STRUCTURAL CHARACTERIZATION OF MOUSE NEUTROPHIL SERINE PROTEASES 
AND IDENTIFICATION OF THEIR SUBSTRATE SPECIFICITIES: RELEVANCE TO 
MOUSE MODELS OF HUMAN INFLAMMATORY DISEASES

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ABSTRACT
It is widely accepted that NSPs play a critical role in neutrophil-associated lung inflammatory and tissue-destructive diseases. To investigate NSP pathogenic role(s), various mouse experimental models have been developed that mimick acutely or chronically injured human lungs. We and others are using mouse exposure to cigarette smoke as a model for chronic obstructive pulmonary disease (COPD) with or without exacerbation. However, the relative contribution of NSPs to lung disease processes as well as their underlying mechanisms remain still poorly understood. And the lack of purified mouse NSPs and their specific substrates have hampered advances in these studies. In this work, we compared mouse and human NSPs and generated 3D models of murine NSPs based on 3D structures of their human homologs. Analyses of these models provided compelling evidence that peptide substrate specificities of human and mouse NSPs are different in spite of their conserved cleft and close structural resemblance. These studies allowed us to synthesize for the first time novel sensitive fluorescence resonance energy transfer (FRET) substrates for individual mouse NSP. Our findings and the newly identified substrates should better our understanding about the role of NSPs in the pathogenesis of cigarette-associated COPD as well as other neutrophils-associated inflammatory diseases.

INTRODUCTION
Neutrophil serine proteases (NSPs), neutrophil elastase (NE), cathepsin G (CG), and proteinase 3 (Pr3), are mainly stored in neutrophil primary granules in readily active forms. NSPs are structurally related and share the conserved charge-relay triad, His\(^57\)-Asp\(^{102}\)-Ser\(^{195}\) where Ser is the active residue (chymotrypsinogen numbering) (1). NSPs contribute to neutrophil oxygen-independent system mediated protection of the host against invading pathogens (2). Indeed, NSPs serve a physiological role for killing of microbes (3). Activated neutrophils are also known to release NSPs in the setting of inflammation. *In vitro*, NSPs are capable of cleaving a panoply of substrates. These include extracellular matrix proteins, pro-inflammatory mediators, coagulation factors, and immunoglobulins (4). NE degrades pro-inflammatory mediators such as TNF\(\alpha\) and IL-1\(\beta\), hence could alter the inflammatory response. The enzyme is capable of inducing the secretion of GM-CSF and IL-8, which could amplify the inflammation. Consequently, the release of these potent proteinases in diseased situations could create a proteolytic environment where degradation of different host molecules might occur and result in inappropriate inflammatory response. Because of their large substrate repertoire, NSPs have been implicated in the pathogenesis of various inflammatory and tissue-destructive diseases including acute lung injury (ALI), cystic fibrosis (CF), and COPD (5-7)

COPD is recognized as a major health problem whose worldwide incidence is increasing dramatically. Cigarette smoke represents the major etiologic factor contributing to the development and progression of COPD. Advances in therapy against COPD have been limited due in part to poor understanding of the pathogenesis of this disease. Various animal models and injurious stimuli mimicking human COPD are nowadays available to help understand the development of this pathology (reviewed in(8)). For example, several inbred strains of mice spontaneously develop emphysema, the main component of COPD, due to genetic abnormalities (reviewed in (8)). And mice exposed to cigarette smoke provide a valuable model of human COPD (9).

The contribution of NSPs to COPD was first evidenced by the observation that patients lacking \(\alpha\)1-Pi, the main physiological inhibitor of neutrophil elastase, were at greater risk of developing emphysema (10,11). Subsequently, various studies reported the induction of emphysema in animal models following instillation of NSPs. Intratracheal instillation of mice with human neutrophil elastase or proteinase 3 leads to tissue destruction and airspace enlargement, characteristic features of emphysema (12). In recent years, it has been shown that mice deficient in NE are considerably protected against cigarette-induced emphysema. Despite these compelling observations, the mechanisms by which NSPs injure the lung are not understood. Part of this concern may be because full characterization of
mouse NSPs is still lacking. While we and others have cloned and characterized murine NSPs genes/cDNAs (13-15), their corresponding proteins have not been purified. More importantly, unlike human NSPs, no specific peptides substrates for mouse NSPs are available in order to detect and quantify their specific activities especially in disease models.

To address these concerns, we purified the three mouse NSPs and carried out a detailed analysis by comparison to human NSPs. Our studies revealed critical differences between mouse and human NSPs, which prompted us to develop new specific FRET peptides substrates for mouse NSPs. Molecular modeling approach in association with kinetic studies enabled us to generate/synthesize new sensitive substrates to both detect and measure all three mouse NSPs activities in the subnanomolar range.

