INFECTION AND IMMUNITY, Jan. 2007, p. 164–174 Vol. 75, No. 1
0019-9567/07/$08.00+0 doi:10.1128/IAI.01239-06

Serratia marcescens Serralysin Induces Inflammatory Responses through Protease-Activated Receptor 2[▽]

Yutaka Kida,[1] Hiroyoshi Inoue,[2] Takashi Shimizu,[1] and Koichi Kuwano[1]*

Department of Bacteriology[1] and Radioisotope Institute for Basic and Clinical Medicine,[2] Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan

Received 4 August 2006/Returned for modification 14 September 2006/Accepted 2 October 2006

The *Serratia marcescens*-derived protease serralysin is considered to play an important role in the pathogenesis of infection. Protease-activated receptor 2 (PAR-2) is activated by trypsin and also several other trypsin-like serine proteases, leading to the modulation of inflammatory and immune responses. However, little is known about the activation of PAR-2 by bacterial proteases and its roles in bacterial infection. In this study, we investigated whether *S. marcescens* serralysin activates host inflammatory responses through PAR-2. Our results demonstrated that serralysin induces interleukin-6 (IL-6) and IL-8 mRNA expression in a human lung squamous cell carcinoma, EBC-l cells. In addition, serralysin activated activator protein 1 (AP-1)-, CCAAT/enhancer-binding protein (C/EBP)-, and nuclear factor-κB (NF-κB)-driven promoters in EBC-1 cells. An electrophoretic mobility shift assay showed that serralysin activates the binding of AP-1, C/EBPβ, and NF-κB in the cells. Inactivation of serralysin resulted in the failure of transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters in the cells. Furthermore, serralysin activated AP-1-, C/EBP-, and NF-κB-driven promoters via PAR-2 in HeLa cells. PAR-2 antagonist peptides decreased serralysin-induced transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells. Considered together, these results suggest that serralysin requires PAR-2 to activate the critical transcription factors AP-1, C/EBPβ, and NF-κB for host inflammatory responses.

Serratia marcescens is a gram-negative enteric bacterium, often isolated from respiratory and urinary tracts, that can function as an opportunistic pathogen in immunocompromised hosts (18). *S. marcescens* is a source of nosocomial infections, in part because the organism readily adheres to invasive hospital instrumentation, such as catheters, endoscopes, and intravenous tubing (21), and is relatively resistant to standard sterilization and disinfection protocols (10, 63). *S. marcescens* causes a wide spectrum of infections such as pneumonia, meningitis, septicemia, urinary tract infection, endocarditis, conjunctivitis, and wound infection (21, 63). Despite numerous reported *S. marcescens* infections and the emergence of antibiotic-resistant strains (21, 62), the virulence mechanisms of this organism are poorly understood. *S. marcescens* secretes many known extracellular proteins, including chitinase, lecithinase, hemolysin, siderophore, lipase, protease, and nuclease (5, 22). Although *S. marcescens* produces various proteases, a zinc metalloprotease, serralysin, is especially produced in the largest amounts from pathogenic clinical isolates (40). Interestingly, Maeda and coworkers reported that serralysin plays a critical role in pathogenesis of this organism (37, 38, 41). Purified serralysin has been used in in vivo models of keratitis with rabbits and guinea pigs (26), and its enzymatic property has been shown to rapidly degrade a wide range of structural and serum proteins (42). Accordingly, bacterial proteases such as serralysin appear to play an important role as a virulence factor.

Protease-activated receptors (PARs) belong to a family of G-protein-coupled seven transmembrane receptors (36). Rather than being stimulated through ligand receptor occupancy, the activation of PARs is initiated by proteolytic cleavage of the amino-terminal domain of the receptor, resulting in the generation of a new tethered ligand that interacts with the receptor within extracellular loop 2 (23, 36). To date, four PARs have been identified; three of them (PAR-1, PAR-3, and PAR-4) are activated mainly by thrombin, and the fourth (PAR-2) is activated by trypsin, as well as several other trypsin-like serine proteases, including factor Xa, neutrophil protease 3, and mast cell tryptase (47, 51). The PAR activation contributes to a variety of physiological and pathophysiological roles in various tissues and cells, including circulatory, gastrointestinal, respiratory, and central nervous systems (23, 36, 47). In particular, the activation of PAR-2 is generally considered to result in inflammatory responses according to the experimental data, including various in vivo models of inflammation with PAR-2-deficient mice (11, 17, 20, 29, 35, 53, 55). Earlier studies have shown that trypsin cleaves the amino-terminal extracellular domain of human PAR-2 at $SKGR^{36} \downarrow S^{37}LIGKV$ (where the "↓" designates the trypsin cleavage site), unmasking the amino-terminal intramolecular tethered ligand SLIGKV (47). Accordingly, the synthetic peptide corresponding to this sequence, which is a specific agonist, can activate PAR-2 without the need for receptor cleavage.

