Anti-Inflammatory Effects of Mutant Forms of Secretory Leukocyte Protease Inhibitor

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The secretory leukocyte protease inhibitor (SLPI) is found in a variety of secreted fluids in mammals and is a known inhibitor of serine proteases. Wild-type (WT) SLPI has recently been shown to block nuclear factor κ B (NF- κ B) activation in rat lungs and to interfere with the ensuing inflammatory response and recruitment of neutrophils after an intrapulmonary deposition of IgG immune complexes. In this study, WT SLPI and SLPI mutants with various degrees of protease-inhibitory capacity (for trypsin, chymotrypsin, and elastase) were evaluated for their ability to suppress the lung-vascular leak, neutrophil accumulation, and NF- κ B activation in the lung inflammatory model. The SLPI mutant with Gly⁷² (replacing Leu⁷²) lost its ability to block *in vivo* activation of NF- κ B, as well as its ability to suppress the lung vascular leak and neutrophil recruitment. The Phe⁷² and Gly²⁰ mutants were as effective as the WT SLPI in suppressing NF- κ B activation and neutrophil recruitment. The Lys⁷² mutant had the most suppressive effects of the lung vascular leak and for neutrophil recruitment into the lung. The *in vivo* suppressive effects of SLPI mutants on lung vascular permeability, neutrophil recruitment, and NF- κ B activation appear to be most closely related to their trypsin-inhibiting activity. These data suggest that the suppressive effects of SLPI on the intrapulmonary activation of NF- κ B and neutrophil recruitment into the lung may be linked to their antiprotease activity, directed, perhaps, at the intracellular proteases. (Am J Pathol 2000, 156:1033–1039)

Although the secretory leukocyte protease inhibitor (SLPI) was originally defined as an inhibitor of serine proteases,^{1,2} it was eventually recognized to have other effects, such as antagonizing lipopolysaccharide-induced production of tumor necrosis factor- α (TNF- α) in stimulated phagocytic cells³ and interfering with the entry of the human immunodeficiency virus into susceptible

cell lines.^{4,5} In rats, SLPI has since been found to inhibit inflammatory lung injury caused by an intrapulmonary deposition of IgG-immune complexes.⁶ This model is characterized by an intensely damaging lung-inflammatory response featuring the roles of cytokines and chemokines and the recruitment of neutrophils.⁷ The manner by which SLPI inhibits these inflammatory reactions and protects the lung was recently found to be related to its ability to prevent nuclear factor κ B (NF- κ B) activation within the lung in a manner related to preservation of the NF- κ B-inhibitory protein, I κ B β .⁸ Further details of how this activation pathway is blocked by SLPI are not known.

In structure-function studies, mutant forms of SLPI have been evaluated *in vitro*, using techniques to induce site-directed mutations involving Leu⁷² of wild-type (WT) SLPI. Leu⁷² is known to be a critical site for the binding of chymotrypsin, elastase, and trypsin to SLPI.⁹ In the current studies, we investigated the effects of various mutants of SLPI to gain insights into the anti-inflammatory nature of this molecule. There appears to be a relationship between the protease inhibitory activity of SLPI mutants, especially correlating with the trypsin-inhibiting activity, and their ability to demonstrate *in vivo* protective effects through the reduction of the lung albumin leak, neutrophil accumulation, and suppression of intrapulmonary activation of NF- κ B.

Materials and Methods

Reagents

Unless otherwise indicated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human SLPI and SLPI mutants were used in all studies. The SLPI mutants were prepared by site-specific mutagenesis using the Mutagene *in vitro* mutagenesis kit purchased from Bio-Rad (Hercules, CA). The plaques were screened initially by hybridization using the mutagenic oligonucleotide as the probe, and those clones that were positive by hybridization were plaque-purified and

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sequenced using modified T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, OH). Mutants having the desired sequence were grown in JM109 cells and M13 RF DNA was prepared as described elsewhere.⁹

