Neutrophil Serine Proteinases Inactivate Surfactant Protein D by Cleaving within a Conserved Subregion of the Carbohydrate Recognition Domain*

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Surfactant protein D (SP–D) plays important roles in innate immunity including the defense against bacteria, fungi, and respiratory viruses. Because SP–D specifically interacts with neutrophils that infiltrate the lung in response to acute inflammation and infection, we examined the hypothesis that the neutrophil-derived serine proteinases (NSPs)1: neutrophil elastase, proteinase-3, and cathepsin G degrade SP–D. All three human NSPs specifically cleaved recombinant rat and natural human SP–D dodecagons in a time- and dose-dependent manner, which was reciprocally dependent on calcium concentration. The NSPs generated similar, relatively stable, disulfide cross-linked immunoreactive fragments of ~35 kDa (reduced), and sequencing of a major catheptic fragment definitively localized the major sites of cleavage to a highly conserved subregion of the carbohydrate recognition domain. Cleavage markedly reduced the ability of SP–D to promote bacterial aggregation and to bind to yeast mannan in vitro. Incubation of SP–D with isolated murine neutrophils led to the generation of synthetic and natural serine proteinase inhibitors. In addition, neutrophils genetically deficient in neutrophil elastase and/or cathepsin G were impaired in their ability to degrade SP–D. Using a mouse model of acute bacterial pneumonia, we observed the accumulation of SP–D at sites of neutrophil infiltration coinciding with the appearance of ~35-kDa SP–D fragments in bronchoalveolar lavage fluids. Together, our data suggest that neutrophil-derived serine proteinases cleave SP–D at sites of inflammation with potential deleterious effects on its biological functions.

The lung relies on numerous mechanisms to defend itself against various infectious or toxic agents. When the first lines of defense against microorganisms are breached, neutrophils rapidly accumulate in the lung. The primary purpose of this neutrophil infiltration is the killing of microorganisms and resolution of the associated inflammation (1). Among neutrophil antimicrobial molecules are three active neutrophil serine proteinases (NSPs)1: neutrophil elastase (NE), proteinase-3 (PR3), and cathepsin G (CG) (2). NE, PR3, and CG are structurally related endopeptidases with different but overlapping specificities whose catalytic activities rely on the triad, His72, Asp102-Ser195 (chymotrypsinogen numbering), where Ser is the active amino acid (3).

NSPs are stored in active forms at high concentrations in the primary granules and are rapidly discharged into the phagocytic vacuoles following bacterial uptake by neutrophils (4). Cell surface localization and/or extracellular release of active NSPs by activated neutrophils has also been documented (5). NSPs can cleave many substrates that include extracellular matrix proteins, coagulation factors, cytokines, and immunoglobulins (6). As a consequence, unchecked release of NSPs can mediate tissue injury. In particular, NSPs contribute to the pathogenesis of tissue destruction in such diverse diseases as acute respiratory distress syndrome, emphysema, and cystic fibrosis (7–9).

There is accumulating evidence that collagenous lectins (collectins) play critical roles in the innate host defense, as well as surfactant homeostasis (10). Surfactant protein D (SP–D), a member of the collectin family, is synthesized and secreted in the lung by alveolar type II cells and Clara cells; however, it is also expressed at mucosal surfaces and other extrapulmonary sites. The antimicrobial host defense activities of SP–D are diverse and include microbial agglutination, opsonization, modulation of phagocyte function, antigen presentation, and direct effects on microbial growth (11–13). SP–D consists of trimeric subunits and each subunit is composed of four domains. The NH2-terminal domain contains two conserved cysteine residues that stabilize the association of trimeric sub-

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1 The abbreviations used are: NSPs, neutrophil serine proteinases; SP, surfactant protein; hSP–D, human surfactant protein D; RrSP–D, recombinant rat SP–D; Rs-SP–D, biotinylated recombinant rat SP–D; Rs-SP–Dmut, maltosyl-agarose eluted recombinant rat SP–D; CRD, carbohydrate recognition domain; NE, neutrophil elastase; PR3, proteinase-3; CG, cathepsin G; PMSF, phenylmethylsulfonyl fluoride; AEBSF, 4-[2-aminoethyl]benzenesulfonyl fluoride; SLPI, recombinant human secretory leukocyte proteinase inhibitor; MNEI, recombinant human monocyte/neutrophil elastase inhibitor; FMLP, formyl-methionyl-leucyl-phenylalanine; LPS, lipopolysaccharide; HPLC, high-pressure liquid chromatography; OD, optical density; WT, wild type; BAL, bronchoalveolar lavage; hBAL, human bronchoalveolar lavage; PBS, phosphate-buffered saline.

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was purchased from R&D Systems (Minneapolis, MN). Recombinant human elastase/neutrophil elastase inhibitor (MNE) was provided by T. Ley (Washington University). Rabbit serum was purchased from Vector Laboratories (Burlingame, CA). Polyclonal rabbit antibody to mouse SP-D was purchased from Chemicon (Ab3434; Temecula, CA). Polyclonal rabbit antibody to mouse CG was kindly provided by T. Ley (Washington University). Rabbit serum was purchased from Vector Laboratories (Burlingame, CA). Except where indicated, all other chemicals were reagent grade and purchased from Sigma.

Human Samples, Mice, and Bacteria—Natural human SP-D (hSP-D) dodecamers were isolated from bronchoalveolar lavage (BAL) fluids from alveolar proteinosis patients as previously described (37). Normal human BAL (hBAL) fluids were obtained in conjunct with a study of surfactant protein A as described in Rubio and co-workers (39). Remains of BAL specimens were selected for study of SP-D that had normal levels of surfactant proteins and lacked detectable signs of infection.

