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Functional study of elafin cleaved by *Pseudomonas aeruginosa* metalloproteinases

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Abstract

Elafin is a 6-kDa innate immune protein present at several epithelial surfaces including the pulmonary epithelium. It is a canonical protease inhibitor of two neutrophil serine proteases [neutrophil elastase (NE) and proteinase 3] with the capacity to covalently bind extracellular matrix proteins by transglutamination. In addition to these properties, elafin also possesses antimicrobial and immunomodulatory activities. The aim of the present study was to investigate the effect of *Pseudomonas aeruginosa* proteases on elafin function. We found that *P. aeruginosa* PAO1-conditioned medium and two purified *Pseudomonas* metalloproteases, pseudolysin (elastase) and aeruginolysin (alkaline protease), are able to cleave recombinant elafin. Pseudolysin was shown to inactivate the anti-NE activity of elafin by cleaving its protease-binding loop. Interestingly, antibacterial properties of elafin against PAO1 were found to be unaffected after pseudolysin treatment. In contrast to pseudolysin, aeruginolysin failed to inactivate the inhibitory properties of elafin against NE. Aeruginolysin cleaves elafin at the amino-terminal Lys6-Gly7 peptide bond, resulting in a decreased ability to covalently bind purified fibronectin following transglutaminase activity. In conclusion, this study provides evidence that elafin is susceptible to proteolytic cleavage at alternative sites by *P. aeruginosa* metalloproteinases, which can affect different biological functions of elafin.

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Introduction

Elafin, also known as SKALP (Skin-derived AntiLeukoProtease) or ESI (Elastase-Specific Inhibitor), is a small-size (6 kDa, 57 amino acids), cationic protease inhibitor first discovered in the skin (Schalkwijk et al., 1990; Wiedow et al., 1990) and the lung (Sallenave and Ryle, 1991). Similar to SLPI (secretory leucoprotease inhibitor), elafin belongs to the chelonianin family, one of the 18 families of canonical inhibitors. Structurally, this inhibitor is a member of the WAP (whey acidic protein) family which is characterised by the presence of one or more domain that contains eight highly conserved cysteine residues organised into four disulphide bridges. Elafin is generated from a longer molecule known as trappin-2 or pre-elafin (10 kDa) possessing, in addition to the WAP domain, an amino-terminal cementoin domain, rich in transglutaminase substrate motifs with the consensus sequence GQDPVK. These sequences allow the inhibitor to covalently bind to a number of extracellular matrix (ECM) proteins following the catalytic action of transglutaminases (Nara et al., 1994; Guyot et al., 2005b). The presence of elafin *in vivo* indicates that the inhibitory WAP domain (elafin) is released from trappin-2 by proteolytic cleavage. A previous study showed that the mast cell protease tryptase could be involved in this process as the enzyme is able to generate elafin from trappin-2 (Guyot et al., 2005a). Both elafin and trappin-2 are potent inhibitors of two neutrophil serine proteases: neutrophil elastase (NE) and proteinase 3 (Pr3) (Ying and Simon, 1993; Zani et al., 2004). Trappin-2/elafin is mainly produced by epithelial cells and its expression is upregulated during inflammation. IL-1β, TNF-α, lipopolysaccharide (LPS) and NE are inflammatory mediators known to increase expression of the inhibitor in lung and skin epithelial cells (Sallenave et al., 1994; Reid et al., 1999; Pfundt et al., 2000; Bingle et al., 2001; Simpson et al., 2001; Meyer-Hoffert et al., 2003). The inhibitory properties of trappin-2/elafin confer a protective role for this protein in shielding mucosal surfaces against excessive neutrophil-mediated proteolysis during inflammation. Although elafin and trappin-2 were originally identified as antiproteases, recent studies indicate that elafin/trappin-2 possesses immunomodulatory (Henriksen et al., 2004; Butler et al., 2006), antibacterial (Simpson et al., 1999; Baranger et al., 2008), antifungal (Baranger et al., 2008) and antiviral activities. Readers are directed to a recent review of the functional activities of elafin/trappin-2 (Moreau et al., 2008).

The regulation of excessive proteolytic activity of neutrophil serine proteases during lung inflammation is crucial to

prevent serious tissue damage. Multiple factors in the pulmonary environment can inactivate endogenous protease inhibitors [α-1-antitrypsin (AAT), SLPI, elafin] and affect the local protease-antiprotease balance. Two distinct mechanisms are known to inactivate neutrophil serine protease inhibitors: oxidation and proteolytic inactivation. The lung environment during inflammation is oxidative as a result of reactive oxygen species produced by neutrophils. AAT, SLPI and elafin contain a methionine residue in their inhibitory loop that can be oxidised into a methionine sulphoxide. This modification was shown to considerably decrease the affinity of the antiproteases for their target enzymes (Beatty et al., 1980; Boudier and Bieth, 1994; Nobar et al., 2005). Host and exogenous proteases can also mediate the inactivation of inhibitors. Several matrix metalloproteases are known to cleave and inactivate AAT (Banda et al., 1980; Michaelis et al., 1990; Mast et al., 1991). SLPI was shown to be cleaved and inactivated by elastolytic cysteine cathepsins B, L and S in the epithelial lining fluid from emphysematous patients (Taggart et al., 2001). Elafin and AAT (but not human SLPI) are susceptible to proteolytic activity of the house dust mite protease Der p 1 (Brown et al., 2003). We have recently demonstrated that excessive levels of NE activity in the *Pseudomonas*-infected cystic fibrosis lung can degrade and inactivate activities associated with elafin function (Guyot et al., 2008). Bacterial proteases can also be involved in maintaining inflammation by breaking down the lung anti-elastase screen. *Pseudomonas aeruginosa*, an opportunistic pathogen involved in several severe human infections, produces metalloproteases able to cleave a large variety of substrates including ECM proteins, cytokines, chemokines, immunoglobulins, complement components, antimicrobial peptides/proteins and protease inhibitors. Among the metalloproteases secreted by *P. aeruginosa*, pseudolysin (also called *Pseudomonas* elastase or LasB) has previously been reported to cleave and inactivate AAT and SLPI (Morihara et al., 1979; Johnson et al., 1982). To date, the proteolytic inactivation of elafin by bacterial proteases remains poorly studied. Only one recent study reports the cleavage of elafin within its protease-binding loop by the cysteinyl protease RgpB from *Porphyromonas gingivalis* (Kantyka et al., 2009). Given the virulence of *P. aeruginosa* and the vulnerability of the pulmonary anti-elastase screen to proteolytic inactivation, the aim of the present study was to evaluate the effect of *Pseudomonas* proteases on elafin.

Results

Effect of PAO1-conditioned medium on the integrity of elafin, SLPI and AAT

PAO1-conditioned medium was used as a complex medium to evaluate the effect of proteases secreted by *P. aeruginosa* on elafin. The elastase inhibitor was incubated with the PAO1-conditioned medium and analysed by Western blot using a biotinylated anti-elafin antibody. As shown in Figure 1A, elafin was quickly cleaved following 1-h exposure to the *Pseudomonas* medium. The class of protease(s) involved in the degradation of elafin was investigated by pretreating the medium with protease inhibitors. Figure 1B shows that only metalloprotease inhibitors (EDTA, GM6001 and phosphoramidon) could prevent the cleavage of elafin by PAO1-conditioned medium. Interestingly, phosphoramidon, an inhibitor of the *Pseudomonas* metalloprotease pseudolysin, was able to prevent elafin degradation after 2 h incubation but not 24 h. These results indicate that one or several *Pseudomonas* metalloprotease(s) including pseudolysin are involved in this process. The effect of PAO1-conditioned medium on SLPI and AAT was determined as positive controls as both inhibitors were previously shown to be cleaved by pseudolysin. As demonstrated in Figure 1C and D, SLPI and AAT were cleaved after 24 h incubation with *Pseudomonas*-conditioned medium. Interestingly, none of the individual non-specific inhibitors used in the study could prevent the cleavage of these antiproteases. However, the use of the serine protease inhibitor Pefabloc combined to the metalloprotease inhibitor EDTA was able to protect both SLPI and AAT against proteolysis (Figure 1C, D).