**EXPERIMENTAL PROCEDURES**

**Reagents.** Human neutrophil elastase (hNE) (EC 3.4.21.37), cathepsin G (hCG) (3.4.21.20), α1-antichymotrypsin, and α1-protease inhibitor (α1-Pi) were obtained from Biocentrum Ltd. (Krakow, Poland) and Proteinase 3 (hPR3) (EC 3.4.21.76) from Athens Research and Technology (Athens, Georgia). All other chemicals were reagent grade and purchased from Sigma-Aldrich, unless otherwise stated.

**Mice and neutrophils.** Wild-type mice (n=15) had C57/BL6 genetic background and were 8-10 weeks old. Mice were maintained in the animal barrier facility with 12 h light/dark cycle and provided with water and food ad libitum. All procedures were approved by the Animal Studies Committee of our institute.

Mouse neutrophils were isolated from peritoneal lavage fluids after they were attracted to the peritoneum by intraperitoneal injection of 15% glycogen (1 ml/mouse) (16). At 16 h post-challenge, mice were sacrificed by cervical dislocation. The peritoneal cavities were lavaged with 5 ml of PBS and the lavage fluids were spun and resuspended in a hypotonic lysis solution to remove red cell contamination. Next, the number and viability of cells in the lavage fluid were determined by hemacytometer and trypan blue exclusion, respectively. For differential counts, cells were cytospun (Cytospin 4, Thermo Shandon, Pittsburgh, PA) and stained with a modified Wrights stain (Hema 3, Fisher Scientific, Middletown, VA). The cell pellet was resuspended in PBS supplemented with 0.15% Brij 35.

**Purification of murine neutrophil serine proteases.** Cell pellet was subjected to three cycles of freezing and thawing. Following the first cycle, the cell suspension was sonicated for 3x1min with 2x1 min intervals on ice. The cell suspension was centrifuged after each cycle and supernatants were collected and diluted in two volumes of 50 mM PBS pH 7.4 to adjust the Brij concentration to 0.05%. Pooled supernatants were run on a heparin-Sepharose column (10mL) at 4°C. Recovered fractions from column washing step, and fractions eluted with a 0.05-0.8M NaCl gradient (2x25mL) were assayed against a polyvalent fluorogenic substrate sensitive to both trypsin-like and chymotrypsin-like proteases, Abz-HPVPVYAFSPQ-EDDnp. Fractions with peptidase activity from the column washing step and following elution with a 0.05-0.8M NaCl gradient were pooled separately. The pool derived from the column washing fractions was loaded onto a phenyl-Sepharose column pre-equilibrated in 0.3M sodium acetate (NaOAc), pH 5.5. Protein elution was carried out with a linear gradient (0-50%) of 2-propanol in 0.05M NaOAc, pH 5.5. Active fractions against Abz-HPVPVYAFSPQ-EDDnp were pooled and equilibrated in 0.3M NaOAc, pH 5.5, 25% 2-propanol and stored at -20°C. An aliquot was subjected to HPLC on a C4 column (2.1mm x 30mm) eluted (0.2 mL/min) with a linear 0-60% (v/v) gradient of acetonitrile in 0.075% TFA. The N-terminal amino acid sequences of selected protein fractions were determined by automated Edman degradation as described below. The pool of active fractions recovered from the NaCl gradient was run on SP-Sepharose pre-equilibrated in 0.05M phosphate buffer, pH 7.4, 150 mM NaCl, 0.05% Brij 35. Buffers containing 0.5M NaCl buffer and 0.8M NaCl were employed to elute trypsin-like protease fractions and chymotrypsin-like protease fractions respectively. Aliquots from the pooled trypsin-like protease fractions and chymotrypsin-like protease fractions were subjected to HPLC on a C4 column. The N-
terminal amino acid sequences of selected protein fractions were determined by automated Edman degradation as described below.

**Immunoblotting.** Prior to NSP purification, cell lysates were subjected to immunoblotting. Aliquots of protein fractions were resolved by SDS-PAGE and processed for Western blotting experiment. The membranes were sequentially incubated with rabbit polyclonal antibody specific to individual mouse NSPs (NE (1:2,000), PR 3 (1:2,000), and CG (1:500) followed by goat anti-rabbit horseradish peroxidase (17). Immunoreactive fragments were visualized by enhanced chemiluminescence as previously described.

**N-terminal sequencing.** N-terminal amino acid sequencing of selected peak were determined using an Applied Biosystems Procise 494 sequencer attached to a Model 140C Micro-gradient System and a 610A Data Analysis System with the chemicals and program recommended by the manufacturer (Applied Biosystems, Courtlaboeuf, France).

**Synthesis of FRET peptide substrates:** FRET Fluorogenic Abz-peptidyl-EDDnp substrates for human and mouse neutrophil serine proteases were synthesized as previously reported (18). Substrates were purified by semipreparative reverse phase chromatography using a 50-min linear (0–100%) gradient of acetonitrile in 0.1% trifluoroacetic acid and checked for homogeneity by analytic RP-HPLC on a C18 column and MALDI-TOF mass spectrometry (TofSpec-E; Micromass, Manchester, UK), or peptide sequencing. Stock substrate solutions (2–5 mM) were prepared in 30% (vol / vol ) N, N’-dimethylformamide and diluted to 0.5 mM with 50 mM Hepes buffer pH 7.4. Stock solutions of Cys-containing substrates were supplemented with 10 mM dithiothreitol (DTT).