IgG Immune Complex-Induced Alveolitis

Pathogen-free male Long-Evans rats (275–300 g; Harlan Sprague-Dawley, Indianapolis, IN) were anesthetized with ketamine HC1 (150 mg/kg, i.p.). The rats received an intratracheal administration of phosphate-buffered saline, pH 7.4, or 2 mg of rabbit IgG antibody to bovine serum albumin (BSA) from ICN Biomedicals (Costa Mesa, CA) in a total volume of 300 μ l, followed by an intravenous infusion of 10 μ g of BSA. When the permeability index was measured,^{125I}-BSA was also injected intravenously.⁸ When SLPI proteins were used, 400 μ g were added to the anti-BSA preparation. This amount has been shown to be suppressive of the lung inflammatory response.⁸ When α_1 -antiproteinase (α_1 -PI) was used, 1.0 mg was added to the anti-BSA preparation before the intratracheal instillation. Four hours after the IgG-immune complex deposition in the lung, the rats were exsanguinated. For the measurement of lung vascular permeability, the pulmonary artery was perfused with 10 ml of phosphate-buffered saline to remove any residual blood in the pulmonary vasculature. The total radioactivity of the lungs was measured and compared with the amount of radioactivity present in 1.0 ml of blood obtained from the inferior vena cava at the time of sacrifice. This ratio was the computed permeability index.⁸ For the measurement of pulmonary neutrophil accumulation, bronchioalveolar lavage (BAL) was performed with three repetitive washes with 5 ml of sterile saline. The BAL-fluid neutrophil counts were determined by microcytometry. For each experimental group, $n = 7$.

Assessment of NF- κ B Activation by Electrophoretic Mobility Shift Assay

Nuclear extracts of whole-lung tissues were prepared by the method of Deryckere and Gannon.¹⁰ Protein concentrations were determined by a bicinchoninic-acid assay with TCA precipitation using BSA as a reference standard (Pierce, Rockford, IL). The NF- κ B consensus oligonucleotide (5'-AGTGAGGGGACTTTCCAGGC-3'; Promega, Madison, WI) was end-labeled with [γ -³²P]ATP (3000 Ci/nmol/L at 10 mCi/ml; Amersham, Arlington Heights, IL). The binding reactions containing equal amounts of protein (10 μ g) and 35 fmol (~50,000 cpm, Cherenkov counting) of oligonucleotide were performed for 30 minutes in binding buffer (4% glycerol, 1 mmol/L MgCl₂, 0.5 mmol/L ethylenediaminetetraacetic acid, pH 8.0, 0.5 mmol/L dithiothreitol, 50 mmol/L NaCl, 10 mmol/L Tris, pH 7.6, 50 μ g/ml poly (dl-dC); Pharmacia, Piscataway, NJ). For supershift assays, 1 μ l of polyclonal antibody to p50, p52, p65, p68, or p75 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the binding reactions. The reaction volumes were held constant to 15 μ l. The reaction products were separated in a 4% polyacryl-

amide gel and analyzed by autoradiography. The NF- κ B activation was quantitated from digitized autoradiography films using image analysis software (Adobe Systems, Inc., San Jose, CA).

Assessment of Inhibitory Activities of SLPI and α_1 -PI

Serine proteases were assayed using specific chromogenic peptide-p-nitroanilide (pNA) substrates in a 96-well microtiter plate format. Each protease was incubated with various concentrations of human WT SLPI or α_1 -PI for 15 minutes at 37°C in specific assay buffer. The residual protease activity was measured after the addition of the appropriate substrate. The pNA product of proteolysis was quantified at 405 nm on a SpectraMAX 340 plate reader (Molecular Devices, Sunnyvale, CA). The human neutrophil elastase (Calbiochem-Novabiochem International, San Diego, CA) was assayed using pyroGlu-Pro-Val-pNA (Pharmacia Hepar Inc., Franklin, OH) in 100 mmol/L Tris-HCl, pH 8.3, 0.96 mmol/L NaCl, 1% BSA.¹¹ Bovine pancreatic trypsin (-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated; Sigma) was assayed using *N*- α -benzoyl-L-Arg-pNA (Boehringer Mannheim Corp., Indianapolis, IN) in 50 mmol/L Tris-HCl, pH 8.2, 20 mmol/L CaCl₂.¹² Bovine pancreatic chymotrypsin (Boehringer Mannheim) was assayed using *N*-Suc-Ala-Ala-Pro-Phe-pNA (Sigma) in 100 mmol/L Tris-HCl, pH 7.8, 10 mmol/L CaCl₂.¹³ The dissociation constants (K_i) of human WT SLPI and α_1 -PI against each proteolytic enzyme were determined as previously described.¹⁴

Statistical Analyses

All values are expressed as mean $\pm$ SEM. Data were analyzed with a one-way analysis of variance and subsequent Student-Newman-Keuls test. The differences were considered significant when $P < 0.05$. For calculations of the percent change, negative control values were subtracted from the positive control and treatment group values.