Effect of PAO1-conditioned medium on the antiprotease activities of elafin

As PAO1-conditioned medium was capable of cleaving elafin, we investigated whether this cleavage had any effects on the ability of elafin to inhibit NE using the chromogenic substrate N-(methoxysuccinyl)-Ala-Ala-Pro-Val-*para*-nitroanilide (MeOSuc-AAPV-pNA). Full-length elafin was approximately 100% inhibitory towards NE but its efficacy decreased to approximately 15% inhibition of NE activity after 1-h exposure to PAO1-conditioned medium (Figure 2). This decrease in inhibition was completely prevented by pretreating the medium with EDTA and phosphoramidon. Our result indicates that *Pseudomonas* metalloprotease(s) play a crucial role in cleaving and inactivating elafin and its ability to inhibit its cognate protease NE.

Effect of purified *Pseudomonas* metalloproteases on elafin integrity and antiprotease activity

Given the previous findings, purified *Pseudomonas* metalloproteases were investigated for their ability to cleave and inactivate elafin. Dose-response incubations of pseudolysin, staphylolysin (LasA) or aeruginolysin (*Pseudomonas* alkaline protease) were carried out for 2 h at 37°C and analysed by SDS-PAGE. As shown in Figure 3, recombinant elafin is cleaved by 10 nM pseudolysin (Figure 3A) and by 400 nM aeruginolysin (Figure 3C). In contrast, staphylolysin does not cleave elafin even at the highest concentration tested (10 μM; Figure 3B).

The effect of proteolytic cleavage on the inhibitory activity of elafin was investigated by measuring the anti-NE activity of elafin preincubated with various concentrations of pseudolysin (5–500 nM) and aeruginolysin (200–2000 nM) for various times (0, 1, 2 and 6 h) at 37°C. As shown in Figure 4A (i), pseudolysin gradually inactivated the antiprotease activity of elafin as a function of the enzyme concentration and the incubation time. Although the inhibition of

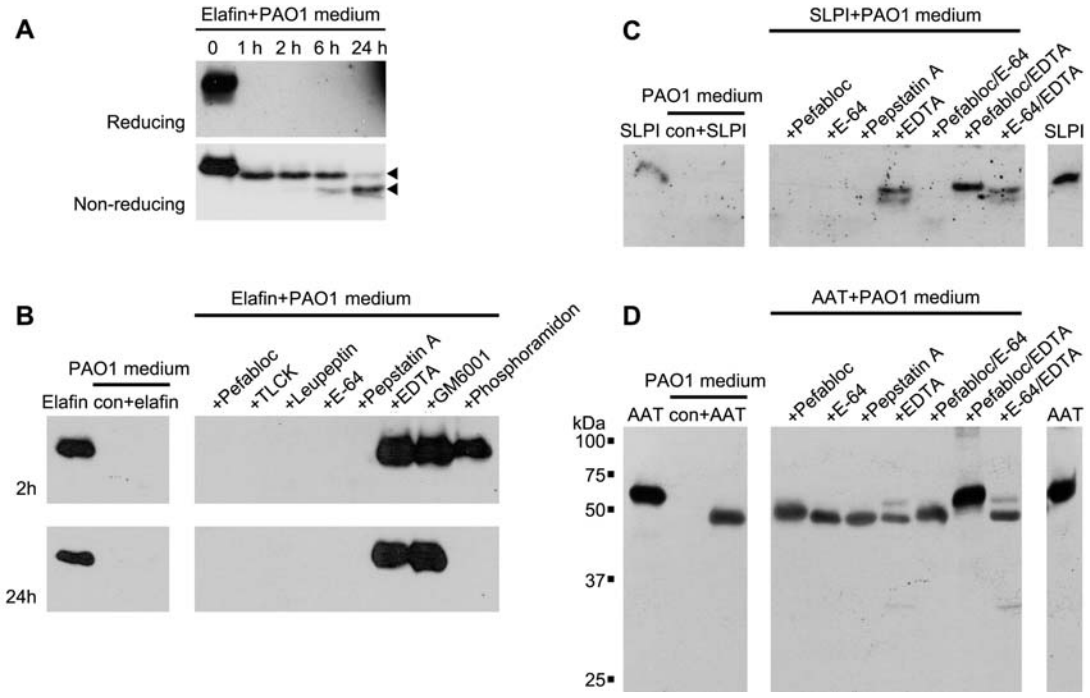

Figure 1 Effect of *Pseudomonas aeruginosa* (PAO1)-conditioned medium on the integrity of elastase inhibitors elafin, SLPI and AAT. In total, 8.5×10^{-7} M of elastase inhibitors (elafin, SLPI and AAT) were incubated with 10 μl PAO1-conditioned medium at 37°C and analysed by Western blotting. (A) Time-course incubation of recombinant elafin with PAO1-conditioned medium for 0, 1, 2, 6 and 24 h. Incubation products were analysed by Western blotting under reducing (upper panel) and non-reducing (lower panel) conditions. Arrowheads indicate cleavage products of elafin. (B) Identification of the *Pseudomonas* proteases involved in elafin cleavage. PAO1-conditioned medium was preincubated with the indicated protease inhibitors (right panels) and mixed with recombinant elafin for 2 h (upper panels) and 24 h (lower panels). Elafin alone, PAO1-conditioned medium incubated in absence (Con) or presence of elafin (+Elafin) are indicated in the left panels. Effect of PAO1-conditioned medium on (C) SLPI and (D) AAT integrity, and identification of the *Pseudomonas* proteases involved in their cleavage are shown. PAO1-conditioned medium was preincubated with the indicated protease inhibitors (middle panels) before incubation with SLPI and AAT for 24 h. SLPI or AAT alone, PAO1-conditioned medium incubated in absence (Con) or presence of SLPI (+SLPI) or AAT (+AAT) are indicated in the left and right panels.

NE by elafin was approximately 94–96% at the initial time of incubation and approximately 92% after 6 h at 37°C, we found that both 50 and 500 nM pseudolysin led to the rapid inactivation of elafin as NE inhibition was reduced to 21% and 15% after 2 h of incubation, respectively, and still continued to decrease to 5–8% inhibition after 6 h. Preincubation of 500 nM pseudolysin with EDTA completely inhibited pseudolysin-mediated inactivation of elafin as 95% of NE inhibition by elafin remained after 6 h at 37°C. Western blot analysis of samples with 50 nM of pseudolysin was performed to correlate the loss of activity with proteolytic cleavage. As shown in Figure 4A (ii), the majority of elafin was cleaved within 1 h of incubation. A fragment with an apparent mass slightly lower to that of native elafin was detectable under non-reducing conditions. Further cleavage of this fragment was detected with prolonged exposure as the amount of this fragment decreased from 1 h to 6 h incubation, whereas another fragment with a lower apparent mass was generated during the same period of time. Taken together, these results suggest that 50 nM pseudolysin rapidly cleaved elafin in such a way as to inactivate its anti-NE activity.