**Enzyme Assays.** Protease activities were assayed in 50 mM Hepes, pH 7.4, 150 mM NaCl, supplemented with 0.05% Igepal CA-630 (vol/vol) to prevent protease sticking to plastic and glass surfaces. Purified mouse NE (mNE) and mouse Pr3 (mPr3) were titrated with human α1-PI and mouse CG (mCG) with human ACT (19,20).

The activities of mNE, mPr3 and mCG were first examined using the polyvalent FRET substrate Abz-HPVPVYAFSPQ-EDDnp. Substrate hydrolysis was followed by measuring the fluorescence at ex = 320 nm and em = 420 nm, as reported earlier (18,21).

Specificity constants (kcat/Km) were determined under first-order conditions using substrate concentrations far below the Km (max 1 µM). The final concentration of all 3 enzymes was in the nanomolar range. The sites in the FRET substrates cleaved by mouse and human proteases were identified by mass spectrometry and/or N-terminal sequencing. Fluorogenic substrates (5–8 µM final) were incubated with human or mouse Pr3, NE and CG (1–10 nM) in reaction buffer at 37 °C. The reaction was stopped by adding 4 volumes of absolute ethanol and incubating for 15 min on ice. Precipitated protein was removed by centrifugation at 13,000 x g for 10 min. The supernatant containing the hydrolysis products was dried under vacuum and dissolved in 200 µl 0.01% trifluoroacetic acid (vol/vol). Hydrolysis fragments were purified by reverse phase chromatography on a C18 column (2.1mm x 30 mm) eluted at 0.3 ml/min with a linear (0–60%, vol/vol) gradient of acetonitrile in 0.01% trifluoroacetic acid for 20 min. Eluted peaks were monitored at three wavelengths (220, 320, and 360 nm) simultaneously to directly identify EDDnp-containing peptides prior to sequencing.

**Modeling of murine neutrophil serine proteases:** Because human and mouse NSPs share about 70% sequence identity, we used various automated comparative modeling tools to generate models of murine NSPs based on the 3D structure templates of HNE, hPr3 and hCG respectively. These include Swiss-Model (http://swissmodel.expasy.org/SWISS-MODEL.html), (PS)² (http://ps2.life.nctu.edu.tw/) and ModWeb (http://modbase.compbio.ucsf.edu/ModWeb20-html/modweb.html). The sequences of mNE (UniProtKB entry name ELNE_MOUSE), mPr3 (UniProtKB entry name PRTN3_MOUSE) and mCG (UniProtKB entry name CATG_MOUSE) were used as inputs for the automatic modeling mode. All the models
were then evaluated using ANAOLEA (http://swissmodel.expasy.org/anolea/) to analyze the non-local environment of each heavy atom in the protein structure and EVAL123D (http://bioserv.cbs.cnrs.fr/HTML_BIO/valid.html), which allows evaluation of 3D protein structures by different methods (Verify3D, Eval23D, ProsaII, EvTree, SFE) in a single run. Based on this analysis, we found that SwissModel provides the best models for murine NSPs. All subsequent studies were carried based on these models.

**Electrostatic potential calculations:**
The electrostatic potentials of mNE, mPr3 and mCG were calculated using the finite difference Poisson Boltzmann (FDPB) method as implemented in DELPHI software (INSIGHT II suite, Accelrys Inc). The ionic strength was set to 0.15 M and the dielectric constant to 4 for the protein interior and 80 for the solvent. Calculations were performed at pH 7.4 at a temperature of 300°C. Formal charges, rather than all charges, were assigned as follows: arginine, lysine, N-terminus, +1; glutamate, aspartate, C-terminus, -1; and histidine, neutral. Contours of electrostatic potentials were displayed at ± 5 kTe⁻¹ using INSIGHT II. The locations of the various subsites in the active site of each protease were inferred from the positions of the side-chains of the corresponding residues of the OMT3 inhibitor complexed with HNE (PDB entry code 1PPF). The solvent-accessible molecular surface was calculated and colored according to the electrostatic potential using Insight II (Accelrys Inc.).

**RESULTS AND DISCUSSION**
In the present work, we purified mouse neutrophil serine proteases and characterized them by comparison to their human counterparts. This allowed us to generate sensitive peptide substrates to individual murine proteases namely mNE, mCG and mPr3.