Mice deficient in NE (NE−/−) or CG (CG−/−) were generated by targeted mutagenesis as previously described (40, 41), and backcrossed at least eight generations into 129/SvJ-C57/BL6 genetic background. Mutant and wild type (WT) mice were maintained in the animal barrier facility with a 12-h light/dark cycle and provided with water and food ad libitum. All procedures were approved by the Washington University Animal Studies Committee.

In this work, we used an uncapsulated phase variant of Klebsiella pneumoniae, K-50 cap- (42), or Pseudomonas aeruginosa H103 (kindly provided by R. Hancock, University of British Columbia, Vancouver, Canada). Overnight cultures were diluted 1:100 and grown aerobically in Luria Bertani broth (10 ml) at 37 °C to late exponential phase (3 h). Bacteria were collected by centrifugation (5000 × g, 10 min), washed twice, and resuspended in 1 ml of phosphate-buffered saline (pH 7.4). Bacteria were quantified by optical density (OD) at 600 nm (OD 1–109 bacteria/ml) (40).

Purification of SP-D—Recombinant rat SP-D (RrSP-D) was expressed in Chinese hamster ovary-K1 cells as previously described (43). SP-D was initially purified by affinity chromatography on maltosylagarose. Following elution from EDTA, dodecamers were isolated by gel filtration chromatography on A-15M-agarose in the presence of EDTA. Numerous studies have shown that these molecules are ultrastructurally indistinguishable from the wild-type proteins (43). In addition, there is no evidence for differences in the specificity or potency of comparably multimemerized preparations of the recombinant and natural proteins in various in vitro assays of host defense functions (25).

Where indicated, RrSP-D was eluted from maltosyl-agarose with 10 mM maltose and used without subsequent gel filtration chromatography (RrSP-Dmaltose). Although RrSP-Dmaltose contains a very small fraction of higher order multimers, which are associated at their amino termini, both preparations are pure as assessed by SDS-PAGE and silver staining. Protein preparations used for these experiments contained low endotoxin (< 300 pg/ml) as measured by chromogenic assay (QLC-1000; Cambrex, East Rutherford, NJ). For binding studies, RrSP-D was biotinylated (RrSP-Dbiotin) at its glycosylation sites within the collagen domain as recently described (44).

Degradation of SP-D by Neutrophil Serine Proteinases—RrSP-D, RrSP-Dmaltose, RrSP-Dbiotin, hSP-D, or human BAL fluids were incubated alone or in the presence of the indicated concentrations of NE, PR3, or CG at 37 °C for the designated times. Unless specified, incubations were performed in a 20-μl reaction volume in PBS, in the absence or presence of the indicated concentration of CaCl2, at a final pH of 7.4, which should approximate the pH in the extracellular milieu of the lungs. At this pH, the cysteine proteinase inhibitor is effective and proteinase inhibitors are fully active under basic conditions (45).

In some experiments, NSPs were preincubated at 37 °C for 5 min with the serine proteinase inhibitors PMSF, AEBSF, or diisopropyl fluorophosphate prior to addition of SP-D. Reaction products were heat-inactivated in SDS sample buffer and resolved by SDS-PAGE (12 or 15%) followed by fluorography. The membranes were then examined for detection of reduction of sulfhydryl bonds with 2 mM β-mercaptoethanol as indicated. Protein bands and proteolytic fragments were visualized by Coomassie Blue staining.

Immunodetection of Cleavage Products—RrSP-D cleavage products or murine and human BAL fluids were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The membranes were sequentially incubated with rabbit polyclonal antibody to rat (dilution, 1:10,000) or mouse (dilution, 1:2,000) SP-D followed by goat anti-rabbit horseradish peroxidase. Immunoreactive fragments were visualized by enhanced chemilumines-
ence (ECL, Amersham Biosciences) (38). For quantitative experiments examining the effects of Ca\textsuperscript{2+}, proteins were reacted with rabbit anti-body to rat SP-D followed by incubation with 125I-labeled goat anti-rabbit IgG. Labeled proteins or fragments were detected with the Storm 860 PhosphorImager (Amersham Biosciences) and quantified using ImageQuant 1.2 (Amersham Biosciences).

**High Pressure Liquid Chromatography (HPLC) and NH\textsubscript{2}-terminal Sequencing of SP-D Degradation Products—**RrSP-D (2.5 μg) was incubated alone or in the presence of CG (2.5 μg) at 37 °C in a 20-μl reaction containing PBS (pH 7.4) and CaCl\textsubscript{2} (10 mM). Following incubation for the designated times at 37 °C, the reactions were diluted to 1 ml with PBS and immediately analyzed by reverse phase HPLC (System Gold Analyzer, Beckman-Coulter, Fullerton, CA). The NH\textsubscript{2}-terminal amino acid composition of predicted protein fragments were determined by electrophoresed Edman degradation (Sequenator 472A; Applied Biosystems, Foster City, CA).

