A.). Recombinant baculovirus was generated by homologous recombination in Sf9 *Spodoptera frugiperda* cells. Fresh monolayers of 10^7 *Trichoplasia 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 Ni^{2+}-nitrilotriacetate chromatography and characterized by Western blotting and silver staining. The muramidase activities of lysozyme M and P were compared with native and His_6-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.

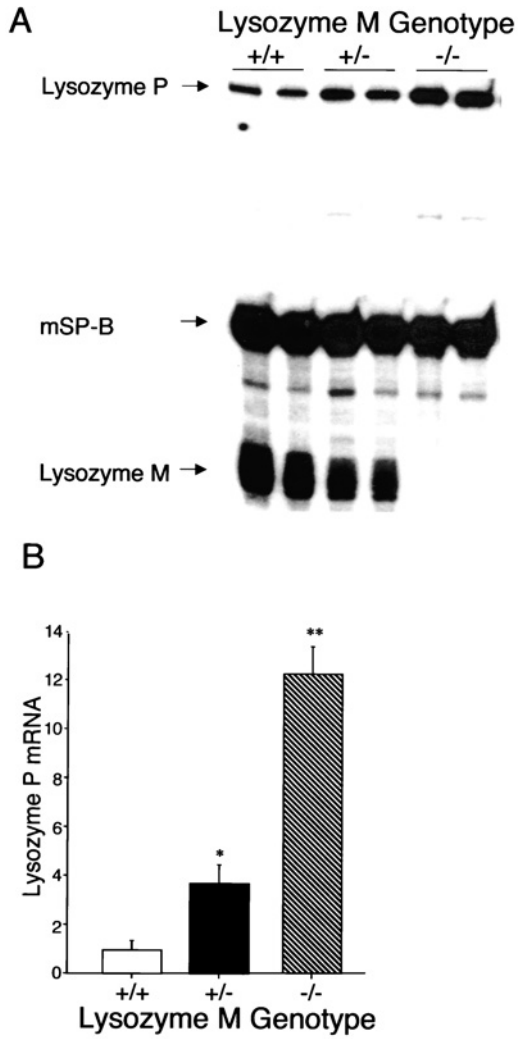

Figure 2 Analysis of lysozyme RNA expression in the lungs

(**A**) S1 nuclease analyses were performed on 15 μg of total lung RNA isolated from 5-week-old mice using ^{32}P-labelled cDNA probes specific for lysozyme M, lysozyme P or SP-B. The 400-nt fragment corresponds to lysozyme P, the 154-nt fragment to lysozyme M and the 186-nt fragment to SP-B. (**B**) The relative abundance of lysozyme P was assessed by scanning densitometry of the blot in (**A**) Values were standardized to SP-B levels and were normalized to levels in wild-type mouse lungs. $^*P < 0.03$ and $^{**}P < 0.01$ relative to wild-type mice.

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).

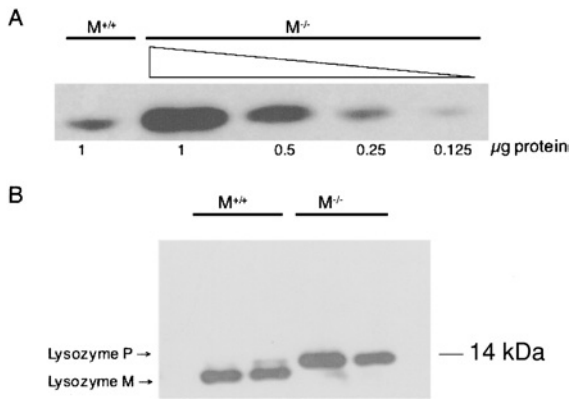

Figure 3 Concentration of lysozyme protein in BALF

(**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.