**Purification of mouse NE, CG and Pr3.** As shown in Figure 1A, intraperitoneal instillation of glycogen resulted in massive recruitment of inflammatory cells. In accordance with our previous work and as judged by morphologic criteria, the cellular infiltrate was predominately neutrophils. Mouse peritonea were lavaged and collected cells were processed for NSPs purification as outlined in methods. To ensure the presence of mouse NSPs, aliquots of cell protein extracts were subjected to Western blotting using antibodies specific to mNE, mCG, and mPr3 (Figure 1B). Next, mouse NSPs were extracted/purified as outlined in Figure 1C. Following loading of neutrophil protein extracts on heparin-Sepharose column, we detected peptidase activity in both flow through and NaCl gradient-eluted fractions using our polyvalent FRET substrate Abz-HPVPVYAFSPQ-EDDnp, a peptide sensitive to both trypsin- and chymotrypsin-like human proteases. Eluted fraction showed an asymmetric peak and was subjected to further fractionation using a cation exchange chromatography. This step allowed us to identify/separate peptidase activities into two fractions. The flow through fraction of the heparin column was loaded on phenyl-Sepharose and peptidase activity was found in the fraction eluted with 50% isopropanol (Figure 1C). All three fractions were HPLC-purified and processed for N-terminal amino acid sequencing. Peptide sequence analyses found that the phenyl-Sepharose fraction corresponded to mPr3 while each of the two fractions with peptidase activity eluted from the cation exchange column corresponded to mNE or mCG (Figure 1D). Comparison of the net charges of mouse and human NSPs showed that the murine NSPs were less cationic than their human counterparts with mCG and mPr3 being the most positively and negatively charged enzymes. Of interest, amino acid sequence analyses identified, in addition to mouse NSPs, a protein called calgranulin. This protein is a member of the EF-hand calcium binding protein family, also known as cystic fibrosis antigen. It expressed in neutrophils, macrophages and epithelial cells (22). While its presence might suggest potential interaction with cationic proteases, in vivo significance of this finding is awaited. Interestingly, analysis of the net charge of murine NSPs calculated from their cDNA- deduced amino acid sequences support our purification strategy (Table 1). Next, the kinetic properties of all three isolated proteases were determined.
Characterization of purified mouse neutrophil serine proteases. In recent years, we have generated highly specific FRET substrates enabling us to detect human NSPs in the 0.1 nanomolar range. Because of the conservation of the active site triad and the physicochemical property closeness between human and mouse NSPs, we first investigated kinetic properties of purified mNE, mCG and mPr3 using human NSP substrates. While incubation of hCG FRET peptide with mCG resulted in high recorded fluorescence value, no changes in fluorescence were detected with hNE and hPr3 substrates in the presence of mNE or mPr3 (Tables 2, 3). These data indicate that mNE and mPr3 substrate specificities differ from those of their human counterparts. In support of these differences in substrate recognition between human and mouse NSPs, the potent recombinant human elastase inhibitor EPI-hNE4 did not interact with mNE (23). However, the human serpin α1-Pi inhibited both mNE and mPr3, and human ACT inhibited mCG. In this regard, we were able to titrate the activities of the three murine proteases using Abz-HPVPVYAFSPQ-EDDnp, against mNE and mPr3, and the hCG substrate Abz-TPFSGQ-EDDnp against mCG (Figure 2). The titrated mouse proteases were then used to raise new specific mouse NSPs substrates and to determine the kinetic parameters of their hydrolysis. To this end, we first identified the structural differences between mouse and human proteases that could potentially account for their differences in substrate specificity. We constructed molecular models of the three mouse NSPs using the 3D crystal structures of their human counterparts as templates, and compared the structures of the critical subsites previously identified as participating in substrate binding.

Design and testing of sensitive mNE substrate. As reported in Table 2, hNE-specific FRET substrates were not cleaved by mNE. We hypothesized that the inability of mNE to target such peptides could be due to the presence of charged residues at P2-P3 and P2'-P3'. In support of this hypothesis, the peptide substrate Abz-HPVPVYAFSPQ-EDDnp, which is cleaved by both hNE and mNE at the VY bond, bears no charge at these positions as well (Table 2). Of interest, when we compared the electrostatic surface potentials of mNE and hNE, we found that the S side of mNE active site was far more negative than that of hNE, (Figure 3) which may explain why mNE does not accommodate the E-E pair at P2-P3 in hNE substrates (Table 2). On the S' side, the two L → R amino acid substitutions at position 143 and position 35 in mNE, also create a positively charged environment close to S2’ and possibly S3’ subsites (Figure 3B). This may explain why hNE substrates with positively charged residues at P2’ and/or P3’ are not accomodated by mNE. But substitution in hNE substrates of one or the two positively charged residues at P2’ and P3’ by negative or neutral residues did not allow cleavage by mNE (Table 2). Because Abz-HPVPVYAFSPQ-EDDnp is cleaved by all three mouse NSPs, we sought to produce a mNE-specific FRET substrate from a sequence in a natural NE substrate, thus has neither charged residues at P2-P3 and P2’-P3’ nor a putative cleavage site for cathepsin G. We identified such a sequence in both mouse and human leukemogenic PML-RARα protein (24). The resulting substrate Abz-QPMAVVQSVQPQ-EDDnp was indeed rapidly hydrolysed by mNE with a kcat/Km in the 10⁶ M⁻¹ s⁻¹ range (Table 2). While this peptide was resistant to cleavage by mCG, it was cleaved at the V-V bond 34 times more slowly by mPr3. When we attempted to prevent mPr3-mediated hydrolysis by introducing a negatively charged residue at P2 position (Abz-QPMDVVQSVQPQ-EDDnp), the resulting substrate was no longer cleaved by mNE probably due to the electronegative surroundings of mNE S2-S3 subsites. Our strategy to introduce a single negative charge at P3 failed as well, but did confirm that the hNE APEEIMDRQ-EDDnp substrate was not cleaved by mNE because of its unprimed negative sequence.