**Bacterial Agglutination Assay—**The agglutination assay was performed as previously described with the following modifications (46). RrSP-D (2.5 μg) was incubated alone or in the presence of CG (2.5 μg) at 37 °C in a 20-μl reaction containing PBS (pH 7.4) and CaCl\textsubscript{2} (10 mM). Following incubation for 4 h, the reactions were stopped by addition of PMSF (1 mM) and mixed with equal numbers of freshly prepared bacteria (10\textsuperscript{7}) in a total volume of 1 ml of PBS/CaCl\textsubscript{2}. These reactions were performed in duplicate. In one set of reactions, the OD of the bacterial suspension was recorded at 0 and 60 min post-incubation and considered as an index for bacterial agglutination. The other set of reactions were stained for fluorescence microscopy using 4',6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) (47). Reactions containing bacteria alone were included as controls.

**Yeast Mannan Binding Assay—**RrSP-D \textsubscript{Lance} (1 μg), labeled within the collagen domain, as described above, was incubated alone or in the presence of CG (1 μg) for 4 h at 37 °C in a 20-μl reaction containing PBS (pH 7.4) supplemented with CaCl\textsubscript{2} (10 mM). The reactions were stopped by addition of PMSF (1 mM) and transferred to albumin-blocked, mannan-coated wells. Following incubation for 2 h at room temperature, samples were washed and bound proteins were detected using strepta-vidin-horseradish peroxidase conjugate and tetramethylbenzidine peroxidase substrate (Kirkegaard & Perry, Gaithersburg, MD) (44). Differences in absorbance were determined at 450 nm using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

**Degradation of SP-D by Neutrophils—**Neutrophils were isolated from mutant or WT mice as previously described (40). Briefly, neutrophils were attracted to the peritumour of mice by intraperitoneal injection with 15% glycogen (1 ml/mouse). Four hours later, mice were sacrificed by CO\textsubscript{2} narcosis. The peritoneal cavities were lavaged with 5 ml of Hank's balanced salt solution (pH 7.4, without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}), and the lavage fluids were spun and resuspended in a hypotonic solution to remove red cell contamination. Neutrophils represented >95% of the cell population and >98% were viable as judged by differ- ences in trypan blue dye exclusion.

To investigate SP-D cleavage by neutrophils, aliquots of 1 × 10\textsuperscript{6} cells in 200 μl of PBS (pH 7.4) were primed and stimulated by addition of LPS (10 μg/ml) and formyl-methionyl-leucyl-phenylalanine (fMLP, 1 μM) (48). Next, RrSP-D (1 μg) was added and the reactions were incubated overnight at 37 °C at pH 7.4. In some experiments, stimulated WT neutrophils were preincubated with the indicated serine proteinase inhibitors for 5 min at 37 °C prior to addition of SP-D. Controls included RrSP-D incubated alone or with purified CG. Following incubation, samples were acetone-treated for protein extraction, and subjected to SDS-PAGE and Western blotting as described above. Under these experimental conditions, overall >50% of the cells were dead following overnight incubation as judged by trypan blue dye exclusion. However, there were no significant differences in viability between unstimulated or LPS/MLP-stimulated neutrophils. The NSF release was determined using the peptide substrates corresponding to the individual enzymes as described elsewhere (49).

**Mouse Model of Pneumonia—**Mice were intranasally challenged with a subcutaneous dose of F. oxysporum (H103) bacteria or sterile saline as previously described (50). Briefly, mice were anesthetized by intraperitoneal injection of ketamine hydrochloride (75 mg/kg) and medetom-dine hydrochloride (1 mg/kg) followed by intranasal administration of bacteria (4 × 10\textsuperscript{8} colony forming units/mouse) in 50 μl of PBS. Groups of mice were sacrificed at designated time points, and their lungs were either fixed with Hanks balanced salt solution buffered (pH 7.4) or infused with 10% buffered formalin, excised, and processed for histology (51). Cell counts from BAL fluids were immediately performed by hemacytometer, and aliquots of BAL fluids were cyospun for differential counting or immunostaining. The remaining BAL samples were centrifuged for 10 min at 4 °C. The cell pellets were resuspended (1 × 10\textsuperscript{6} cells) and lyzed with 0.1% Triton X-100. BAL supernatants and cell lysates were snap-frozen and stored at −80 °C until use.

**Immunostaining and Confocal Microscopy—**Immunohistochemistry on serial lung tissue sections was performed as previously described (51). Briefly, formalin fixed tissues were paraffin embedded, sectioned (5 μm), deparaffinized, rehydrated, and microwave-treated for 7 min in PBS to enhance antigen retrieval. Endogenous peroxidase and nonspecific binding sites were blocked by incubation with H\textsubscript{2}O\textsubscript{2} (0.3%) and blocking reagent (Background Eraser; Biocare Medical, Walnut Creek, CA) for 30 min. Sections were incubated with antibodies specific for mouse SP-D (dilution, 1:750) or CG (dilution, 1:200) at 4 °C overnight. Equivalent concentrations of rabbit preimmune serum were used as a negative control. Next, samples were incubated for 20 min with biotinylated secondary antibody and labeled for 15 min with horseradish peroxidase or alkaline phosphatase-conjugated streptavidin. Immune complexes were visualized using 3,3-diaminobenzidine or Vulcan Fast-Red chromogen (Biocare Medical) as substrates for horseradish peroxidase or alkaline peroxidase, respectively, and counterstained with Mayer's hematoxylin.