PAR-2 is widely distributed in the mammalian body and is also expressed in various cells, including epithelial cells, endothelial cells, T cells, neutrophils, and neurons (8, 15, 24, 25, 45,

* Corresponding author. Mailing address: Department of Bacteriology, Kurume University School of Medicine, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan. Phone: 81-942-31-7548. Fax: 81-942-31-0343. E-mail: kuwano@med.kurume-u.ac.jp.

▽ Published ahead of print on 16 October 2006.

46). Particularly in the respiratory system, proteases from the house dust mite *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus*, as well as trypsin and also several other trypsin-like serine proteases, activate PAR-2, thereby modulating host inflammatory and immune responses (4, 28, 51, 58). Interestingly, *Porphyromonas gingivalis*-derived proteases gingipains have been shown to upregulate the expression of the antimicrobial peptide β-defensin 2 via PAR-2 in human gingival epithelial cells (12). Thus, the activation of PAR-2 appears to play a crucial role in such responses. However, not much is available on the activation of PAR-2 by bacterial proteases and the role of PAR-2 in bacterial infection. Accordingly, we speculated that host inflammatory responses would be affected by bacterial proteases such as serralysin, as well as by proteases from mites and fungi.

In the present study, we determined whether serralysin activates host inflammatory responses via PAR-2. Our results indicated that serralysin induces interleukin-6 (IL-6) and IL-8 mRNA expression in a respiratory cell line, EBC-1. Moreover, we demonstrated that serralysin activates the critical transcription factors activator protein 1 (AP-1), CCAAT/enhancer-binding protein β (C/EBPβ), and nuclear factor-κB (NF-κB) for host inflammatory and immune responses via PAR-2. These results suggest that serralysin would require PAR-2 to modulate various host responses against bacterial infection.

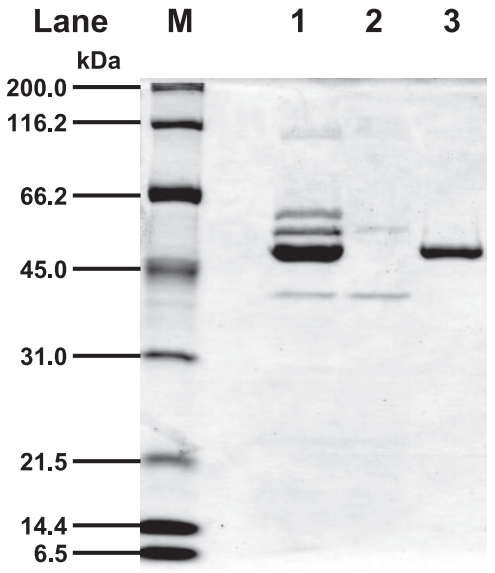


FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of serralysin purified by anion-exchange column chromatography. The gel was stained with Coomassie blue G-250. Lane M, molecular weight markers; lane 1, supernatant proteins were precipitated with ammonium sulfate; lane 2, flowthrough fraction from Q-Sepharose anion-exchange column; lane 3, purified serralysin.

MATERIALS AND METHODS

Cells. A human lung squamous cell carcinoma cell line, EBC-l cells (Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University, Sendai, Japan), was maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented 10% heat-inactivated fetal bovine serum (Gibco, Rockville, MD), 2 mM L-glutamine, 0.2% sodium bicarbonate, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (33). A human cervical epithelioid carcinoma cell line, HeLa cells, was maintained in Dulbecco modified Eagle medium (DMEM; Nissui Pharmaceutical) supplemented 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 0.35% D-glucose, 0.37% sodium bicarbonate, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Cells were passaged without the use of trypsin by nonenzymatic cell dissociation solution (Gibco) to minimize the proteolytic activation of the PARs.