Generation of a mPr3 substrate following specific amino acid substitution in hPr3 substrates. We have previously identified critical residues in the reactive site of human Pr3 that contribute to its substrate specificity. These are K99 at the S2 subsite, D61 at both S1’ and S3’, and most importantly R143 at the S2’ subsite (21). Interestingly, D61 and R143 remained conserved while K99 was substituted
with N99 in mPr3 (25) (Figure 4A). Features of hPr3 substrates are characterized by a negatively charged residue at P2 that is accommodated within the S2 lysyl-containing subsite (Table 3). We replaced the P2 aspartyl residue by an arginyl residue in the lead human Pr3 substrate, Abz-VA(D→R)CRDRQ-EDDnp. Hydrolysis of the new substrate, Abz-VARDCRDRQ-EDDnp, resulted in a 12 fold increase of mPr3 kcat/Km while hPr3 kcat/Km decreased by 160 fold (Table 3). Its cleavage site, C-R bond, remained unchanged as judged by comparison of the reverse phase HPLC retention time of the EDDnp-containing peptide with that of hPr3-cleaved Abz-VADCRDRQ-EDDnp (21). Of note, neither mNE nor mCG cleaved Abz-VARDCRDRQ-EDDnp. To improve the sensitivity of this substrate to mPr3 we substituted the P’ sequence -RDRQ- with the sequence -ADYQ-, which increases cleavage susceptibility of hPr3 substrates (21) (Table 3). The kcat/Km values for both mPr3 and hPr3 increased significantly suggesting that the S’ subsites are less important for discriminating between hPr3 and mPr3. Mouse Pr3 did not cleave the hPr3 substrate derived from the NFkB internal sequence that also contains an aspartyl residue at P2 (21) further highlighting the importance of the S2 subsite for discriminating between human and mouse Pr3. In support of these observations, analysis of the 3 D model of mPr3 confirmed that the main difference between the mouse and human Pr3 resides in the S2 subsite (Figure 4B); The R60Q and K99N substitutions generate an overall negative environment surrounding the S2 subsite in mPr3. This is also reflected by the fact that mouse Pr3 has a more negative net charge than human Pr3 (Table 1). Unlike their S subsites, the overall conformations of the S’ subsites of the mouse and human Pr3 seem to be very similar (Figure 4B).

**Targeted changes in hCG amino acid substrates explains mCG substrate specificity.** Contrary to mNE and mPr3, mCG cleaved hCG substrates particularly the FRET peptide Abz-TPFSGQ-EDDnp with a far better kcat/Km value (Table 4). This latter peptide is derived from hCG protein substrate, the protease-activated-thrombin receptor PAR-1 (26). Of interest, hCG-mediated cleavage at F-S bond was conserved in mCG. The other sensitive hCG peptide, Abz-EPFWEDQ-EDDnp, a peptide derived from the reactive loop of the human serpin ACT, was also cleaved by mCG at F-W bond (Table 4). Of note, human ACT inhibits not only hCG but also mCG.

To better our understanding of mCG-FRET peptide substrate interaction, molecular modeling studies revealed that only slight differences in the partition of charged residues between mCG and hCG reside in the surrounding of active site (Figure 5B) indicating that mCG and hCG might exhibit similar substrate specificity. There remains, however, some obvious difference namely the hCG Arg at position 41 in the vicinity of the S2’ binding sites that is replaced with Ala in mCG (Figure 5B). Such difference does not prevent mCG and hCG from accommodating a negatively charged residue at P2’ as observed using the Abz-EPFWEDQ-EDDnp substrate. But, if a positive residue was present at P2’, it could possibly impair interaction with hCG, but not (or to a lesser degree ) mCG. To test this hypothesis, we replaced the P2’ D residue in the polyvalent MNEI-derived Abz-GIATFCDLMEQ-EDDnp substrate cleaved at the F-C bond by hCG, by an R residue. Analysis of substrate cleavage found that the Arg-containing substrate was cleaved 10 times more slowly by hCG, and ~3 times faster by mCG suggesting that the S2’ subsite in cathepsin G is important for substrate binding and that residue at position 41 is involved in the P2’ specificity of cathepsin G (Table 5). Another difference between hCG and mCG G is the absence of Glu 226 at the bottom of the S1 pocket in mCG (Figure 5C). This negatively charged residue could explain why both P1-Phe and P1-Lys residues are accommodated in the hCG S1 pocket (27). Consequently, unlike its human homolog, mCG should not cleave after Lys residues. This assumption was tested by comparing cleavage rates of two FRET substrates that differed only by Phe or Lys at P1, by hCG and mCG. Under similar experimental conditions, mCG cleaved only Abz-TPFSALQ-EDDnp at a significant rate, while hCG hydrolysed both Abz-TPFSALQ-EDDnp and Abz-TPKSAQ-EDDnp, although the latter peptide was cleaved ~10-times slower (Table 5).
Potential in vivo implications of our findings.