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

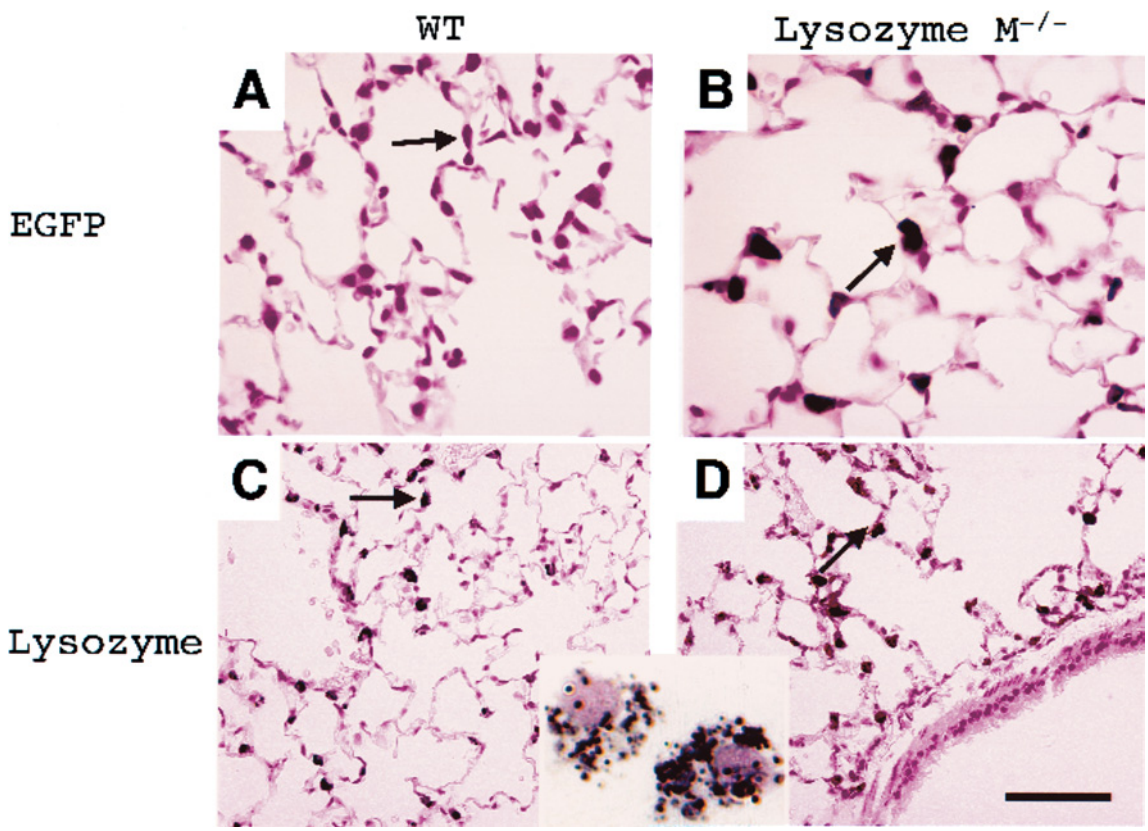

Figure 4 Cellular expression of lysozyme

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.

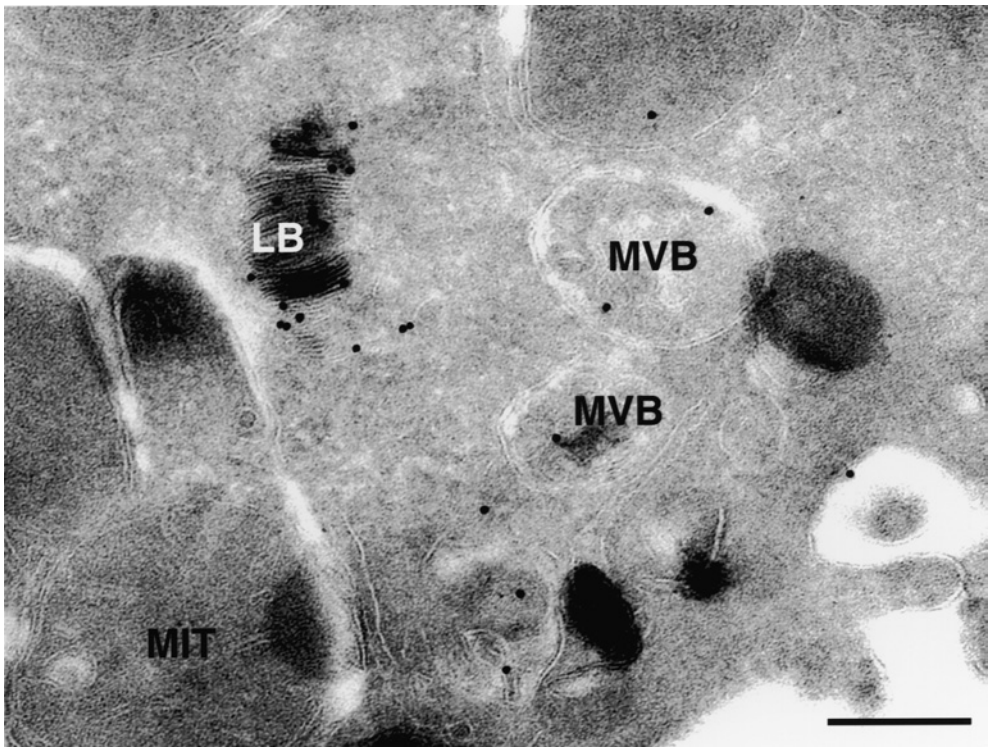

Figure 5 Subcellular localization of lysozyme P

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, His_6 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