Reagents. Rabbit anti-C/EBPβ, anti-c-Jun/AP-1, and anti-NF-κB p65 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-NF-κB p50 polyclonal antibody was obtained from Rockland (Gilbertsville, PA). The plasmid pC/EBP-Luc, containing the firefly luciferase reporter gene, which is controlled by a synthetic promoter that contains three repeats of the binding site (TTGCGCAAT) for the C/EBP was obtained from Stratagene (La Jolla, CA). The plasmid pNF-κB-Luc, containing the firefly luciferase reporter gene, which is controlled by a synthetic promoter that contains five repeats of the binding site (GGGGACTTTCC) for the NF-κB, was obtained from Stratagene. The plasmid pAP-1-Luc, containing the firefly luciferase reporter gene, which is controlled by a synthetic promoter that contains six repeats of the binding site (TGAGTCAG) for the AP-1, was obtained from Clontech (Palo Alto, CA). Bovine pancreas-derived endotoxin-free trypsin was purchased from Calbiochem (San Diego, CA). The peptides of human PAR-2 agonist (SLIGKV-NH_2) and antagonist (FSLLRY-NH_2 and LSIGRL-NH_2) were synthesized with amidated carboxyl terminus by solid-phase methods at Invitrogen (Carlsbad, CA) (2). All peptides were >95% purity as determined by high-pressure liquid chromatography and mass spectrometry analysis. Stock solutions were prepared in 50% dimethyl sulfoxide and stored at −20°C.

Purification of serralysin. A pigment-forming *S. marcescens*, which is a clinically isolated strain, was grown in Tryptosoy broth (Difco, Detroit, MI) for 20 h at 35°C with rotary shaking at 100 rpm (AT-12R shaker; Thomas, Tokyo, Japan). Bacterial cells were removed from the medium by centrifugation (8,000 × *g*, 30 min, 4°C), and serralysin was purified from the supernatant (39). Briefly, proteins in the supernatant were precipitated with ammonium sulfate (80% saturation). The precipitate was dissolved in 20 mM Tris-HCl (pH 8.0), dialyzed, and loaded onto a Q-Sepharose anion-exchange column (Amersham-Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer. The column was washed with 20 mM Tris-HCl (pH 8.0) containing 100 mM NaCl until unbound material was removed. Serralysin was eluted with 20 mM Tris-HCl (pH 8.0) containing 200 mM NaCl. The purified serralysin was then dialyzed against phosphate-buffered saline (PBS). The purity of serralysin was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A single band was detected in the purified serralysin (Fig. 1). The level of endotoxin was determined with a *Limulus* amebocyte lysate QCL-1000 (Cambrex, Walkersville, MD) and was revealed to be <0.5 pg/ml when suspended in PBS at a protein concentration of 1 nM. The protein concentration was determined with a Coomassie protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard. The amino-terminal amino acid sequences of serralysin were determined by using an automated protein sequencer (PSQ-1; Shimadzu, Kyoto, Japan) at Hipep Laboratories (Kyoto, Japan). The sequences have been confirmed to show the same sequences, as described previously by Nakahama et al. (44).

EDTA treatment of serralysin. Serralysin was treated with 50 mM EDTA (pH 7.4) in PBS for 24 h at 4°C. As a control, serralysin was treated with PBS for 24 h at 4°C. The treated materials were dialyzed against PBS at 4°C, with three changes of buffer (100× volume). The enzyme activities of the dialyzed materials were then determined with a protease assay kit (Calbiochem) according to the instructions provided by the manufacturer. After EDTA treatment, the enzyme activity was reduced less than 1%.

Real-time PCR analysis. EBC-1 cells maintained as described above were detached from culture flask with nonenzymatic cell dissociation solution and washed three times in serum-free DMEM–F-12 (Gibco). The cells were then seeded into six-well plates (Costar, Cambridge, MA) at a density of 10^6 cells/well in serum-free DMEM–F-12 and incubated for 24 h. One hour before stimulation, the growth medium was replaced by fresh serum-free DMEM–F-12. Cells were stimulated with serralysin at 1 nM for 2 h. Total RNA was then purified by using an RNeasy minikit (QIAGEN, Chatsworth, CA) and treated with an RNase-free DNase set (QIAGEN) to remove contaminated DNA according to the instructions provided by the manufacturer. Synthesis of cDNA was performed with an RNA PCR kit (Takara, Ohtsu, Japan) according to the manufacturer's protocol. Real-time PCR analysis was performed by using an iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) on a DNA engine Opticon 2 real-time PCR detection system (Bio-Rad Laboratories). The following human IL-6-specific primers were used: sense, 5′-AAGCCAGAGCTGTGCAGATGAGTA-3′; and antisense, 5′-TGTCCTGCAGCCACTGGTTC-3′. The following human IL-