Cytopsin from BAL fluids were fixed for 10 min in 3% (w/v) paraformaldehyde and permeabilized for 5 min with 0.1% Triton X-100, each step followed by three washes in PBS. Nonspecific binding was blocked with 3% bovine serum albumin in PBS for 30 min, and cells were incubated with antibodies specific for mouse SP-D for 1 h at room temperature for bacterial staining, samples were either horseradish peroxi-dase/3',3-diaminobenzidine-immunostained as described above, or goat anti-rabbit Alexa 488-immunostained (dilution 1:200, Molecular Probes) for confocal microscopy. The cells were washed and the nuclei were stained with TO-PRO-3 iodide (Molecular Probes) for 10 min. Following a final wash, cells were mounted in Vectashield (Vector Laboratories) and coverslipped using Secure Seal imaging spacers (Sigma). Specimens were examined by confocal microscopy using a LSM 510 Meta laser scanner microscope (Carl Zeiss Inc., Thornwood, NY).

**Protease Activities in BAL Fluids—**NE activity was determined by κ-elastin zymography (40). Briefly, cell-free BAL fluids or cell lysates (20 μl) were electrophoresed under non-reducing conditions at 4 °C on 12% SDS-PAGE gels containing 1 mg/ml elastin. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 30 min, rinsed briefly, and incubated at 37 °C for 48 h in 50 mM Tris-HCl (pH 8.2), containing 5 mM CaCl\textsubscript{2}. The gels were then stained with Coomassie Blue and destained in 5% acetic acid and 10% methanol. Active NE appears as a transparent band at ~30 kDa. Protease activities in cell-free BAL fluids were further confirmed using conventional chromogenic peptide assays as described above.

**RESULTS**

**Neutrophil Serine Proteinases Specifically Degrade SP-D—**To determine whether SP-D is susceptible to degradation by NSPs, we incubated RrSP-D dodecamers with purified NE, PR3, or CG. All three enzymes cleaved SP-D and generated distinct fragments of ~35 kDa when examined by protein electrophoresis and Coomassie staining (Fig. 1A) and Western blotting using polyclonal antibodies specific for rat SP-D (Fig. 1B). Identical fragmentation patterns were obtained when incubations were performed in PBS (pH 7.4) or Tris-buffered saline containing 0.05% Tween 20 (data not shown). Significantly, SP-D degradation was prevented when the NSPs were first exposed to the specific serine proteinase inhibitor PMSF, indicating that the catalytic activity of the enzyme is required to cleave SP-D (Fig. 1C). In other experiments, specific inhibition was also achieved with AEBSF and disopropyl fluorophos-phate (data not shown).

Recalcification of the SP-D stock with CaCl\textsubscript{2} in excess of EDTA resulted in less marked but a qualitatively similar pattern of proteolysis (Fig. 1D). Degradation of SP-D in the presence of CaCl\textsubscript{2} was also dependent on time and temperature (Fig. 2). Of note, there was no evidence of SP-D degradation in the absence of enzymes even with prolonged incubations at 37 °C (Fig. 2B).

Various enzyme concentrations can be encountered in vivo including very high concentrations near sites of granule exocytosis (52). Accordingly, we examined a wide range of enzyme:
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Neutrophil serine proteinases cleave SP-D. RrSP-D (1 μg) was incubated alone or in the presence of NE, PR3, or CG (1 μg each) for 8 h. The reactions were resolved by SDS-PAGE under reducing conditions and visualized by Coomassie staining or immunoblotting. A, all three NSPs degraded SP-D at physiologic pH, ionic strength, and temperature with the generation of major ~35-kDa fragments (asterisk). B, the fragments react with antibodies to SP-D. The arrow points to the position of NSPs. C, the addition of PMSF (1 mM) prevented SP-D degradation. D, SP-D cleavage was decreased when the reactions were incubated in the presence of CaCl2 (10 mM). Mr standards are at the left. The findings are illustrative of at least three independent experiments.

Degradation of SP-D Is Highly Dependent on the Calcium Concentration—RrSP-D-maltose preparations, which have not been exposed to EDTA, were used to evaluate the effect of different free Ca2+ concentrations including physiological values. Quantification of the extent of cleavage was achieved by immunoblotting and phosphorimaging as described under “Experimental Procedures.” Incubation of RrSP-D-maltose with CG, NE, or PR3 revealed a dramatic reciprocal relation between CaCl2 levels and the extent of cleavage (Fig. 3). In other experiments we compared the dose response for each proteinase in the absence and presence of CaCl2. Although the extent of cleavage was greater, considerably higher enzyme concentrations and/or times of incubation were required to achieve the same extent of cleavage (data not shown). Of note, there was no significant change in the activity of the three proteinases in standard chromogenic peptide assays when CaCl2 was varied over the range of 0.01 to 10 mM (Table I). Thus, the decreased degradation seen at relatively high Ca2+ concentrations can be attributed to effects on the substrate rather than the proteinases. Also, the pattern of RrSP-D-maltose cleavage was not appreciably different from that observed for RrSP-D and the extent of cleavage was slightly increased, rather than decreased (data not shown). This increase is unlikely to reflect the presence of small amounts of residual saccharide ligand (1.5 mM in these reactions), which is significantly below the I50 in most binding assays. Moreover, cleavage of RrSP-D-maltose by CG was not altered when the maltose concentration was incrementally increased to 40 mM (data not shown).

Human SP-D Is Cleaved by Neutrophil Serine Proteinases—To directly confirm that our findings with recombinant rat SP-D could be extrapolated to human SP-D, we incubated NE, PR3, or CG with human SP-D dodecamers that we previously isolated from alveolar proteinosis BAL fluids (37). As shown in Fig. 4A, all three NSPs degraded hSP-D and generated a ~35-kDa fragment with the same mobility as the major fragment generated with RrSP-D.