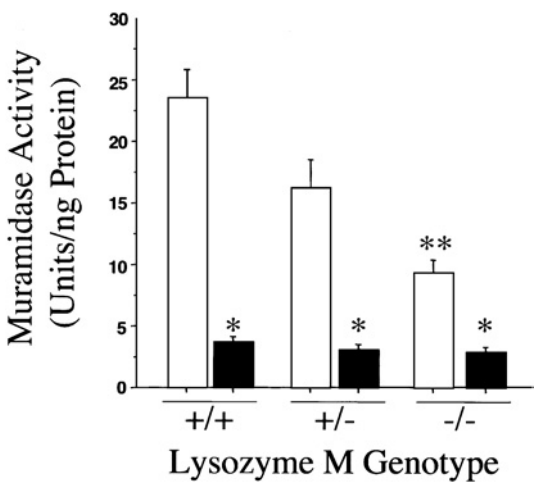

Figure 6 Muramidase activity in BALF

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. lysodeiktikus* 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.

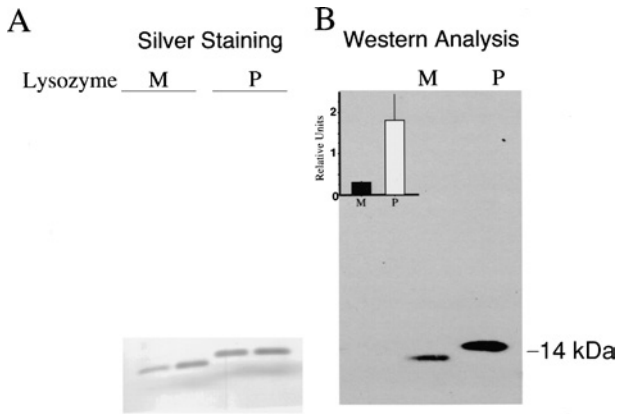

Figure 7 Generation of recombinant lysozyme M and P

cDNA encoding lysozyme M or P was cloned in-frame with a C-terminal His_6 tag and was expressed in Sf9 insect cells. Recombinant lysozyme M or P was purified from medium using a Ni^{2+}-nitrilotriacetate column. The purity of recombinant proteins was assessed by resolving equal amounts of protein by SDS/PAGE and silver staining of eluted fractions (**A**) or Western blotting with anti-(human lysozyme) antibody (**B**). The inset shows scanning densitometric analysis of the immunoblot in (**B**).

the hypothesis that the His_6 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 (*Ps. 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. aeruginosa* and *K. pneumoniae* ($P < 0.05$). Less than 40 pmol of lysozyme M was required to kill 50 % of *Ps. aeruginosa* and *K. pneumoniae* compared with more than 120 pmol for lysozyme P. Collectively, these results suggest that lysozyme M was more effective than lysozyme P in killing Gram-negative bacteria, and that both enzymes were equally effective in killing Gram-positive organisms.

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 lysozme 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 nice 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