Results

Effects of SLPI Mutants on the Lung Permeability Index

The ability of WT SLPI and SLPI mutants to affect the permeability index 4 hours after the initiation of the lung inflammatory injury is shown in Figure 1. As expected, the permeability index values for the negative and positive controls were different by a factor of more than threefold. The presence of WT SLPI reduced the permeability index by nearly 47% ($P < 0.001$). The Gly²⁰ mutant was similarly protective ($P < 0.001$). The Gly⁷² mutant had no protective effects (P not significant). The Phe⁷² mutant was statistically as protective as the WT SLPI ($P = 0.002$), whereas the Lys⁷² mutant was the most protective of all the forms of SLPI ($P = 0.001$). In addition, the Lys⁷²

Figure 1. Effects of mutant forms of SLPI in reducing the lung vascular permeability index in rats after intrapulmonary deposition of IgG immune complexes. When used, WT SLPI and SLPI mutants (400 μ g) were instilled into the airways together with 2 mg of IgG anti-BSA. *P* values were compared with the positive control group not otherwise treated and to the WT SLPI group. Values represent mean $\pm$ SEM with *n* = 7 for each group.

mutant was the only form of SLPI that possessed protective activities that exceeded those of the WT SLPI (*P* = 0.02; Figure 1).

Effects of SLPI Mutants on Lung Recruitment of Neutrophils

The SLPI and its mutant forms were evaluated for their effects on the neutrophil accumulation in BAL fluids from rat lungs 4 hours after the intrapulmonary deposition of the IgG immune complexes. The neutrophils are largely responsible for the damage occurring in this model.¹⁵ As expected, when the BAL content of neutrophils was compared in the negative and positive control groups, there was a nearly 20-fold increase in the number of neutrophils recruited into inflamed lungs (Figure 1). In the presence of 400 μ g WT SLPI, there was a 48% reduction (*P* = 0.001) in neutrophil accumulation in the inflamed lungs. The Gly²⁰ mutant had inhibitory effects similar to WT SLPI, decreasing the BAL neutrophil counts by 50% (*P* < 0.001). In the presence of the Gly⁷² mutant of SLPI, the inhibitory effects were lost, compared with the positive control group (*P*, not significant). The Phe⁷² mutant of SLPI had inhibitory effects (40% decrease, *P* = 0.001) that were indistinguishable from those of WT SLPI. The Lys⁷² mutant of SLPI caused the greatest reduction in BAL neutrophils compared with the positive control group (82% decrease, *P* < 0.001). When compared with WT SLPI for the effect on the BAL accumulation of neutrophils, Lys⁷² had significantly greater inhibitory properties (*P* = 0.001).

Effects of SLPI Mutants on NF- κ B Activation in Lung

Using this lung-inflammatory model, we have recently shown that the anti-inflammatory effects of WT SLPI are

Figure 2. Effects of WT SLPI and SLPI mutants on accumulation of neutrophils in bronchoalveolar (BAL) fluids obtained from lungs of rats 4 hours after intrapulmonary deposition of IgG immune complexes. When used, WT SLPI and SLPI mutants (400 μ g) were instilled into the airways together with 2 mg of IgG anti-BSA. Values represent mean $\pm$ SEM with *n* = 7 for each group.