In contrast to pseudolysin, aeruginolysin did not inactivate the antiprotease activity of elafin, despite its ability to cleave the protein. Indeed, Figure 4B (i) shows that over 90% of NE still remained inhibited by elafin when the inhibitor was incubated alone or with 200 nM aeruginolysin. A slight decrease of NE inhibition to 80% was, however, observed with the highest concentration of enzyme (2000 nM) used in our experiment after 6 h incubation [Figure 4B (i)]. Western blot analysis of samples with 200 nM aeruginolysin demonstrated that elafin was completely cleaved after 1 h into a unique cleavage product [Figure 4B (ii)] that retained inhibitory properties as shown in Figure 4B (i).

Identification of pseudolysin and aeruginolysin generated cleavage sites

Elafin fragments generated by pseudolysin and aeruginolysin were separated by reverse phase HPLC (Figure 5) and identified by electrospray mass spectrometry. HPLC analysis of elafin incubated with 10 nM pseudolysin resulted in the generation of three main peaks (Figure 5A, peaks 1, 2 and 3). As shown in Table 1, product peptides of elafin were identified in peaks 1 and 2, whereas peak 3 was exclusively composed of the full-length elafin. A single peptide was identified in peak 1; it contained residues 26–57. The major

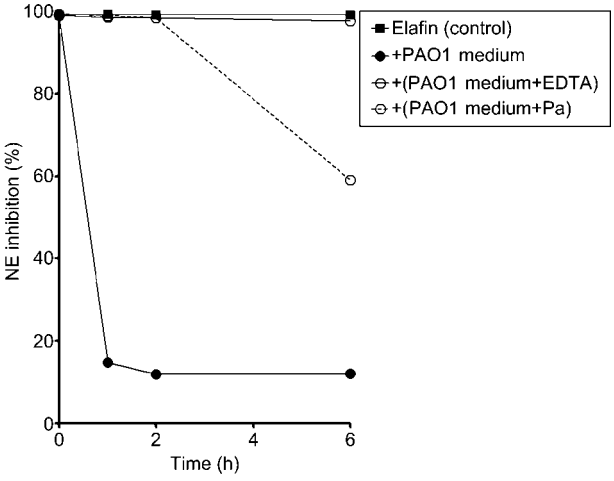

Figure 2 Effect of *Pseudomonas aeruginosa* (PAO1)-conditioned medium on the anti-elastase activity of elafin.
Recombinant elafin was incubated with Tryptic Soy Broth (control) or PAO1-conditioned medium [preincubated in the absence or in presence of the metalloproteinase inhibitors EDTA and phosphoramidon (Pa)] for 0, 1, 2 and 6 h and analysed for its inhibitory activity against neutrophil elastase (NE) by spectrophotometry using the NE substrate MeOSuc-AAPV-pNA.

(94%) elafin product detected in peak 2 was identified as containing residues 1–23. A peptide containing residues 1–24 constituted 5% and one with residues 1–25 was detected at 0.4%. These results indicate that 10 nM pseudolysin cleaved the inhibitor between Met25 and Leu26. The use of a higher concentration of the pseudolysin (100 nM) led to the elimination of native elafin (peak 3) and an increase in peaks 1 and 2 (Figure 5A). A HPLC chromatogram of elafin incubated with 400 nM aeruginolysin also displayed three major peaks (Figure 5B). Measured masses for each peak are indicated in Table 2 with the corresponding assigned sequence. The measured masses of peaks 1 and 3 allowed their identification as elafin residues 1–6 and 7–57. Peak 2 was identified as the native elafin. These data indicate that 400 nM aeruginolysin cleaved elafin at the Lys6-Gly7 peptide bond within 2 h incubation. Cleavage sites of elafin by pseudolysin and aeruginolysin are summarised in Figure 5C.

Effect of PAO1-conditioned medium on the anti-neutrophil elastase activity of normal bronchoalveolar lavage (BAL) fluid

PAO1-conditioned medium was incubated with bronchoalveolar lavage (BAL) fluid from healthy patients to mimic the first steps of colonisation of the lung by *P. aeruginosa*. As shown in Figure 6, normal BAL fluid exhibited anti-NE activity. The incubation of normal BAL fluid with PAO1-conditioned medium led to a rapid decrease in the anti-NE activity of the BAL fluid as 63%, 37% and 17% of inhibition of NE were detected after 1, 2 and 6 h of incubation, respectively, whereas normal BAL fluid displayed 78% of inhibition of NE after 6 h. Pretreatment of PAO1-conditioned medium with the metalloprotease inhibitors EDTA and phosphoramidon (a specific inhibitor of pseudolysin) resulted in the retention of anti-NE activity of the normal BAL fluid to a similar level of that observed for control: 73% and 82% of NE still remained inhibited by BAL fluid after 6 h incubation with PAO1-conditioned medium pretreated with phosphoramidon and EDTA, respectively.

Effect of aeruginolysin on the transglutaminase-mediated crosslinking of elafin to fibronectin

In contrast to our findings with pseudolysin, aeruginolysin cleaved recombinant elafin within its amino terminal extremity at Lys6-Gly7 and this cleavage did not alter its inhibitory properties. However, the amino terminal extremity of elafin contains a transglutaminase substrate motif (AQEPVK) that could be involved in crosslinking the inhibitor to ECM proteins by transglutamination. We thus hypothesised that the

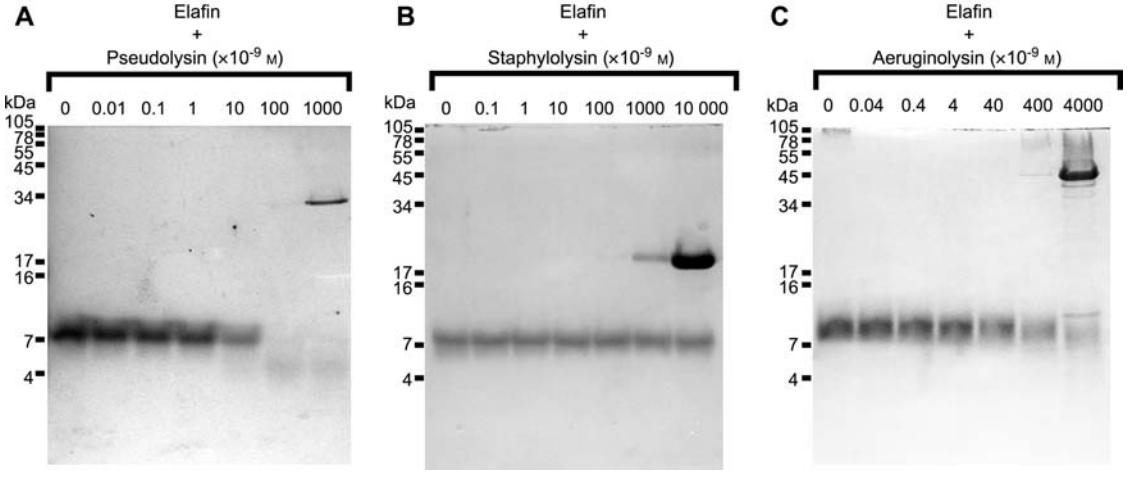

Figure 3 Effect of purified *Pseudomonas aeruginosa* metalloproteases on the integrity of recombinant elafin.
Recombinant elafin (3 μg, 25 μM) was incubated with various concentrations of purified (A) pseudolysin (0–1 μM), (B) staphylolysin (0–10 μM) and (C) aeruginolysin (0–4 μM) for 2 h at 37°C. Incubation products were separated by SDS-PAGE and visualised by Coomassie Blue staining.