To further exclude the possibility that our recombinant or natural substrates were altered during isolation, we examined the effects of purified NSPs on cleavage of human SP-D in unprocessed BAL fluids (39). As shown in Fig. 4B, addition of CG resulted in efficient and dose-dependent cleavage of SP-D with the generation of the expected cleavage product.

Specific Cleavage of SP-D Occurs within the COOH-terminal Region—Under physiological conditions, SP-D exists as multimers of trimeric subunits stabilized by interchain disulfide bonds within the amino-terminal peptide domains. Accordingly, we examined the migration of the SP-D fragments by SDS-PAGE under non-reducing conditions. In the absence of sulfhydryl reduction, the proteolytic fragments of RrSP-D migrated as high molecular weight complexes only slightly faster.
than the disulfide cross-linked SP-D trimers, indicating the presence of intact interchain disulfide bonds (Fig. 5A). Given the large sizes of these fragments and the retention of the NH$_2$-terminal interchain bonds, we readily inferred that initial cleavage must occur at relatively COOH-terminal sites probably within the CRD. As noted for the reduced monomeric products, there are also slight differences in mobility of the non-reduced fragments liberated by each proteinases (Fig. 5A), indicating that the three NSPs cleave at distinct neighboring sites within a subregion of the CRD.

**Identification of a Major Catheptic Cleavage Site within the SP-D Carboxyl-terminal Domain**—To more precisely localize the sites of cleavage, we first isolated the relatively stable ~35-kDa peptides for NH$_2$-terminal sequence analysis. Because RrSP-D has a blocked NH$_2$ terminus that is not effectively de-blocked using the usual procedures (43), no NH$_2$-terminal sequence should be obtained in the absence of NH$_2$-terminal degradation. Consistent with our expectation, no sequence was obtained for the ~35-kDa fragments generated in the presence of NE, PR3, or CG.

Given these findings, we proceeded to isolate transient intermediates that included disulfide-bonded COOH-terminal fragments suitable for sequencing. As shown in Fig. 2A, CG gave comparatively rapid cleavage that reproducibly proceeded through a transient intermediate, which was slightly larger than the stable ~35-kDa fragment. Given the particularly high reproducibility of catheptic cleavage, we chose this NSP for further biochemical characterization of the cleavage site. RrSP-D was incubated alone or in the presence of CG for varying times, and the reactions were analyzed by protein electrophoresis or reverse phase HPLC followed by NH$_2$-terminal sequencing of selected peak fractions (Fig. 5, B and C). An unambiguous and unique NH$_2$-terminal amino acid sequence was obtained from a discrete but transient peak that was generated 2 h after incubation of SP-D with CG: Thr-Tyr-Pro-Thr-Gly-Glu-Ala-Leu-Val. This sequence is identical to residues 305–313 of the mature rat SP-D. Comparison to known SP-D structures of other mammalian species by sequence alignment revealed that cleavage occurs within a highly conserved subregion of the CRD (Fig. 5D). Examination of the primary structure of mature SP-D monomer demonstrates that the sequenced catheptic fragment is located within the designated loop 1 and the minimal functional CRD (Fig. 6A). Moreover, when viewed in projection to the crystal structure of a trimeric human SP-D molecule, the proposed cleavage site is at the surface of the CRD but distant from the ligand binding
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Cleavage by CG Alters the Biological Activity of SP-D—To determine whether exposure of SP-D to NSPs alters its biological functions, we employed two well characterized in vitro assays: bacterial agglutination and mannan binding, both of which are known to involve the C-type lectin domain (26, 46).

Because agglutination is mediated by bridging interactions that require at least two functional subunits, it should be very sensitive to cleavage of the CRD domains. Whereas intact SP-D agglutinates K. pneumoniae, there was no significant agglutination following incubation of RrSP-D with CG under conditions that generate the ~35-kDa fragment (Fig. 7A). Thus, cleavage within the CRD by NSPs interferes with the lectin activity of SP-D.

We also examined a more stringent solid-phase mannan binding assay. Although we have frequently used biotinylated SP-D labeled at the amino groups for binding studies, for these experiments we used an alternative biotinylation procedure that selectively labels carbohydrates, which are restricted to the collagen domain, well amino-terminal to the sites of cleavage (Fig. 7B, left). Because isolated trimeric subunits can bind to mannan and because two or three intact CRDs of a single trimeric subunit of the dodecamer are probably necessary to mediate binding of the labeled substrate to the plate, a decrease in binding activity should reflect the functional inactivation of all four trimeric subunits of a SP-D molecule. Using the same cleavage conditions described for the bacterial agglutination assay, we found that treatment of SP-D with CG decreased its binding to mannan by more than 50% as compared with the incubated control (Fig. 7B).

Isolated Neutrophils Degrade SP-D—We next sought to determine whether neutrophils mediate similar cleavage of SP-D. Neutrophils were isolated from the peritonea of WT mice and stimulated with LPS and fMLP. The number of neutrophils added per reaction was calculated to yield concentrations of liberated proteinases comparable with those shown in Figs. 1 and 2, and the release of NSP under these assay conditions was determined using the peptide substrates corresponding to the individual enzymes (data not shown). Incubation with WT neutrophils resulted in complete cleavage of RrSP-D with the generation of an immunoreactive band that migrated near the

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We also examined a more stringent solid-phase mannan binding assay. Although we have frequently used biotinylated SP-D labeled at the amino groups for binding studies, for these experiments we used an alternative biotinylation procedure that selectively labels carbohydrates, which are restricted to the collagen domain, well amino-terminal to the sites of cleavage (Fig. 7B, left). Because isolated trimeric subunits can bind to mannan and because two or three intact CRDs of a single trimeric subunit of the dodecamer are probably necessary to mediate binding of the labeled substrate to the plate, a decrease in binding activity should reflect the functional inactivation of all four trimeric subunits of a SP-D molecule. Using the same cleavage conditions described for the bacterial agglutination assay, we found that treatment of SP-D with CG decreased its binding to mannan by more than 50% as compared with the incubated control (Fig. 7B).