8-specific primers were used: sense, 5′-ACACTGCGCCAACACAGAAATTA-3′; and antisense, 5′-TTTGCTTGAAGTTTCACTGGCATC-3′. The following human β-actin-specific primers were used: sense, 5′-ATTGCCGACAGGATG CAGA-3′; and antisense, 5′-GAGTACTTGCGCTCAGGAGGA-3′. The PCR profile included denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. To control for the specificity of the amplification products, a melting-curve analysis was performed. No amplification of unspecific products was observed. The threshold cycle, C_T, which correlates inversely with the level of the target mRNA, was measured as the cycle number at which the reporter fluorescent emission increased above the midpoint of the logarithmic increasing phase along the amplification curve. A standard curve relating C_T to a serial dilution of standard cDNA was used to compute the relative abundances of IL-6, IL-8, and β-actin mRNA each sample. In the quantitation of IL-6, IL-8, and β-actin mRNA, cDNA prepared from EBC-1 cells treated with human PAR-2 agonist peptide (25 μM for 2 h) was used as the standard. The relative abundance of β-actin mRNA in each sample of cells was used to normalize the IL-6 and IL-8 mRNA levels.

Transient transfection of EBC-1 cells and luciferase assay. EBC-1 cells prepared as described above were washed three times in serum-free DMEM–F-12 (Gibco). The cells were then seeded into 24-well plates (Costar) at a density of 2×10^5 cells/well in serum-free DMEM–F-12 and incubated for 24 h. One hour before transfection, the growth medium was replaced by fresh serum-free DMEM–F-12. Transient transfections were performed with 95 ng of appropriate plasmids: pAP-1-Luc, pC/EBP-Luc, and pNF-κB-Luc as reporter plasmids and 5 ng of phRG-TK (Promega, Madison, WI) as an internal control plasmid using the FuGENE6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. After 32 h, transfected cells were stimulated with or without serralysin. To antagonize PAR-2, human PAR-2 antagonist peptides were added directly to the culture medium at a final concentration of 200 μM at 1 h prior to stimulation. After a further 16 h of incubation, cells were lysed and assayed for luciferase activity using a dual-luciferase reporter assay system (Promega). Both firefly and *Renilla* luciferase activities were monitored with a Lumat LB9507 luminometer (Berthold, Wildbad, Germany). Normalized reporter activity was expressed as the firefly luciferase value divided by the *Renilla* luciferase value. The relative fold induction was calculated as the normalized reporter activity of the stimulated samples divided by the unstimulated samples.

Construction of human PAR-2 expression plasmid. EBC-1 cells prepared as described above were washed twice in PBS. Preparation of total RNA and synthesis of cDNA was performed as described above. PCR was performed with a KOD Plus DNA polymerase (Toyobo, Osaka, Japan) according to the protocol recommended by the manufacturer. The following human PAR-2-specific primers were used: sense, 5′-GGAAACAAGCTTCCACCATGCGGAGCCCCAGC GCGGCGTG-3′ (underlining indicates the HindIII restriction site); and antisense, 5′-GGAAACGGATCCTCAATAGGAGGTCTTAACAGTGG-3′ (underlining indicates the BamHI restriction site). The PCR profile included denaturation at 96°C for 3 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 57°C for 30 s, and extension at 68°C for 90 s, with a final extension at 68°C for 5 min. The PCR products were digested with HindIII and BamHI and cloned into a pcDNA3.1 (Invitrogen). The plasmids were purified with the QIAGEN plasmid kit (QIAGEN) and used for transient transfection.

Transient transfection of HeLa cells and luciferase assay. HeLa cells, which were maintained as described above, were detached from culture flask with nonenzymatic cell dissociation solution and washed three times in serum-free DMEM–F-12 (Gibco). The cells were then seeded into 24-well plates (Costar) at a density of 10^5 cells/well in serum-free DMEM–F-12 and incubated for 24 h. One hour before transfection, the growth medium was replaced by fresh serum-free DMEM–F-12. Transient transfections were performed with 40 to 250 ng of human PAR-2 expression plasmid, 95 ng of the appropriate plasmids (pAP-1-Luc, pC/EBP-Luc, and pNF-κB-Luc as reporter plasmids), and 5 ng of phRG-TK (Promega) as an internal control plasmid using the FuGENE6 transfection reagent (Roche). After 48 h, transfected cells were stimulated with or without 1 nM serralysin. After a further 6 h of incubation, cells were lysed and assayed for luciferase activity as described above.