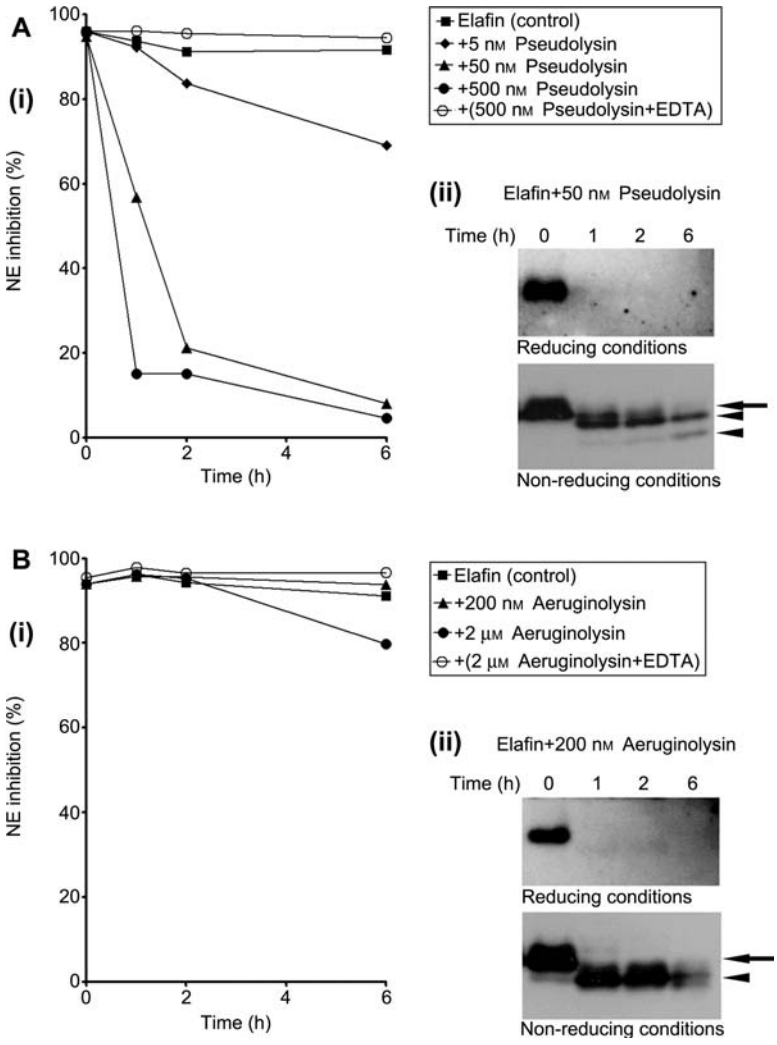

Figure 4 Effect of purified pseudolysin and aeruginolysin on the inhibitory activity of elafin.
Recombinant elafin was incubated alone (control) or with various concentrations of (A) pseudolysin or (B) aeruginolysin for 0, 1, 2 and 6 h at 37°C. Samples were analysed (i) by spectrophotometry for their inhibitory activity toward neutrophil elastase (NE) using the NE substrate MeOSuc-AAPV-pNA and (ii) by Western blotting under reducing (upper panels) and non-reducing (lower panels) conditions to confirm elafin cleavage in the presence of pseudolysin and aeruginolysin. Arrows and arrowheads indicate native elafin and cleavage products of elafin, respectively.

removal of this motif by aeruginolysin could suppress the transglutaminase-mediated binding of elafin to ECM proteins. Elafin has previously been shown to crosslink a number of ECM proteins including fibronectin. Therefore, we investigated the ability of the amino terminal truncated form of elafin to crosslink fibronectin by transglutamination. Recombinant elafin was pretreated with increasing concentrations of aeruginolysin and then incubated with GM6001. Samples were then incubated with plasma fibronectin in the presence or absence of guinea pig liver transglutaminase. Results were analysed by Western blotting using anti-elafin antibodies (Figure 7A) and anti-fibronectin antibodies (Figure 7B). As shown in Figure 7A (lanes 1–6), native recombinant elafin had an apparent mass of 7 kDa under non-reducing conditions. However, in the presence of fibronectin and transglutaminase (lanes 2, 4 and 6), part of the inhibitor was also detected at high molecular mass (>188 kDa) suggesting that the inhibitor was covalently crosslinked to fibronectin. Treatment of elafin with 100 nM aeruginolysin cleaved elafin resulted in the inhibition of its ability to crosslink to fibronectin by transglutamination (Figure 7A, lane 8). This effect is due to the truncation of elafin rather than fibronectin degradation as samples were treated with GM6001 to suppress metalloproteinase activity (Figure 7B).

Effect of pseudolysin on the antibacterial activity of elafin

The ability of pseudolysin to inactivate the inhibitory activity of elafin by cleaving the protease-binding loop prompted us to investigate the effect of such cleavage on the antibacterial