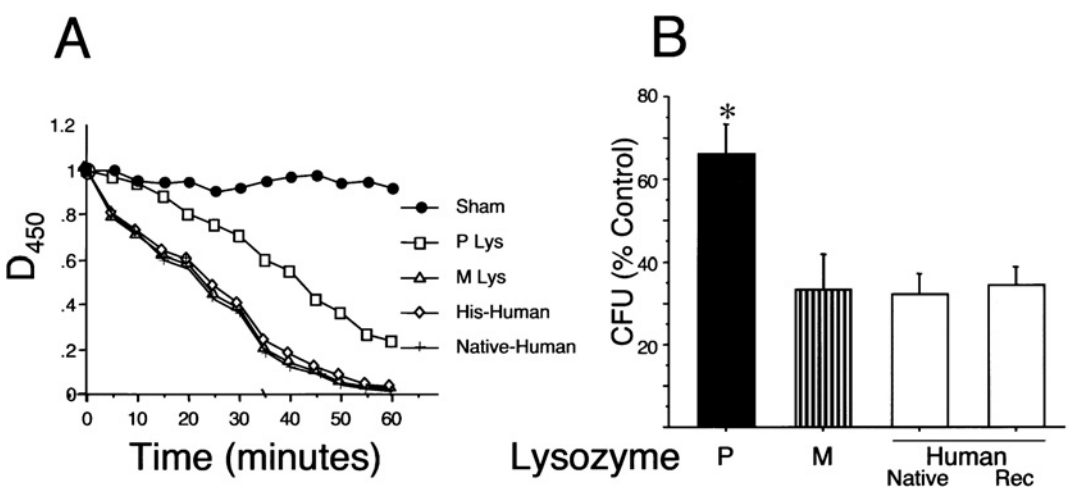

Figure 8 Muramidase activity of recombinant lysozyme M and P

(**A**) Recombinant lysozyme M, lysozyme P, native human lysozyme or His_6-tagged recombinant human lysozyme (0.5 μg) was incubated with a suspension of *M. lysodeikticus* and the change in D_{450} over time was recorded. (**B**) Bacteriolytic activity of recombinant lysozyme M and lysozyme P. Recombinant lysozyme M, lysozyme P, His_6-tagged recombinant human lysozyme (Rec; 50 pmol), native human lysozyme or buffer were incubated with 10^2 CFU of *K. pneumoniae* at 37 °C for 3 h. Residual bacteria were assessed by qualitative culture. Sham indicates treatment with fractions from media of *T. ni* cells infected with empty vector. Experiments were performed in triplicate. $*P < 0.02$.

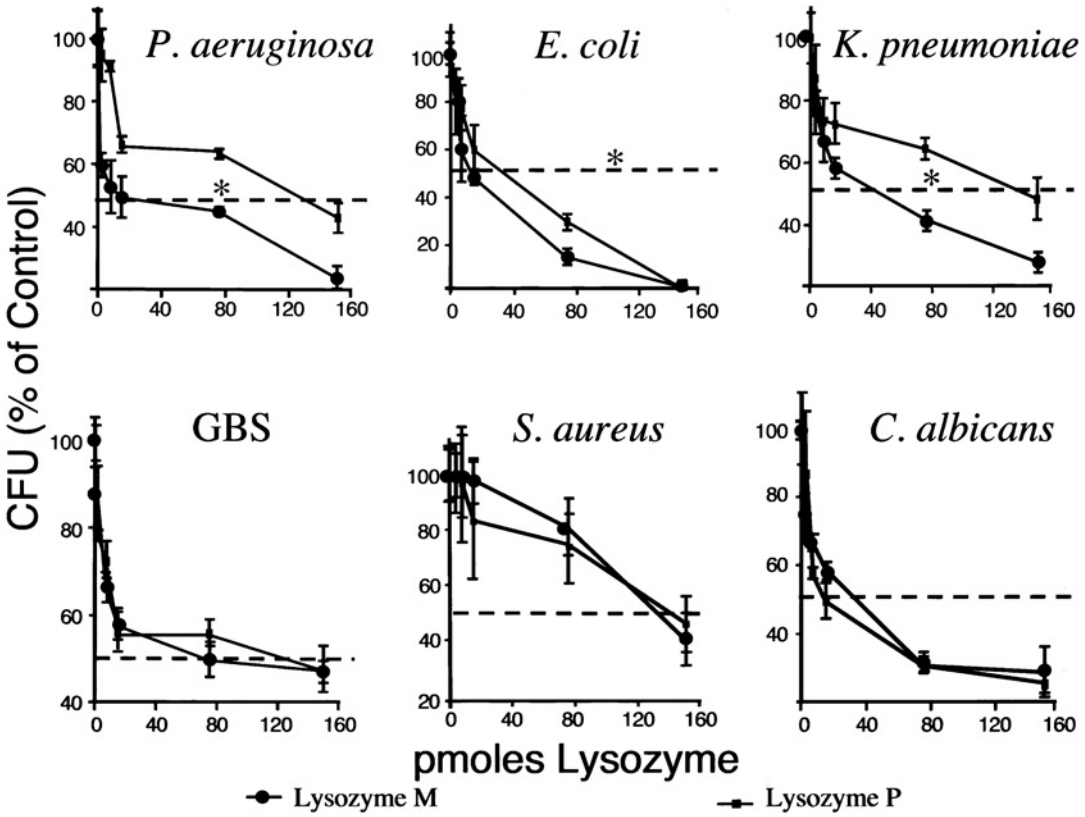

Figure 9 Antimicrobial properties of recombinant lysozyme M and P

Group B streptococci (GBS), *E. coli*, *Ps. 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. $^*P < 0.05$.

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. Lyso-

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.

zyme 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

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.

This work was supported by NIH (National Institutes of Health) grant A1 050797. The authors thank Ann Maher for secretarial assistance.