Isolated Neutrophils Degrade SP-D—We next sought to determine whether neutrophils mediate similar cleavage of SP-D. Neutrophils were isolated from the peritonea of WT mice and stimulated with LPS and fMLP. The number of neutrophils added per reaction was calculated to yield concentrations of liberated proteinases comparable with those shown in Figs. 1 and 2, and the release of NSP under these assay conditions was determined using the peptide substrates corresponding to the individual enzymes (data not shown). Incubation with WT neutrophils resulted in complete cleavage of RrSP-D with the generation of an immunoreactive band that migrated near the

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position of the major fragment liberated by purified CG (Fig. 8A). The major fragments retained interchain disulfide bonds, indicating cleavage within the CRD, similar to the sites of cleavage for the purified enzymes (data not shown).

SP-D cleavage by WT neutrophils was largely abrogated when incubations were performed in the presence of AEBSF, a relatively stable broad spectrum serine proteinase inhibitor (Fig. 8A) strongly suggesting that degradation is mediated by neutrophil-derived serine proteinases. Similar but less reproducible results were obtained with PMSF (data not shown) probably because of the comparatively low stability of this inhibitor in aqueous solution. SP-D was also incubated with WT neutrophils in the presence of more physiological polypeptide inhibitors including MNEI, which has been shown to efficiently inhibit all three NSPs (36). Interestingly, treatment of neutrophils with increasing concentrations of MNEI, a stoichiometric serpin inhibitor, led to progressive inhibition of SP-D degradation (Fig. 8A). Significant inhibition was also observed with SLPI and α1-antitrypsin (Fig. 8A and data not shown). Incubations in the presence of EDTA gave no appreciable inhibition (data not shown), a finding consistent with the limited ability of neutrophil metalloelastases to cleave rat SP-D decamers in vitro (33). To assess the roles of specific NSPs in neutrophil-mediated degradation, we incubated RrSP-D decamers with neutrophils isolated from mice deficient in NE<sup>−/−</sup>, CG<sup>−/−</sup>, or NE-CG<sup>2/2−</sup>− mice, and the reactions were processed for immunoblotting using SP-D-specific antibody. Incubation with WT neutrophils resulted in complete cleavage of the SP-D starting material and generation of an immunoreactive band that migrated near the position of the major fragment liberated by purified CG (lanes 2 and 3). SP-D cleavage by WT neutrophils was largely abrogated when incubations were performed in the presence of the synthetic serine proteinase inhibitor AEBFSF (1 mM) (lane 4). Preincubation of neutrophils with the physiologic serine proteinase inhibitor SLPI (10 μg) prevented considerably SP-D degradation (lane 5). Interestingly, another physiologic inhibitor, MNEI (10, 30, or 100 μg), inhibited SP-D degradation in a dose-dependent fashion (lanes 8–10). Neutrophils from WT and mutant mice degrade SP-D and generate major ~35-kDa fragments (asterisk). However, deficiency in one or two NSPs reduces the ability of the neutrophil to cleave SP-D (lanes 4–6) when compared with WT neutrophils (lane 3). The SP-D antibody does not cross-react with neutrophil proteins (lane 7); the arrow indicates the position of CG. The findings are illustrative of three independent experiments per condition.

In Vivo Co-localization of SP-D and Neutrophil Serine Proteases—To determine whether SP-D degradation by neutrophils can occur in vivo, we used a previously described mouse <i>P. aeruginosa</i> pneumonia model (50). Of note, SP-D binds to both smooth and rough forms of <i>P. aeruginosa</i> through interactions with lipopolysaccharides (53), and SP-D has been shown to enhance alveolar macrophage-mediated uptake and killing of <i>P. aeruginosa in vitro</i> (54). To circumvent the confounding effect of <i>P. aeruginosa</i> metalloelastase, known to degrade SP-D...
Neutrophil Serine Proteinases Cleave and Inactivate SP-D

Fig. 9. In vivo co-localization of CG and SP-D. (A) Lung tissue sections from saline control mice (n = 2) were immunostained for mouse SP-D and visualized using 3,3-diaminobenzidine chromogen (brown precipitate). Under physiologic conditions, SP-D is restricted to epithelial surfaces and alveolar type II cells (arrow), consistent with the known localizations of SP-D production. B–D, representative serial lung tissue sections from mice (n = 6) following intranasal challenge with P. aeruginosa. Immunostaining for SP-D reveals that the protein comes in close spatial contact with the cellular infiltrates during inflammation (B). Immunostaining with antibody specific for mouse CG and visualization by alkaline peroxidase–Fast Red chromogen identified neutrophils as the predominant cell type (C). No staining was seen when the primary antibody was replaced by preimmune serum (D).