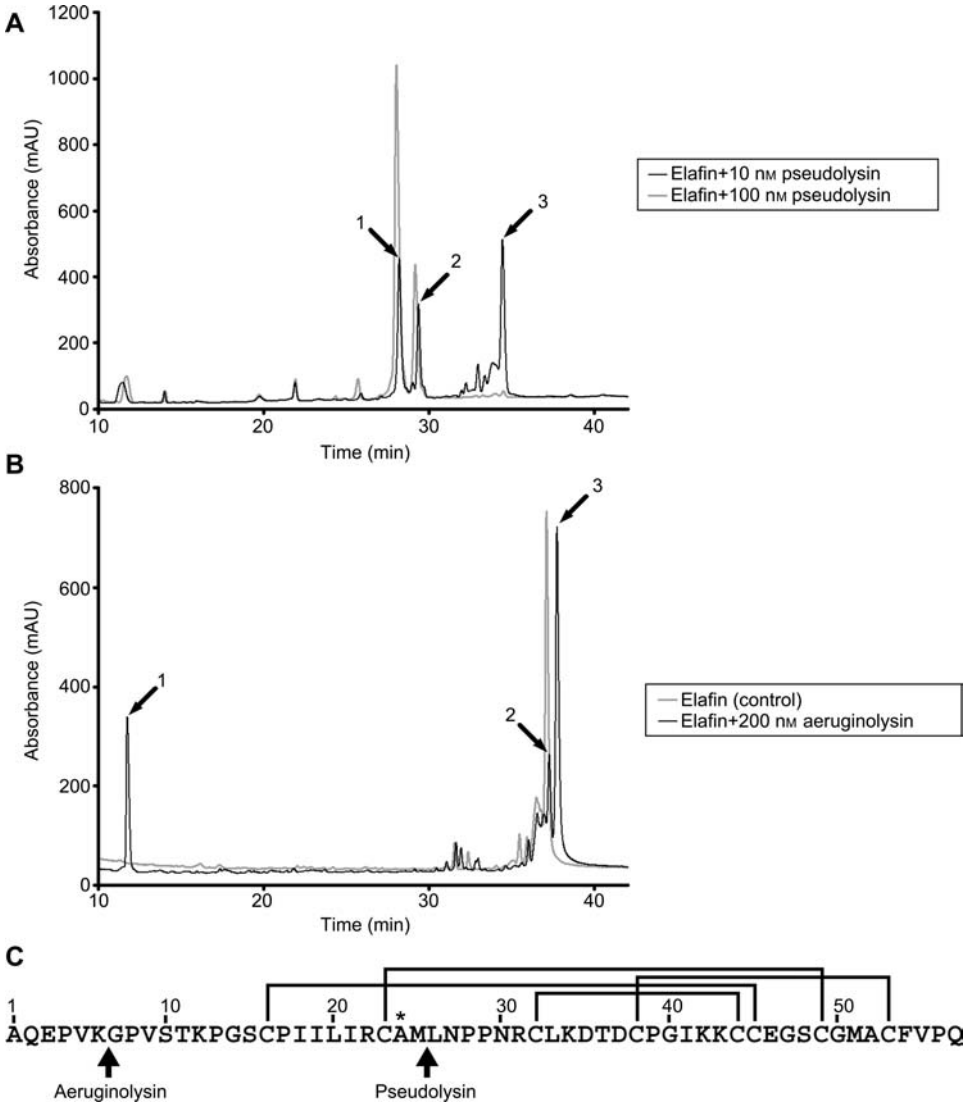

Figure 5 HPLC-mass spectrometry analysis of elafin incubated with pseudolysin and aeruginolysin.
Recombinant elafin (3 μg, 25 μM) was incubated with various concentrations of pseudolysin or aeruginolysin for 2 h at 37°C. Samples were then analysed by reverse phase HPLC coupled to electrospray mass spectrometry. (A) HPLC analysis of elafin incubated with 10 nM (black line) and 100 nM (grey line) pseudolysin. (B) HPLC analysis of elafin incubated alone (grey line) or with 200 nM (black line) aeruginolysin. (C) Identification of the cleavage sites in elafin. The amino acid sequence of elafin is represented from the amino-terminus (Ala1) to the carboxy-terminus (Gln57). The lines indicate the position of the disulphide bridges and the asterisk identifies the residue in P1 position within the protease-binding loop. Arrows highlight the cleavage sites generated by pseudolysin (at Met25-Leu26) and aeruginolysin (at Lys6-Gly7).

activity of elafin (Figure 8). Previous studies have demonstrated antibacterial activities of elafin against various bacteria including *P. aeruginosa* and were confirmed in our study by dose-response experiments using recombinant elafin and *P. aeruginosa* PAO1. PAO1 survival was diminished as a function of elafin concentration used (Figure 8A). To ensure that the observed effect was only due to the recombinant elafin, we assessed the purity of the elafin preparation by SDS-PAGE. As shown in Figure 8B, no contamination was observed as only one single band corresponding to elafin was detected at around 7 kDa after silver staining. The antibacterial assay was then performed using elafin treated with 30 and 3000 nM pseudolysin to generate cleaved forms of elafin. Our results presented in Figure 8C demonstrate that elafin, even cleaved by 30 and 3000 nM pseudolysin, retained its antibacterial properties against *P. aeruginosa* PAO1.

Discussion

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium causing severe infections in vulnerable hosts. Such infections can affect various tissues such as lung, cornea, skin or urinary tract. In the lung, *P. aeruginosa* is responsible for acute respiratory infections common in ventilated and immunocompromised patients and causes chronic respiratory

Table 1 Monoisotopic mass of elafin fragments generated by pseudolysin.

Peak	Observed mass (Da)	Calculated mass (Da)	Assignment
1	3426.53	3426.51	P_{26-57}
2	2392.31	2392.30	P_{1-23}
3	6002.86	6002.88	Full-length P_{1-57}

Elafin fragments obtained with pseudolysin and identified by HPLC (peaks 1, 2 and 3, Figure 5A) were analysed by mass spectrometry. The observed and calculated monoisotopic masses for the components constituting over 90% of each peak are represented.

Table 2 Monoisotopic mass of elafin fragments generated by aeruginolysin.

Peak	Observed mass (Da)	Calculated mass (Da)	Assignment
1	670.36	670.36	P_{1-6}
2	6002.86	6002.88	Full-length P_{1-57}
3	5350.52	5350.52	P_{7-57}

Elafin fragments obtained with aeruginolysin and identified by HPLC (peaks 1, 2 and 3, Figure 5B) were analysed by mass spectrometry. The observed and calculated masses and assigned sequence for each peak are represented.

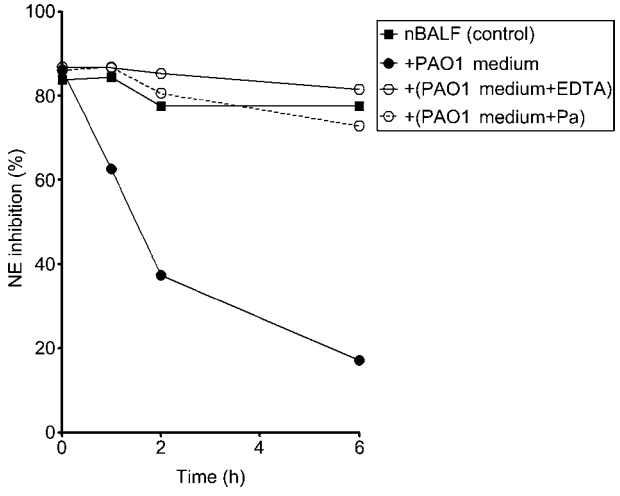

Figure 6 Inactivation of the anti-NE activity of normal BAL fluid by *Pseudomonas aeruginosa* PAO1-conditioned medium.
Normal BAL fluid was incubated with Tryptic Soy Broth (control) or PAO1-conditioned medium, preincubated in the absence or presence of EDTA and phosphoramidon (Pa) for 0, 1, 2 and 6 h and analysed for its inhibitory activity against neutrophil elastase (NE) by spectrophotometry using the NE substrate MeOSuc-AAPV-pNA.

infections in patients with cystic fibrosis. The severity of *Pseudomonas* infections is due to the ability of the pathogen to secrete extracellular factors associated with virulence, including toxins (exotoxin A, exoenzyme S), hemolysins (phospholipase C, lecithinase) and proteolytic enzymes. *Pseudomonas* proteases can cleave and inactivate a variety of lung proteins with important biological functions and are thus thought to play a role in promoting bacterial invasion. To date, one serine protease (arginyl peptidase) and three metalloproteases (pseudolysin, staphylolysin, aeruginolysin) have been associated with virulence of *P. aeruginosa*. Among these proteases, pseudolysin (*Pseudomonas* elastase, LasB) has been demonstrated to cleave and inactivate AAT and SLPI, two inhibitors of neutrophil serine proteases found in the lung. We confirmed in our study that both inhibitors are cleaved by proteases secreted by *P. aeruginosa* PAO1 following 24 h incubation. Interestingly, we found that only a combination of inhibitors of metalloproteases (EDTA) and serine proteases (Pefabloc) prevented this cleavage of SLPI and AAT. These results demonstrate that, in addition to pseudolysin, a serine protease secreted by *P. aeruginosa* PAO1 is able to cleave these inhibitors. We hypothesise that arginyl peptidase (protease IV) and/or LasD could be involved in this process. However, there is no evidence to date that *Pseudomonas* serine protease(s) can inactivate SLPI or AAT.