REFERENCES

1 Thompson, A. B., Bohling, T., Payvandi, F. and Rennard, S. I. (1990) Lower respiratory tract lactoferrin and lysozyme arise primarily in the airways and are elevated in association with chronic bronchitis. J. Lab. Clin. Med. **115**, 148–158
2 Gordon, S., Todd, J. and Cohn, Z. A. (1974) *In vitro* synthesis and secretion of lysozyme by mononuclear phagocytes. J. Exp. Med. **139**, 1228–1248
3 Power, J. H. T., Barr, H. A. and Nicholas, T. E. (1993) Characterization and immunohistochemical localization of the 15 kD protein isolated from rat lung lamellar bodies. Am. J. Respir. Cell Mol. Biol. **8**, 98–105
4 Konstan, M. W., Chen, P. W., Sherman, J. M., Thomassen, M. J., Wood, R. E. and Boat, T. F. (1981) Human lung lysozyme: sources and properties. Am. Rev. Respir. Dis. **123**, 120–124
5 Singh, G., Katyal, S. L., Brown, W. E., Collins, D. L. and Mason, R. J. (1988) Pulmonary lysozyme–a secretory protein of type II pneumocytes in the rat. Am. Rev. Respir. Dis. **138**, 1261–1267
6 Ibrahim, H. R., Aoki, T. and Pellegrini, A. (2002) Strategies for new antimicrobial proteins and peptides: Lysozyme and aprotinin as model molecules. Curr. Pharm. Design **8**, 671–693
7 Ibrahim, H. R., Matsuzaki, T. and Aoki, T. (2001) Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett. **506**, 27–32
8 Laible, N. J. and Germaine, G. R. (1985) Bactericidal activity of human lysozyme, muramidase-inactive lysozyme, and cationic polypeptides against *Streptococcus sanguis* and *Streptococcus faecalis*: inhibition by chitin oligosaccharides. Infect. Immun. **48**, 720–728
9 Cross, M., Mangelsdorf, I., Wedel, A. and Renkawitz, R. (1988) Mouse lysozyme M gene: isolation, characterization, and expression studies. Proc. Natl. Acad. Sci. U.S.A. **85**, 6232–6236
10 Cortopassi, G. A. and Wilson, A. C. (1990) Recent origin of the P lysozyme gene in mice. Nucleic Acids Res. **18**, 1911
11 Ganz, T., Gabayan, V., Liao, H. I., Liu, L. D., Oren, A., Graf, T. and Cole, A. M. (2003) Increased inflammation in lysozyme M-deficient mice in response to *Micrococcus luteus* and its peptidoglycan. Blood **101**, 2388–2392
12 Akinbi, H. T., Epaud, R., Bhatt, H. and Weaver, T. E. (2000) Bacterial killing is enhanced by expression of lysozyme in the lungs of transgenic mice. J. Immunol. **165**, 5760–5766
13 Markart, P., Korfhagen, T. R., Weaver, T. E. and Akinbi, H. T. (2004) Mouse lysozyme M is important in pulmonary host defense against *Klebsiella pneumoniae* infection. Am. J. Respir. Crit. Care Med. **169**, 454–458
14 Faust, N., Varas, F., Kelly, L. M., Heck, S. and Graf, T. (2000) Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. Blood **96**, 719–726
15 Yei, S. P., Bachurski, C. J., Weaver, T. E., Wert, S. E., Trapnell, B. C. and Whitsett, J. A. (1994) Adenoviral-mediated gene transfer of human surfactant protein B to respiratory epithelial cells. Am. J. Respir. Cell Mol. Biol. **11**, 329–336
16 Voorhout, W. F., Weaver, T. E., Haagsman, H. P., Geuze, H. J. and van Golde, L. M. J. (1993) Biosynthetic routing of pulmonary surfactant proteins in alveolar Type II cells. Microsc. Res. Tech. **26**, 366–373
17 Haller, E. M., Shelley, S. A., Montgomery, M. R. and Balis, J. U. (1992) Immunocytochemical localization of lysozyme and surfactant protein-A in rat Type-II cells and extracellular surfactant forms. J. Histochem. Cytochem. **40**, 1491–1500
18 Ibrahim, H. R., Thomas, U. and Pellegrini, A. (2001) A helix–loop–helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action. J. Biol. Chem. **276**, 43767–43774

Received 25 November 2003/21 January 2004; accepted 16 February 2004
Published as BJ Immediate Publication 16 February 2004, DOI 10.1042/BJ20031810