associated with the inhibition of the intrapulmonary activation of NF- κ B through the preservation of I κ B β .⁸ To determine how mutant forms of SLPI compare to the effects of WT SLPI on pulmonary NF- κ B activation, nuclear extracts from whole lungs that were harvested 4 hours after IgG-immune complex deposition (in the absence or presence of SLPI proteins) were analyzed by electrophoretic mobility shift assay (EMSA). As expected, there was little evidence of NF- κ B activation in the negative control lungs (Figure 3, upper frame, first lane, arrow). The intrapulmonary deposition of IgG immune complexes resulted in a significant increase in the nuclear translocation of NF- κ B (second lane). In the presence of 400 μ g of WT SLPI, NF- κ B activation was greatly reduced (third lane). Image analysis of digitized EMSA autoradiograms indicated that WT SLPI reduced NF- κ B activation by 55% (*P* = 0.002) when compared with positive controls in the absence of SLPI (Figure 3, lower frame). Treatment with the Gly²⁰ mutant of SLPI also had suppressive effects, reducing NF- κ B activation by 47% (*P* = 0.020; fourth lane). The effects of the Gly⁷² mutant of SLPI (fifth lane) were indistinguishable from the positive control group. The effects of the Phe⁷² mutant of SLPI on the lung NF- κ B activation were similar to WT SLPI, decreasing IgG-immune complex-induced NF- κ B activation by 51% (*P* = 0.006; sixth lane). The Lys⁷² mutant had the most suppressive effects, virtually abolishing evidence of NF- κ B activation (*P* = 0.003; seventh lane). Accordingly, there appears to be a correlation between SLPI effects on NF- κ B activation (Figure 3), reductions of the permeability index (Figure 1), and the diminished recruitment of neutrophils (Figure 2).

We have previously shown that the NF- κ B complex activated by the IgG-immune complexes in the lung consists of p50 and p65 subunits.¹⁶ In the present studies it was observed that treatment with WT SLPI and, to a lesser extent, Gly²⁰ and Phe⁷² mutants altered the NF- κ B banding patterns in the EMSA's (Figure 3). Specifically, a

Figure 3. Effects of W. T. SLPI and SLPI mutants on IgG immune complex-induced lung NF- κ B activation as detected by EMSA. **A:** The main NF- κ B activation band (arrow) is shown in whole-lung tissues harvested 4 hours after intratracheal administration of 2 mg of IgG anti-BSA (IgG-IC) followed by intravenous infusion of 10 mg of BSA. WT SLPI and SLPI mutants (400 μ g) were administered intratracheally with the IgG anti-BSA. **B:** Quantitation of EMSA blots by image analysis.

prominent, slower-migrating NF- κ B band was observed in these treatment groups. To identify the components of these EMSA bands, supershift assays were performed on nuclear extracts of inflamed lungs in the presence of 400 μ g WT SLPI. The results are shown in Figure 4. Indicated at the left side of the figure are the positions of the primary NF- κ B band (solid arrow), the slower migrating band (solid arrowhead), and the supershift bands (open arrow). In the presence of the antibody to p50, some supershift of NF- κ B was found (second lane). Supershifts were not observed in the presence of the antibody to p52, p68, or p75. In the presence of the antibody to p65, there was some supershift of the primary NF- κ B band and a complete supershift of the slower-migrating NF- κ B band. These data suggest that the primary NF- κ B band (solid arrow) is composed largely of p50/p65 heterodimers, and that the slower-migrating NF- κ B band observed (solid arrowhead) in SLPI treatment groups is composed of p65/p65 homodimers.

The suppressive effects of SLPI proteins on NF- κ B activation appeared to correlate most closely with the

Figure 4. Supershift analysis of SLPI-induced NF- κ B components. DNA-binding reactions were done with nuclear extracts from whole lungs harvested 4 hours after intratracheal administration of IgG immune complexes, using the 32 P-labeled NF- κ B consensus oligonucleotide in the absence or presence of antibodies to the NF- κ B proteins indicated (p50, p52, p65, p68, p75). Supershifts of p50 and p65 are indicated by the open arrow. The solid arrowhead indicates the position of the higher molecular weight NF- κ B complex induced by SLPI. The solid arrow indicates the position of the primary NF- κ B band.

published trypsin-inhibitory effects of these proteins (Table 1). In view of this possibility, the effects of α_1 -PI on pulmonary NF- κ B activation were also investigated, because α_1 -PI is a potent inhibitor of trypsin and other serine proteases, including chymotrypsin and elastase.^{17,18} The administration of 1 mg of α_1 -PI in rat lungs also failed to reduce lung NF- κ B activation, which is induced by IgG-immune complexes (Figure 5). It should be noted, however, that the amount of α_1 -PI administered was, on a molar basis, less than the amount of SLPI (60 mmol/L versus 110 mmol/L, respectively). The addition of α_1 -PI in the airways of the rats also failed to inhibit either the permeability index (positive control values in the absence or presence of 1.0 mg α_1 -PI were 0.537 ± 0.049 and 0.53 ± 0.024 , respectively) or the numbers of neu-