We employed P. aeruginosa strain H103, which lacks this enzyme (56). Immunostaining of lung sections from unchallenged control mice found SP-D expression restricted to alveolar type II cells and the bronchiolar epithelium, consistent with the known sites of SP-D production. Of note, we also detected significant amounts of NE in the cell-free fraction of the BAL fluids, suggesting the presence of unopposed and active neutrophil-derived serine proteinases within the alveolar spaces (Fig. 10F). The presence of free active NSPs following challenge with bacteria, but not saline, was further confirmed by incubation of cell-free BAL fluids with synthetic proteinase substrates (data not shown). As shown in Fig. 10G, immunoblotting of BAL fluids 24 h post-infection revealed limited degradation of endogenous SP-D with generation of a ~35-kDa cleavage product similar to that generated by purified NSPs. Of note, there was no evidence of SP-D degradation in saline-treated mice.

DISCUSSION

Activated neutrophils accumulate and release their proteolytic enzymes at sites of acute inflammation. Here, we demonstrate that the three active NSPs, NE, PR3, and CG, specifically degrade SP-D within a conserved subregion of the C-terminal lectin domain abrogating its biologic activity. Furthermore, we demonstrate that SP-D is susceptible to specific degradation by NSPs released by isolated neutrophils and that specific deficiency in NSP production decreases the efficiency of neutrophil-mediated cleavage.

The in vivo significance of these findings was confirmed using a murine P. aeruginosa pneumonia model. Both SP-D and active NSPs were detected in neutrophilic infiltrates and in the surrounding tissue. Furthermore, analysis of BAL fluids found free active NSPs and SP-D cleavage product similar to that generated by purified enzymes. Thus, our data strongly suggest that NSPs encounter SP-D in the lungs and contribute to its degradation, at least in the setting of bacterial infection. Our in vivo findings are supported by previous in vitro studies that described NE-mediated SP-D degradation (32). However, our experiments indicate that CG and PR3 are at least as potent as NE. This could be of considerable biological importance given that the proteinases are released at approximately equivalent concentrations from neutrophil granules, but show different susceptibilities to inactivation by physiological proteinase inhibitors such as SLPI (57). With respect to NSP-catalyzed SP-D degradation, the relative contribution of NE, PR3, or CG will be best determined once mice deficient in PR3 or all three NSPs are available. To this end, efforts to generate mice deficient in PR3 and triple NE/PR3/CG mutant mice are in progress.

SP-D is secreted in a mature form that does not require extracellular proteolytic processing (58). In the mouse, instilled SP-D has a half-life of ~13 h (59). Macrophages and/or alveolar type II cells probably play a role in physiological turnover as these cells can internalize and degrade SP-D through unknown mechanisms (60, 61). It has recently been shown that LPS challenge in rats is associated with a nearly 2-fold increase in the clearance of instilled SP-D (23). This was accomplished by increased numbers of SP-D positive tissue-associated neutrophils and a marked increase of labeled SP-D per neutrophil. In addition, in vitro studies showed internalization and increased intracellular degradation of SP-D by LPS-treated lung neutrophils (23). However, the mechanism of this intracellular degradation was not investigated. The present studies provide strong evidence that neutrophils can employ NSPs to degrade SP-D. Because cleavage can be inhibited by physiological pul-

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neutrophil serine proteinases cleave SP-D in vivo. Mice were intranasally infected with *P. aeruginosa* and their lungs were processed for BAL at designated times (*n* = 4 mice/time point). A, cell counts of BAL show a sharp increase of total recovered cells (filled bars) within 24 h post-challenge. Differential cell counts on BAL cytospins (insets, ×200) demonstrate resident alveolar macrophages immediately post-challenge (time 0 h), but a massive influx of neutrophils (open bars) by 24 h. B–E, cytospins from BAL fluids 24 h post-challenge were immunostained for SP-D and visualized using 3,3-diaminobenzidine chromogen (B) or Alexa 488-labeled antibody and laser scanning confocal microscopy (green channel, SP-D; blue channel, nuclei) (C and E, white bar = 10 μm). Immunoreactive SP-D was localized within the cytoplasm of alveolar macrophages (filled arrowhead) and neutrophils (open arrowhead) (B and C), suggesting that both cells actively internalize SP-D. In contrast, neutrophils that were isolated from the peritoneum of mice stained negative for SP-D (D) and no staining was seen when the primary antibody was replaced with rabbit preimmune serum (E). F, elastin zymography shows NE in lysates from BAL cells at 24 h as a single lysis band at ~30 kDa (lane 1) (40). Consistent with the absence of neutrophils at 0 h, no NE is detected at this time point (lane 2). In contrast, increasing amounts of free and active NE were detected in cell-free BAL fluids 4 and 24 h post-infection (lanes 3 and 4). G, BAL fluids from mice at 0, 4, or 24 h post-challenge were reduced and processed for immunoblotting using SP-D antibody. All three time points revealed intact SP-D monomers (lanes 7–9), as compared with SP-D control (lane 5). However, at 24 h, coinciding with the massive influx neutrophils and active NE in the BAL fluid, a distinct cleavage fragment (asterisk) was detected that migrated similar to the cleavage fragment generated by purified CG (lanes 6 and 9). Data in B–G were similar in all challenged mice.

monary NSP inhibitors *in vitro*, it is likely that NSP-dependent cleavage is limited to situations of acute inflammation or infection *in vivo*. In fact, our observations with SLPI and MNEI suggest that the degradation of SP-D *in vivo* will be determined by the balance of active proteinase and proteinase inhibitors within specific microenvironments of the lung. However, consistent with our findings, lung injury or microbial challenge can be associated with the massive recruitment of neutrophils and extracellular release of free, active NSPs at inflamed milieu (62). As we have shown, NSPs can accumulate in close spatial proximity to SP-D at sites of active inflammation, both within cells and in the extracellular space, and at a time when active enzyme is detected in BAL fluids. In this regard, decreased SP-D levels and exaggerated neutrophil influx with increased concentrations of extracellular NE in the lungs have been described in association with acute lung injury and acute respiratory distress syndrome (6, 20, 63). The relative contributions of intracellular and extracellular degradation of SP-D remain to be elucidated. As shown in Fig. 8, the *in vitro* neutrophil-mediated degradation was not completely inhibited, suggesting some contributions of other classes of enzyme. However, it is unlikely that neutrophil matrix metalloproteinases contribute to early cleavage events, given that degradation was observed in the presence of a calcium chelator.