It is widely accepted that NSPs play a critical role in neutrophil-associated lung inflammatory and tissue-destructive diseases. To investigate NSP pathogenic role(s), various mouse experimental models have been developed that mimic acutely or chronically injured human lungs. As alluded to earlier, the relative contribution of NSPs to lung disease processes as well as their underlying mechanisms remain still poorly understood. And the lack of purified mouse NSPs and their specific substrates have hampered advances in these studies. In this work, we provided evidence that peptide substrate specificities of human and mouse NSPs could be different in spite of their conserved cleft and close structural resemblance. We then went on to identify key residues in the vicinity of the active sites of human and mouse NSPs, which account for these specificities. It must be emphasized that the positioning of charged residues contributes, but may not fully explain, the observed substrate specificity in both types of proteases.

In any event, mouse and human NSPs may well generate different peptide profiles from a common substrate. For example, hNE and mNE generate chemotactic elastin peptides that differ structurally (but not functionally) (28). Because of its different S2 specificity mPr3 does not cleave the hPr3 substrate deduced from the internal sequence of NFκB whose cleavage impacts cytokine network and cell viability during inflammation (29). Since mouse NFκB has the same internal sequence as its human counterpart, this raises the question of the role of mPr3 as a NFκB-degrading protease that influences the endothelial cell response during inflammation. We also showed that mCG does not cleave after Lys residues, unlike hCG, because it appear to lack a negatively charged residue at the bottom of the S1 pocket. In addition, hCG poorly accommodates positively charged residues in its S2’ site, while mCG does not. This might significantly influence the physiological function of the mouse protease which may involve the cleavage of protein substrates. Also, enzyme inhibitors may exhibit different affinities towards mouse and human NSPs. In support of this, eglin C inhibits mPr3 but not hPr3 and recombinant hNE inhibitor Val15 aprotinin is a poor inhibitor of mNE (30). Another example is the recently engineered hNE inhibitor, EPI-hNE4. This molecule initially designed as a putative therapeutic for lung inflammatory diseases does not inhibit mNE (23,31). It must be emphasized that crystal/X-ray structures of mouse neutrophil serine proteases need to be determined to ascertain our findings and gain in depth insights on the structural differences with their human counterparts. In any event and in light of our findings, data generated from mouse models studying disease pathogenesis or testing NSP inhibitors should be cautiously interpreted before extrapolation to human situations. Also, inhibition strategies of human NSPs using mice as experimental models (intermediate step) should take into consideration the differences in substrate specificities between mouse and human NSPs.

In conclusion, the availability of mice deficient in NSPs should continue to help define the relative contribution of these proteases in neutrophil-associated lung diseases. The identified structural differences between human and mouse NSPs and the now available peptide substrates to specifically detect/quantify either mNE, mCG, and/or mPr3 should better our understanding of NSP roles in disease pathogenesis.

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**FOOTNOTES**
The nomenclature used for the individual amino acid residues (e.g. P1, P2, etc.) of a substrate and corresponding residues of the enzyme subsites (e.g. S1, S2, and so on) is that of Schechter and Berger (32).

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The abbreviations used are: COPD, chronic obstructive pulmonary disease; NSP, neutrophil serine protease; hNE, human neutrophil elastase, hPr3, human proteinase 3; hCG, human cathepsin G; mNE, mouse neutrophil elastase; mPr3, mouse proteinase 3; mCG, mouse cathepsin G; α1-Pi, α1-protease inhibitor; ACT, antichymotrypsin; FRET, fluorescence resonance energy transfer; PBS, phosphate-buffered saline; rpHPLC, reversed-phase high-performance liquid chromatography. PDB ID codes: HNE (PDB entry code 1PPF), hPr3 (PDB entry code 1FUJ), hCG (PDB entry code 1CGH).

**FIGURE LEGENDS**

**Figure 1:** Purification of mouse neutrophil serine proteases. A) Cytospins for lavage fluids at 16h revealed that neutrophils dominated the cellular infiltrate following intraperitoneal injection of glycogen. Shown is a representative micrograph of glycogen-elicitated cells (x200, inset x400). B) Immunodetection of mNE, mCG, and mPr3 in neutrophil protein extracts. Arrowheads indicate the position of serine proteases. Immunoblotting for MPO was used an internal control. MPO antibody detect the H chain (~59 kDa) and an autocatalytic cleavage product (~45kDa). Molecular standards are shown on the left. C) Scheme of purification strategy of individual mouse neutrophil serine proteases. D) NH2-terminal sequencing of the end product of protease purification resulted in an unambiguous amino-acid sequence identical to mPr3, mNE, or mCG.