Table 1. Relative Protease Inhibitory Effects of SLPI Mutants and α_1 -PI

Form of SLPI	Relative inhibitory effects*		
	Chymotrypsin	Elastase	Trypsin
Leu ⁷² (WT)	1.0	1.0	1.0
Gly ⁷²	0.0006	0.02	0.003
Phe ⁷²	40	0.002	10
Lys ⁷²	0.009	0.0005	>1000
Gly ²⁰	0.80	0.30	0.67

Calculated according to data in reference 9, which describes kinetic dissociation constants.

*As compared with WT SLPI in which the values were arbitrarily set at the value of 1.0.

Figure 5. Effects of α_1 -proteinase inhibitor (α_1 -PI) on IgG immune complex (IgG-IC)-induced lung NF- κ B activation (arrow). NF- κ B activation was evaluated in whole-lung tissues harvested 4 hours after intratracheal administration of 2 mg of IgG anti-BSA followed by intravenous infusion of 10 mg BSA. α_1 -PI (1 mg) was administered intratracheally with the IgG anti-BSA.

trophils (in millions) in the BAL fluids (positive control values in the absence or presence of 1.0 mg α_1 -PI were 5.32 ± 0.582 and 5.70 ± 0.483 , respectively). Thus, although α_1 -PI is a broad inhibitor of serine proteases, in this experimental model it does not suppress NF- κ B activation, the permeability index, or the number of neutrophils recruited into the lung.

Inhibitory Activities of SLPI and α_1 -PI

Because WT SLPI, but not α_1 -PI, blocked NF- κ B activation in the lung (Figures 3 and 5), the kinetic dissociation constants (K_i) of the two protease inhibitors were determined. As shown in Table 2, the protease-inhibitory activity of SLPI revealed a descending rank order of chymotrypsin = elastase $\gg$ trypsin. In the case of α_1 -PI, the descending order of inhibition was chymotrypsin $>$ elastase $>$ trypsin. A comparison of the K_i values for WT SLPI and α_1 -PI revealed that the protease inhibitory activities of SLPI and α_1 -PI are similar in the case of trypsin and chymotrypsin, whereas α_1 -PI is about 1/10 as effective as SLPI for inhibition of elastase.

Table 2. Comparison of the Anti-Protease Activities of SLPI and α_1 -PI

Inhibitor	K_i (μ mol/L)*		
	Chymotrypsin	Elastase	Trypsin
SLPI (WT)	0.0013	0.0016	0.051
α_1 -PI	0.002	0.016	0.044

*Dissociation constant (K_i) values were determined as described in Materials and Methods and in an earlier report (9).

Discussion

SLPI contains two molecular domains (1 and 2) and a total of eight Cys-Cys groups, resulting in a very stable, oxidant-resistant molecule. The substitution of Leu in position 72 significantly affects the protease inhibitory capacity of SLPI.⁹ In the N-terminal domain 1, although it was speculated that Arg²⁰ might be a binding site for trypsin and other serine proteases, substituting Gly for Arg in this position failed to affect the protease-inhibitory capacity of SLPI, implying that this is not a binding site for serine proteases.⁹ Similarly, the Gly²⁰ mutant of SLPI retained the full *in vivo* anti-inflammatory effects when compared with WT SLPI (Figures 1, 2).