Our study indicates that elafin is also susceptible to proteolytic cleavage by *Pseudomonas* proteases, as the inhibitor is rapidly degraded in the presence of the supernatant of *P. aeruginosa* PAO1 culture. Additionally, we found that *Pseudomonas* supernatant is able to decrease the anti-NE activity of elafin. In contrast to SLPI and AAT, we found that only metalloprotease inhibitors (EDTA, GM6001 and phosphoramidon) prevented elafin cleavage. This result demonstrates that elafin is susceptible to one or more *Pseudomonas* metalloproteases. The observed resistance of elafin to proteolysis mediated by *Pseudomonas* serine protease(s) suggests that it is not as sensitive as SLPI and AAT, or could be explained by the capacity of elafin to inhibit serine protease(s) secreted by the pathogenic microorganism. Indeed, a recent study suggests that elafin inhibits growth of *P. aeruginosa* in complex media via the inhibition of a serine protease produced by the pathogen, presumably the arginyl peptidase protease IV (Bellemare et al., 2008). Identification of the *Pseudomonas* metalloprotease(s) involved in the cleavage and inactivation of elafin was carried out using purified *Pseudomonas* metalloproteases: pseudolysin, staphylolysin (LasA) and aeruginolysin (*Pseudomonas* alkaline protease, serralysin). Our study provides evidence that pseudolysin and aeruginolysin, but not staphylolysin, are able to cleave recombinant elafin. Although two *Pseudomonas* metalloproteases were shown to degrade elafin, functional investigations demonstrate that these proteases have distinct effects on the functional activities of elafin.

Elafin is a protein inhibitor belonging to the chelonianin family, one of the 18 families of canonical inhibitors (Laskowski et al., 2000; Krowarsch et al., 2003). The canonical inhibitors are a group of protein inhibitors characterised by a conserved protease-binding loop. The inhibitory loop is convex and matches perfectly with the concave active site of the target enzyme. The inhibition of proteases by the canonical inhibitors is reversible and obeys the standard mechanism of canonical inhibitors well-described elsewhere (Laskowski et al., 2000; Krowarsch et al., 2003). The crystallised structure of the complex between elafin and porcine

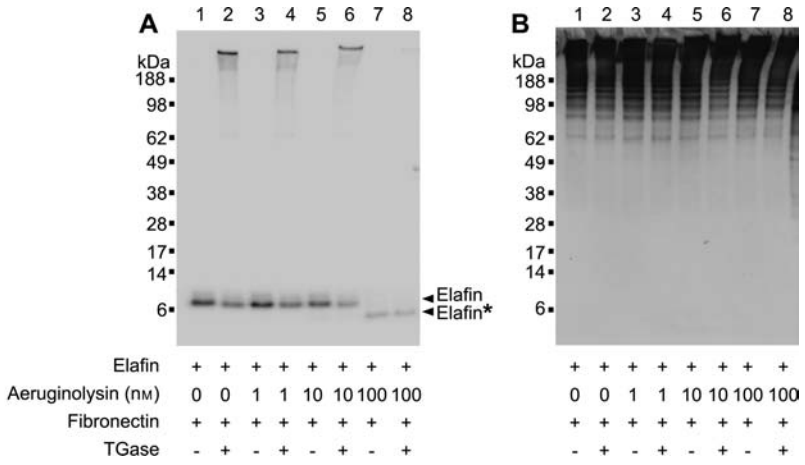

Figure 7 Effect of purified aeruginolysin on transglutaminase-mediated crosslinking of elafin to fibronectin.
Recombinant elafin (100 ng, 3 μM) was incubated for 2 h at 37°C with increasing amounts of aeruginolysin (lanes 1 and 2: no enzyme, 3 and 4: 1 nM, 5 and 6: 10 nM, 7 and 8: 100 nM, 9 and 10: 1000 nM). Samples were treated with GM6001 to inactivate aeruginolysin activity and incubated for 1 h at 37°C with fibronectin in the absence (lanes 1, 3, 5, 7) or presence (lanes 2, 4, 6, 8) of 0.38 mU guinea pig liver transglutaminase (TGase). Samples were analysed by SDS-PAGE followed by Western blotting using (A) a biotinylated anti-elafin antibody and (B) an anti-fibronectin antibody. In panel (A) intact and cleaved elafin (respectively denoted elafin and elafin*) are indicated by arrowheads.

pancreatic elastase (Tsunemi et al., 1996) indicates that seven residues of the inhibitory loop (Leu20–Leu26) are in contact with the enzyme and that the scissile bond is located at Ala24-Met25 also described as P1-P1′ peptide bond according to the nomenclature of Schechter and Berger (Schechter and Berger, 1967). In the present study, we report that pseudolysin cleaves elafin within its protease-binding loop yielding two peptides as major products, P_{1-23} and P_{26-57}. The key consequence of pseudolysin-mediated cleavage of elafin is the removal of both P1 and P1′ amino acid residues (Ala24 and Met25) from the protease-binding loop. Curiously, the elafin fragments, 1–23 and 26–57, generated by pseudolysin were detected by Western blotting under non-reducing conditions but not in the presence of reducing agents. This feature could be explained at least in part by the weak ability of the antibody to detect reduced elafin. The gap generated by the lack of the P1 and P1′ residues within the canonical loop could prevent association with target enzymes and thus prevent elafin inhibiting NE, as demonstrated in our study. The role of pseudolysin in the inactivation of elafin and the pulmonary anti-NE defence is underlined in our study by the capacity of phosphoramidon, a specific inhibitor of pseudolysin with no effect on staphylolysin and aeruginolysin activities, to prevent anti-elastase inactivation in normal BAL by PAO1-conditioned medium. The reason for using normal BAL in the study was to mimic the initial phases of the lung infection by *P. aeruginosa*. However, a severe infection will lead to a rapid and a massive influx of neutrophils into the lungs followed by a secretion of large amounts of neutrophil serine proteases in the airways including the target enzymes of elafin: NE and Pr3. In this context, a competition can then occur between pseudolysin and neutrophil serine proteases for the interaction with elafin. In addition, pseudolysin can inactivate neutrophil elastase making it difficult to evaluate the inactivation of elafin by pseudolysin in the presence of neutrophil elastase (Döring et al., 1985). In contrast to pseudolysin, aeruginolysin does not inactivate elafin. This enzyme was shown to cleave the inhibitor at Lys6-Gly7. This peptide bond is located in the amino terminal extremity of the inhibitor and structural data indicate that the amino terminal region (1–8) of elafin is flexible and is not involved in the antiprotease function of the inhibitor (Tsunemi et al., 1996; Francart et al., 1997). As with pseudolysin cleavage of elafin, the elafin fragment Gly7-Gln57 generated by aeruginolysin was detected by Western blotting under non-reducing conditions but not in the presence of reducing agents. Once again, this feature could be explained at least in part by the weak ability of the antibody to detect reduced elafin. Moreover, this observation could also suggest that the fragment Ala1-Lys6 contains a major epitope for the antibody. Similar observations were previously reported with the elafin fragments Lys6-Gln57 and Ser10-Gln57 generated by neutrophil elastase (Guyot et al., 2008).