**Figure 2:** Titration of purified active mNE and mPr3 against human serpin α1-Pi and mCG against human ACT. Purified proteases were incubated with increasing amounts of titrated inhibitors for 15 minutes before starting the reaction with appropriate FRET substrates. Fluorescence was recorded spectrofluorometrically for 3-5 minutes. Purified protease working solutions were all in the nanomolar range. Working solutions of inhibitors were 6nM, 15nM and 3.6nM for mNE, mPr3 and mCG respectively, in accordance with the protease concentrations.

**Figure 3:** Active site region of murine elastase compared with that of human elastase. A) The sequence of murine neutrophil elastase (mNE) was aligned with its orthologous human counterpart (hNE) using Jalview (http://www.jalview.org/). Identical residues are shown against a gray background. Residue numbers based on the chymotrypsinogen sequence numbering system are indicated above the alignment. Asterisks denote the position of the amino acids of the catalytic triad (His57, Asp102 Ser195). Amino-acid sequences were from UniprotKB database files ELNE_MOUSE and ELNE_HUMAN for mouse and human elastase respectively. B) The solvent-accessible surface of each protease is colored to show its positive (dark blue) and negative (red) electrostatic potential calculated with DELPHI software. The boxed region of each protease, which corresponds to the active site region, has been enlarged to show the location of S4 to S3’ subsites as inferred from the side-chain location of the P4-P3’ residues of OMTKYIII inhibitor complexed to HNE (PDB accession number: 1PPF). In the close-up view, the catalytically active serine (Ser 195) is coloured yellow. Charged critical residues that lie in the vicinity of the active site are indicated. The main difference lies in residues lining the S2’ subsite of both proteases where the neutral Leu143 in hNE is replaced by a positively charged arginyl residue.
(Arg143) in mNE. The substitution of Leu for Arg at position 35 in mNE may also have an influence on the P3’ specificity.

The contour lines of electrostatic potential are -5 kT/e (red) and +5 kT/e (blue). Model of murine elastase was built as described in Materials and Methods and the atomic coordinates for human NE was extracted from PDB file 1PPF.

Figure 4: Comparison of murine and human proteinase 3 active sites.
A) Alignment of murine (UniprotKB accession name PRTN3_MOUSE) and human (UniprotKB accession name PRTN3_HUMAN) proteinase 3 sequences showing identical (gray background) and substituted residues between both sequences. Amino-acid numbers correspond to those of the chymotrypsinogen sequence. Asterisks denote the position of the amino acids of the catalytic triad (His57, Asp102 Ser195).

B) Values of electrostatic potentials (calculated as in Figure 3) of murine and human proteinase 3 were mapped onto the solvent-accessible surface of each protease. Positive and negative regions of the molecular surface are coloured blue and red respectively. Critical charged residues suspected in substrate specificity are indicated in the close-up view of the protease active site (Boxed region). Note the positive residues Arg60 and Lys99 of the substrate binding region in hPr3 are replaced by the neutral Gln60 and Asn99 in mPr3 respectively.

The structural model of mPr3 was derived from hPr3 X-ray structure (PDF file: 1FUJ) by homology modelling as described in Materials and Methods. Positionning of the S4 to S3’ subsites was assessed as in Figure 3. The catalytic serine (Ser195) is coloured yellow.

Figure 5: Compared structures of the active site regions of murine and human cathepsin G.
A) Sequence alignment for murine (UniprotKB accession name CATG_MOUSE) and human (UniprotKB accession name CATG_HUMAN) cathepsin G. Asterisks denote the position of the amino acids of the catalytic triad (His57, Asp102 Ser195). The critical Glu 226 (chymotrypsinogen numbering) of the S1 pocket of hCG is replaced by an alanyl residue in mCG. Both residues are shown against a red background.

B) The solvent-accessible surface of mCG and hCG has been coloured according to values of electrostatic potentials calculated with DELPHI software (blue: positive; red: negative; yellow: catalytic Ser195). The positively charged Arg41 in hCG active site is substituted by the neutral and small Ala residue in mCG. As seen in the zoomed view of each active site (boxed region), this leads to major differences in the shape and charge of the S2’-S3’ binding region. mCG model was built by comparative modelling techniques (see Materials and Methods) based on the hCG X-ray structure (PDB accession number 1CGH). Location of S4 to S3’ subsites in each protease active site was inferred as described in Figure 3.