The C-terminal domain 2 of WT SLPI contains Leu⁷², which is known to be critical for the binding of serine proteases to SLPI.⁹ The availability of mutant forms of SLPI has allowed us to assess how replacements of Leu⁷² alter the anti-inflammatory activities of SLPI, in turn perhaps providing information on whether the anti-inflammatory effects of SLPI are correlated with its protease inhibitory activity. Such studies could suggest whether proteases are required for the full development of a lung inflammatory injury. In this report, we have evaluated the effects of several substitutions of Leu⁷² SLPI with Gly⁷², Phe⁷², or Lys⁷² and have determined to what extent these mutants demonstrate altered ability to reduce lung-vascular permeability, suppress neutrophil accumulation, and inhibit NF- κ B activation *in vivo*. Substitutions of Leu⁷² are known to affect positively or negatively the anti-protease activities of SLPI (reference 9; Table 1). The replacement of Leu⁷² with Gly⁷² dramatically reduced the binding activity of mutant SLPI for all three proteases (Table 1). Gly⁷² SLPI also lost its *in vivo* protective effects in the inflammatory model, having no effect on lung-vascular permeability (Figure 1), neutrophil accumulation (Figure 2), or NF- κ B activation (Figure 3). The replacement of Leu⁷² with Phe⁷² increased binding interactions of mutant SLPI with chymotrypsin (by 40-fold) and for trypsin (by 10-fold), but the binding for elastase was decreased (by 2.5-fold). This mutant was similar to WT SLPI in terms of the suppression of neutrophil recruitment and intrapulmonary NF- κ B activation. Perhaps the most dramatic effects were found with Lys⁷² SLPI, in which the binding interactions for chymotrypsin and elastase are known to be reduced by 100-fold and 2000-fold, respectively, but the binding interactions for trypsin were increased by more than 1000-fold (Table 1). This mutant form of SLPI was most effective in reducing lung-vascular permeability, in suppressing neutrophil accumulation, and in inhibiting NF- κ B activation *in vivo*.

There are several possible explanations for these observations. Proteases can directly activate phagocytic cells, resulting in the enhanced generation of oxidants and cytokines/chemokines.¹⁹ Trypsin and related proteases (elastase and cathepsin G) are known to be able to cleave the fifth component of the complement (C5), releasing the powerful anaphylatoxin, C5a,²⁰ which is a potent agonist for phagocytic cells.^{21–23} The recent dem-

onstration that a powerful synthetic inhibitor of elastase is protective in the same model of lung injury²⁴ provides additional evidence that the serine proteases are somehow involved in the processes leading to injury in this inflammatory model. It is also possible that SLPI proteins gain access to the intracellular environment of cells, such as lung macrophages, which are known to be essential for the chain of events leading to lung damage²⁵ and blocks intracellular proteases involved in signal transduction and NF- κ B activation events. The cellular internalization of SLPI has been suggested in a report demonstrating that SLPI and SLPI mutants bind with a high affinity to a 55-kd receptor on the surface of monocytes.⁵ Surface binding was followed by a rapid decrease in the ability to detect SLPI on the cell surface or in supernatants.⁵

The activation of the transcription factor, NF- κ B, has been shown to be a required event for the development of lung-inflammatory injury induced by IgG immune complexes^{16,26} and by systemic administration of LPS.²⁷ Under normal conditions, NF- κ B is retained in the cytoplasm complexed to inhibitory proteins of the I κ B family. In response to inflammatory stimuli, I κ B proteins are phosphorylated, ubiquitinated, and degraded by the multicatalytic proteinase complex (MCP).²⁸ Inhibitors of the MCP have been shown to attenuate the activation of NF- κ B.²⁹ The MCP possesses chymotrypsin and trypsin-like activities,³⁰ which may suggest potential points of action for the inhibitory effects of SLPI proteins. If SLPI is able to bind to a cell-surface receptor and be internalized, as has been suggested elsewhere,⁵ it could be processed or complexed, possibly with the MCP. The data contained in the current report suggests that SLPI blocks activation of NF- κ B in association with its protease inhibitory activities. The inability of another serine protease inhibitor, α_1 -PI, to block intrapulmonary activation of NF- κ B suggests that a secreted extracellular serine protease may not be involved in the NF- κ B activation process. Evidence supporting the internalization of uncomplexed α_1 -PI has not been reported. Alternatively, the relatively large size of α_1 -PI (53 kd) compared with SLPI (12 kd), may also prevent this molecule from gaining access to the intracellular environment.

In summary, mutant forms of SLPI with various degrees of antiprotease capacity have been applied to a model of inflammatory lung injury in rats and compared with the effects of WT SLPI, which we have recently shown to have potent anti-inflammatory activity. The protective effects of mutant SLPI forms appear to correlate with their antiprotease activity, especially with a trypsin inhibiting capacity. It is suggested that the anti-inflammatory effects of SLPI may be linked to intracellular protease activity that is required for activation of NF- κ B.

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