CG, which yields a disulfide cross-linked NH₂-terminal fragment similar in size to NE and PR3, cleaves SP-D within a conserved region of the large disulfide loop (designated loop 1) of the CRD. The Phe³⁰⁴-Thr³⁰⁵ bond is well within the minimal functional carbohydrate recognition domain, which begins NH₂-terminal to the first cysteine of the CRD. Inspection of the cleavage site in relation to the known crystal structure of the human SP-D CRD indicates that the cleaved peptide bond is near the surface of the molecule, and near to, but spatially separated from, the sugar-binding site (Fig. 6). As shown in the figure, the aromatic ring of Phe³⁰⁴ is at least partially buried. Although this is likely the site of initial cleavage, prior cleavage at the slightly more COOH-terminal site cannot yet be entirely excluded. Non-aromatic hydrophobic residues, which are preferred at position 1 of NE and PR3 cleavage sites, are present near the Phe³⁰⁴-Thr³⁰⁵ bond of the mature protein. Recently, cleavage sites for NE were demonstrated at Ala²⁹¹-Phe²⁹² and Val³¹³-Tyr³¹⁴ (32), proximal and distal to the site of CG cleav-
age. Together, these data indicate that NSPs cleave SP-D at specific sites within a conserved subregion of the CRD.

There are important functional implications of degradation in the region of the cathodic cleavage site. Because a disulfide cross-linked conformation is required for lectin activity, even single-site cleavage within disulfide loop 1 is likely to inhibit lectin activity and/or modify ligand selectivity of the cleaved chain (16). This is consistent with our finding of decreased yeast mannann binding activity and bacterial agglutination of SP-D following incubation with CG. Interestingly, examination of the cleavage site in relation to the ligand binding surface of the CRD (Fig. 6C) suggests that this site may remain accessible in the presence of bound ligand.

Previous studies have shown increased proteolytic degradation of C-type lectins, including collectins (30, 32, 34, 64); however, this is the first systematic study of the effects of Ca$$^{2+}$$ on SP-D degradation by NSPs. In the present study, the susceptibility of SP-D to degradation was markedly increased in the absence of Ca$$^{2+}$$ for all three NSPs particularly in assays performed at relatively low enzyme/substrate ratios (~1:10–1:100). Importantly, there was no effect of Ca$$^{2+}$$ on proteinase activity under the conditions of our experiments, and addition of CaCl$_2$ rendered SP-D relatively resistant to NSP proteolysis. Because the fragments generated in the presence of low and high Ca$$^{2+}$$ concentrations show the same mobility on SDS-PAGE, it is likely that cleavage occurs at the same site, and that differences in the efficiency of cleavage reflect alterations in the accessibility of the cleavage site.

In this regard, a coordinated Ca$$^{2+}$$ ion is integral to the primary carbohydrate binding site of collectins and most other C-type lectins (14), and residues involved in coordinating this cation are found beginning at Glu321, only slightly COOH-terminal to the demonstrated site. Three additional Ca$$^{2+}$$ binding sites have been identified based on the crystal structure of SP-D (65, 66); two of these sites are even closer to the site of cleavage. Although there is no information regarding the functions of these calcium atoms, they can be expected to influence local protein structure. Furthermore, Ca$$^{2+}$$-dependent alterations in the CD spectrum of SP-D have been described (67). Together, the available data strongly suggest that low Ca$$^{2+}$$ concentrations alter the structure of the CRD and render the molecules more susceptible to degradation by NSPs. Because the calcium binding sites differ in affinity (66), their differential occupancy could also influence susceptibility to degradation.

Little is known about the regulation of extracellular Ca$$^{2+}$$ in the airspaces of the normal or injured lung; however, hypocalcemia or local decreases in free Ca$$^{2+}$$ could facilitate SP-D degradation by NSPs in vitro. Normal levels of ionized Ca$$^{2+}$$ in the blood are ~1.3 mm, only slightly higher than the concentration required to maintain significant resistance of purified SP-D to relatively low concentrations of NSPs in vitro. Interestingly, decreased levels of ionized blood calcium levels are very common among intensive care patients and complicate sepsis syndromes (68, 69), settings in which the levels of SP-D can be at least transiently decreased (20, 22). It should be noted, however, that higher enzyme/substrate ratios of ~1:1 or greater, which might easily be achieved in the vicinity of degranulating neutrophils (52), were associated with significant degradation, even in the presence of supra-physiological Ca$$^{2+}$$ concentrations.

In conclusion, neutrophil-dependent degradation of SP-D is anticipated in any neutrophil-rich inflammatory milieu in which the total NSP burden exceeds inhibitory levels of physiological serine protease inhibitors. Consequently, active NSPs could broadly interfere with SP-D function leading to an acquired deficiency with resultant effects on host defense, innate immunity, and surfactant homeostasis.

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