C) Close-up view of the active sites (ribbon representation) of murine CG and human CG. Glu 226 in hCG, which allows accommodation of a Lys side-chain in the S1 pocket of hCG, is replaced by an Ala residue in mCG. Both views were prepared with PYMOL (www.pymol.org).
Table 1: Net charge of mouse and human neutrophil serine proteases

| Net charge (pH 7.4) | Mouse | Human |
|---------------------|-------|-------|
| Elastase            | +9    | +11   |
| Proteinase 3        | -2    | +1    |
| Cathepsin G         | +16   | +23   |

Table 2: Specificity constants kcat/Km for the hydrolysis of FRET elastase substrates

|          | mNE | hNE | mPr3 |
|----------|-----|-----|------|
| (mM⁻¹s⁻¹)|     |     |      |
| 1-Abz-APEEI MDRQ-EDDnp | nsh | 683 | 12   |
| 2-Abz-APEEI MRRQ-EDDnp | nsh | 531 | nsh  |
| 3-Abz-APEEI MDYQ-EDDnp | nsh | 123 | nsh  |
| 4-Abz-APEEI MDYQ-EDDnp | nsh | 296 | nsh  |
| 5-Abz-HPVPV YAFSPQ-EDDnp | 5230 | 6867 | 192 |
| 6-Abz-QPMAV VQSVQV-EDDnp | 4393 | 1093 | 129 |
| 7-Abz-QPMDV VQSVQV-EDDnp | <1  | nsh | 17   |

Values are means of at least three experiments. The error for kcat/Km is < 15%. Cleavage sites are indicated by a space in the peptide sequence. nsh, non significant hydrolysis.
Table 3: Specificity constants \( k_{cat}/K_m \) for the hydrolysis of proteinase 3-directed FRET substrates

|                | \( k_{cat}/K_m \) (mM\(^{-1}\)s\(^{-1}\)) |
|----------------|------------------------------------------|
|                | mPr3 | hPr3 | mNE |
| 1-Abz-VADC ADQ-EDDnp | nsh   | 614  | nsh |
| 2-Abz-VADC RDRQ-EDDnp | 15    | 6500 | nsh |
| 3-Abz-VADC ADYQ-EDDnp | 18    | 11379| nsh |
| 4-Abz-VARC RDRQ-EDDnp | 181   | 40   | nsh |
| 5-Abz-VARC ADYQ-EDDnp | 1077  | 388  | nsh |

Values are means of at least three experiments. The error for \( k_{cat}/K_m \) is < 15%. Cleavage sites are indicated by a space in the peptide sequence.

Table 4: Specificity constants \( k_{cat}/K_m \) for the hydrolysis of cathepsin G-directed FRET substrates

|                | \( k_{cat}/K_m \) (mM\(^{-1}\)s\(^{-1}\)) |
|----------------|------------------------------------------|
|                | mCG | hCG | mNE | mPr3 |
| Abz-TPF SGQ-EDDnp | 5500 | 162 | nsh | nsh |
| Abz-EPF WEDQ-EDDnp | 543  | 243 | nsh | nsh |

Values are means of at least three experiments. The error for \( k_{cat}/K_m \) is < 15%. Cleavage sites are indicated by a space in the peptide sequence.
Table 5: P2’ specificities of mouse and human cathepsin Gs

| Fluorescence (arbitrary units) | mCG | hCG |
|-------------------------------|-----|-----|
| Abz-GIATF CDLMPEQ-EDDnp       | 29  | 600 |
| Abz-GIATF CRLMPEQ-EDDnp       | 92  | 51  |
| Abz-TPF  SALQ-EDDnp           | 215 | 700 |
| Abz-TPK  SALQ-EDDnp           | <2  | 70  |

Values are means of at least three experiments. Cleavage sites are indicated by a space in the peptide sequence.
Figure 1 Kalupov et al

A
Peritoneal lavage cells

B

\[ \begin{align*}
34 & : NE \\
26 & : PR3 \\
34 & : CG \\
26 & : MPO
\end{align*} \]

C

Purification scheme

Peritoneal mouse neutrophil extract

\[ \quad \downarrow \quad \text{Heparin-Sepharose} \]

\[ \quad \downarrow \quad \text{flow through} \]

Phenyl Seph.

\[ \quad \downarrow \quad 2\text{-propanol gradient} \]

C4 HPLC

\[ \begin{align*}
\text{mAU} & : 50 \\
\text{min} & : 20
\end{align*} \]

\[ \quad \downarrow \quad 0.5\text{M NaCl} \]

SP-Seph.

\[ \quad \downarrow \quad 0.8\text{M NaCl} \]

C4 HPLC

\[ \begin{align*}
\text{mAU} & : 5 \\
\text{min} & : 15
\end{align*} \]

\[ \quad \downarrow \quad \text{N-terminal sequencing} \]

IVGGHEARPH.. = mPr3

IVGGRPARPH.. = mNE

IIGGREARPH.. = mCG
Kalupov et al Fig 2
Figure 3: Kalupov et al.
Figure 4: Kalupov et al.
Figure 5: Kalupov et al.
Structural characterization of mouse neutrophil serine proteases and identification of their substrate specificities: Relevance to mouse models of human inflammatory diseases
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