Several studies have demonstrated that elafin and its precursor trappin-2 have intrinsic antimicrobial activities against various pathogens including bacteria (such as *P. aeruginosa*, *Staphylococcus aureus* and *Klebsiella pneumoniae*) and fungi (*Aspergillus fumigatus*, *Candida albicans*). Nevertheless, the mechanism by which elafin and trappin-2 act as antimicrobials remains unclear. It is thought that the cationic nature of both inhibitors as well as their structure (WAP domain) can play a key role in this process. Moreover, it has been found recently that elafin/trappin-2 can use an antimicrobial mechanism dependent (Bellemare et al., 2008) or independent (Baranger et al., 2008) of their antiprotease properties. It seems that the antimicrobial effect against *P. aeruginosa* and the mechanism of action of elafin/trappin-2 differ depending on the bacterial strain used in the studies. In the present study, we tested the antibacterial properties of elafin against *P. aeruginosa* PAO1. Around 30–40% of control PAO1 was

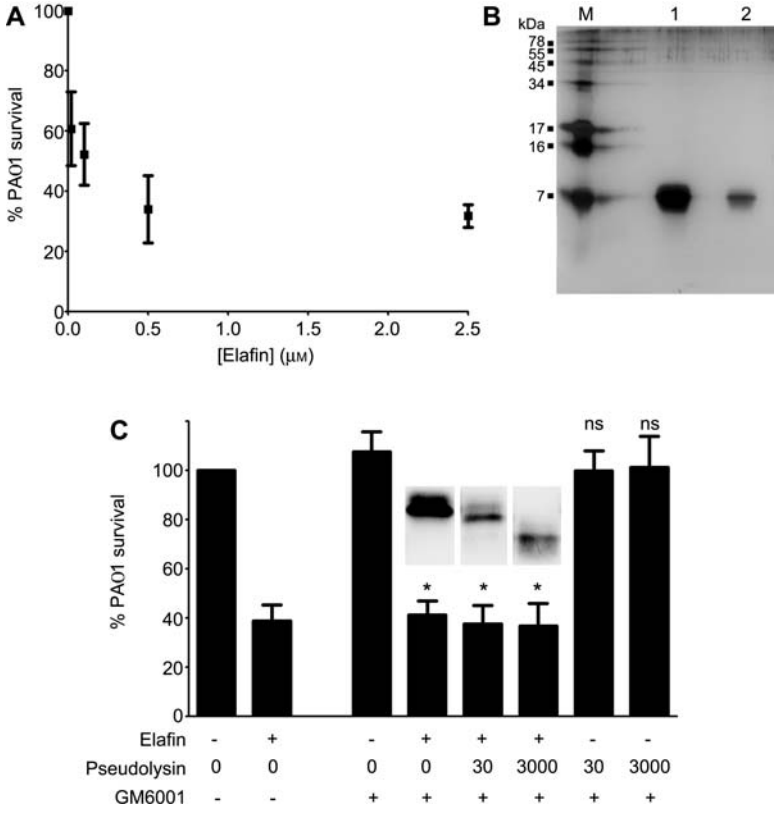

Figure 8 Effect of purified pseudolysin on the antibacterial activity of recombinant elafin against *Pseudomonas aeruginosa* PAO1. (A) Dose-response effect of elafin on *P. aeruginosa* PAO1 survival. Various concentrations of elafin (0–2.5 μM) were incubated with *P. aeruginosa* PAO1 for 2 h. Colonies were counted and the results were expressed as percentage of bacteria survival. Values are means±SEM (n=5). (B) Purity of recombinant elafin assessed by SDS-PAGE and silver staining. M: molecular mass marker; lane 1: 1 μg elafin; lane 2: 200 ng elafin. (C) Effect of pseudolysin-cleaved elafin on *P. aeruginosa* PAO1 survival. Recombinant elafin (0 or 41.6 μM) was treated with increasing concentrations of pseudolysin (0, 30 and 3000 nM) for 2 h and treated with or without GM6001 as indicated in the Figure. Samples (diluted 41.6 times) were incubated with *P. aeruginosa* PAO1 for 2 h. Bacterial survival was determined by counting the colony-forming units. Results are expressed as percentage of PAO1 survival. Values are means±SEM (n=5). One-way analysis of variance was performed to determine the statistical significance of the effects of elafin and concentrations of pseudolysin on PAO1 survival (*$p<0.05$; ns, not significant). Insets represent elafin integrity determined by Western blotting under non-reducing conditions.

recovered after treatment with 1–2.5 μM elafin. These results are similar to those obtained previously by Simpson et al. (1999). Interestingly, our data demonstrate that the cleavage of elafin by pseudolysin within the protease-binding region did not alter antibacterial properties of elafin against PAO1, whereas its anti-NE activity was inhibited. These findings suggest an anti-PAO1 activity independent of its antiprotease properties for elafin, as previously demonstrated for trappin-2 (Baranger et al., 2008).

Elafin and its precursor trappin-2 are substrates of transglutaminases (Molhuizen et al., 1993; Nara et al., 1994; Guyot et al., 2005b). Transglutaminases are enzymes that catalyse the formation of an isopeptide bond between the lateral chains of a lysine residue and a glutamine residue. By this mechanism, trappin-2 and elafin can covalently associate with structural proteins. *In vivo*, elafin/trappin-2 was shown to be covalently crosslinked to components in the tracheal epithelium that have not yet been identified (Nara et al., 1994). In the epidermis, elafin and trappin-2 were shown to associate with a number of structural proteins of the cornified cell envelope (including keratin-1, loricrin, involucrin and desmoplakin I/II among others). *In vitro*, elafin can crosslink to elastin, fibronectin, collagen, laminin, fibrinogen (Nara et al., 1994; Guyot et al., 2005b; Muto et al., 2007). The amino terminal sequence of elafin seems to be important in the capacity of the inhibitor to crosslink by transglutamination, as we have shown that the removal of Ala1-Lys6 sequence by aeruginolysin inhibited crosslinking of elafin to fibronectin following transglutaminase activity. This is in agreement with recent results demonstrating that elafin fragment truncated at its amino-terminus (Ser10-Gln57) is unable to bind fibronectin by transglutamination (Guyot et al., 2005b). Interestingly, elafin crosslinked to fibronectin retains its inhibitory properties and can protect fibronectin against NE (Guyot et al., 2005b) suggesting a role for elafin in the preservation of structural proteins against proteolysis mediated by neutrophil serine proteases. Given that aeruginolysin can inhibit the covalent binding of elafin to fibronectin *in vitro*,

then it is likely that during infection, *P. aeruginosa* can regulate elafin crosslinking and subsequent protection against increased proteolysis mediated by neutrophils.

In conclusion, this study provides evidence that metalloproteases secreted by *P. aeruginosa* can cleave elafin at different sites and specifically alter functions associated with the inhibitor. Among these proteases, pseudolysin is able to inactivate the antiprotease properties of elafin. In contrast, aeruginolysin can potentially perturb the transglutaminase-mediated crosslinking of elafin to ECM proteins. Therefore, both proteases could affect the protective role of elafin against neutrophil-mediated proteolysis in the lung via different mechanisms, shedding further light on the pathogenesis of *Pseudomonas* infections. The inactivation of elafin and pulmonary anti-elastase defence by *P. aeruginosa* could considerably disturb lung epithelial homeostasis. In this regard, these findings could have pathological importance.

Materials and methods

Materials

Recombinant human elafin was obtained from Proteo Biotech AG (Kiel, Germany). Biotinylated anti-human elafin antibody and recombinant SLPI were purchased from R&D Systems (Abingdon, Oxon, UK). N_{α}-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), leupeptin, Pefabloc, pepstatin A, ethylenediamine-tetraacetic acid (EDTA), guinea pig liver transglutaminase and N-methoxysuccinyl-Ala-Ala-Pro-Val-*p*-nitroanilide (MeOSuc-AAPV-pNA) were purchased from Sigma-Aldrich (Dublin, Ireland). *Pseudomonas* elastase (EC 3.4.24.26), plasma fibronectin, E-64, GM6001 and phosphoramidon were purchased from Merck Biosciences (Nottingham, UK). Human neutrophil elastase (NE) (EC 3.4.21.37) was from Elastin Products Company, Inc. (Owensville, MO, USA). Purified α-1-antitrypsin (AAT) was from Athens Research (Athens, GA, USA). Horseradish peroxidase (HRP)-conjugated streptavidin was purchased from Cambridge BioSciences Ltd (Cambridge, UK). SuperSignal West Femto Maximum Sensitivity Substrate was purchased from Pierce (Medical Supply Co., Dublin, Ireland). *Pseudomonas* alkaline protease (EC 3.4.24.40) was from Nagase Biochemicals (Fukuchiyama, Japan). LasA (EC 3.4.24.-) was obtained from Prof. Efrat Kessler (Tel-Aviv University Sackler University of Medicine, Tel-Hashomar, Israel). All other reagents were of analytical grade.

Pseudomonas PAO1-conditioned medium

Pseudomonas aeruginosa strain 01 (PAO1) was a gift from R. Hancock (University of British Columbia). PAO1-conditioned medium was prepared by filter-sterilising culture supernatants from 72 h PAO1 Trypticase Soy Broth cultures.

Incubations and SDS-PAGE analysis

PAO1 conditioned-medium (10 μl) was preincubated for 0.5–1 h at 37°C alone or with the following protease inhibitors: 10 mM Pefabloc, 0.13 mM TLCK, 0.6 mM leupeptin, 0.4 mM E-64, 0.4 mM pepstatin A, 13 mM EDTA, 0.5 mM GM6001 or 0.2 mM phosphoramidon. Recombinant elafin, SLPI or purified AAT (8.5×10^{-7} M each) were mixed with 10 μl of pretreated PAO1-conditioned medium in 30 mM Tris-buffered saline (TBS) to a final volume of 20 μl for 24 h at 37°C. Additionally, recombinant elafin (2.5×10^{-5} M) was incubated with various concentrations of purified *Pseudomonas* proteases for 2 h in 30 mM TBS in a 20 μl final volume at 37°C and analysed by SDS-PAGE. All incubations were stopped by adding sample treatment buffer with or without reducing agent and boiling samples for 5 min at 100°C. Samples were separated by Tricine SDS-PAGE using a 17.5% polyacrylamide gel and proteins were analysed by staining the gel with Coomassie Blue G or by Western blotting.

Western blotting

Samples separated by Tricine SDS-PAGE were blotted onto a 0.2 μM nitrocellulose membrane (Sigma-Aldrich). The membrane was blocked for 1 h at room temperature with 3% bovine serum albumin (elafin), 0.2% Iblock (SLPI) or 5% milk (AAT) in phosphate buffered saline (PBS) containing 0.1% Tween 20. Elafin was detected by using a biotinylated anti-elafin antibody (R&D Systems, Abingdon, UK; 1:500 dilution, overnight at 4°C) followed by peroxidase-conjugated streptavidin (Cambridge BioSciences Ltd, Cambridge, UK; 1:2500 dilution, 20 min at room temperature). SLPI and AAT were detected by incubating the membrane overnight at 4°C with rabbit anti-SLPI (1/1000) and anti-AAT (1/1000) antibodies, respectively, followed by a peroxidase-conjugated anti-rabbit antibody (1/2000) for 1 h at room temperature. Peroxidase activity was detected with chemiluminescent substrates (Pierce, Medical Supply Co., Dublin, Ireland).

HPLC-mass spectrometry analysis

Cleavage of elafin by *Pseudomonas* proteases was assessed by incubating 3 μg elafin with *Pseudomonas* elastase or alkaline protease in 30 mM TBS in 20 μl final volume for 2 h at 37°C. Protease activity was neutralised with 1 μl of 0.1 g/ml EDTA for 30 min at room temperature. Then, 14 μl of each sample was lyophilised for analysis when it was reconstituted in 10 μl 6 M guanidine HCl, 100 mM Tris pH 8.5, 1 mM EDTA, and 10 mM dithiothreitol (DTT). Samples were incubated for 30 min at 37°C to ensure the reduction of disulphide bridges. Then, 1 μl of 10% trifluoroacetic acid was added to each sample to bring the pH below 3. Samples were then analysed by reverse phase HPLC coupled to electrospray mass spectrometry as described elsewhere (Liu et al., 2006). Spectra were deconvoluted with Mass Hunter version 2 (Agilent Technologies, Santa Clara, CA, USA), using peaks whereby heights were 1% or more of the largest peak.

Neutrophil elastase (NE) activity assays

The effect of *Pseudomonas* proteases on the anti-NE activity of elafin was assessed as follows: PAO1-conditioned medium or purified proteases (pseudolysin, aeruginolysin) were pretreated with or without protease inhibitors (10 mM EDTA or 0.17 mM phosphoramidon) for 20 min at 37°C in 30 mM TBS (20 μl final volume) and incubated with recombinant elafin for various times (0, 1, 2 or 6 h) at 37°C. The reaction mix was diluted with 0.1 M HEPES, 0.5 M NaCl, pH 7.5 containing 0.1% Brij 35 and EDTA and incubated with NE in a 100 μl volume in such a way that elafin final concentration was two times higher than NE concentration. The residual activity of NE was determined spectrophotometrically by adding 50 μl of 3 mM MeOSuc-AAPV-pNA and by measuring the absorbance at 405 nm over time at 37°C. Inhibition of NE was expressed as a percentage of the NE present in control samples. The same method was applied to study the effect of PAO1-conditioned medium on the anti-NE activity of normal BAL fluid. Then, 2 μl

of PAO1-conditioned medium and 10 μl of a pooled fraction of normal BAL fluids (n=5) were used in the initial reaction.

Crosslinking of elafin to fibronectin by transglutamination

Recombinant elafin (100 ng, 3 μM) was incubated with increasing concentrations of aeruginolysin (0–100 nM) in PBS for 2 h at 37°C. Aeruginolysin was inactivated by 198 μM GM6001 for 30 min. The resulting mix was incubated in 200 mM Tris-acetate pH 6 containing 5 mM $CaCl_2$ and 0.1 mM DTT for 1 h at 37°C with 5 μg plasma fibronectin and 0.38 mU guinea pig liver transglutaminase (1 unit catalyses the formation of 1.0 μM of hydroxamate per minute from N_α-CBZ-glutaminylglycine and hydroxylamine at pH 6.0 at 37°C). The reaction was stopped by adding sample treatment buffer without reducing agent and by boiling samples for 5 min at 100°C. Samples were separated by 4–12% Bis-Tris SDS-PAGE (Invitrogen, Bio Sciences Ltd., Dun Laoghaire, Ireland) and analysed by Western blotting using a biotinylated anti-elafin antibody as described above.

Antibacterial assay

A single *P. aeruginosa* PAO1 colony was grown in 10 ml Tryptic Soy Broth (Sigma-Aldrich, Dublin, Ireland) at 37°C with agitation (250 rpm). After 4–6 h of growth, the PAO1 culture was centrifuged (4000 *g*, 10 min) and the bacterial pellet was washed and resuspended in PBS. Then, 50 μl of PAO1 diluted 10 000 times was mixed with 50 μl of recombinant elafin (0–2.5 μM) in PBS. The reaction mixture was incubated for 2 h at 37°C with agitation (250 rpm). Then, 10 μl of various serial dilutions of the reaction mixtures were spread on Tryptic Soy Agar plates (Sigma-Aldrich) and incubated overnight at 37°C. The colonies were counted and expressed as PAO1 survival compared with PAO1 alone as control (100%). To study the effect of pseudolysin on the antibacterial activity of elafin, the inhibitor was treated as follows: 41.6 μM recombinant elafin was incubated with increasing concentrations of pseudolysin (0, 30 and 3000 nM) in PBS for 2 h at 37°C and treated with 60 μM GM6001 to stop the reaction. Controls were also carried out using pseudolysin alone and PBS alone without GM6001 in the same conditions. The reaction mixtures were diluted 41.6-fold in 100 μl PBS (final elafin concentration: 0 or 1 μM) containing PAO1 as described above.

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