EMSA. Nuclear extracts were prepared from EBC-1 cells as described previously (30). Synthetic oligonucleotides were used as probe for electrophoretic mobility shift assay (EMSA). The oligonucleotides were designed to generate a single 5′-G overhang to each end after annealing with their complements. The following oligonucleotides were used: AP-1, 5′-GGATAGCCTGAGTCAGGG GATA-3′; C/EBP, 5′-GGATAGGCTTGCGCAATGGATA-3′; and NF-κB, 5′-GGATCCGGGGACTTTCCGCGGAT-3′. The consensus sequence for binding of these transcription factors are underlined. The double-stranded oligonucleotides were end labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I (Amersham-Pharmacia Biotech). Labeled DNA probe was purified by using a QuickSpin column G-25 (Roche). Purified DNA probe was adjusted to 10,000 cpm/μl and stored at 4°C until use. For binding reactions, 3 μg of nuclear extract was incubated in a total volume of 23 μl of binding buffer [10 mM HEPES-KOH (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 5 mM dithiothreitol, 3 μg of poly(dI-dC), protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan), and 1 mM Na_3VO_4] for 10 min at room temperature. Then, α-^{32}P-end-labeled probe (20,000 cpm) was added to the reaction mixture for an additional 30 min at room temperature. For competition assays, excess unlabeled oligonucleotides were preincubated with nuclear extract in the binding buffer described above at room temperature for 10 min prior to the addition of radiolabeled probe (20,000 cpm). For antibody-mediated supershift assays, nuclear extracts were preincubated with 1 μg of appropriate antibodies or normal rabbit immunoglobulin G (IgG; Inter-Cell Technologies, Hopewell, NJ) at 4°C for 60 min before the addition of the radiolabeled probe. The reactions were loaded onto a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA (45 mM Tris [pH 8.3], 45 mM borate, and 1 mM EDTA) and electrophoresed for 2 h at 200 V before being dried. The intensity of the DNA-protein complex bands was measured by using a Phosphor-Imaging system (Fujifilm BAS-2000; Fujifilm, Tokyo, Japan).

Statistical evaluation. Data were analyzed by using the Student paired *t* test. *P* values of <0.05 were considered significant.

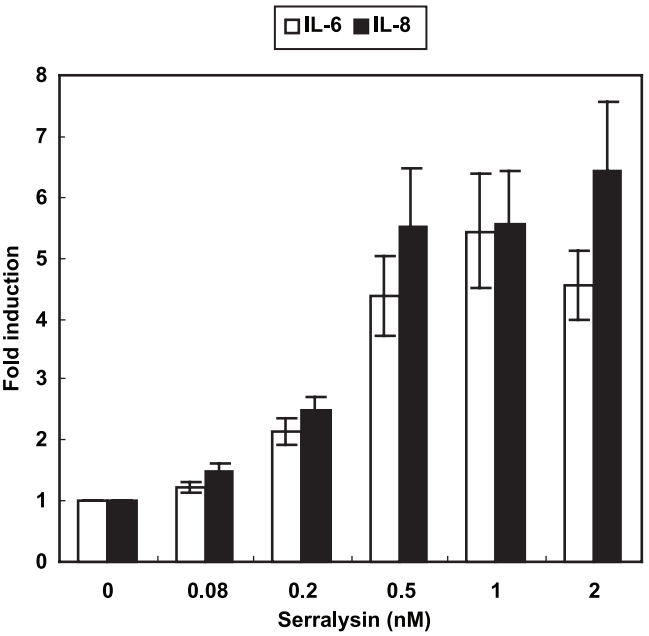


FIG. 2. Serralysin induces IL-6 and IL-8 mRNA expression in EBC-1 cells. EBC-1 cells were stimulated with a range of concentrations of serralysin for 2 h. Total RNA was then extracted, and quantitative real-time reverse transcription-PCR analysis was performed to determine the amounts of IL-6, IL-8, and β-actin mRNA. IL-6 and IL-8 mRNA levels, normalized to those β-actin, are expressed as the fold induction over unstimulated EBC-1 cells. Values represent the mean ± the standard error of the mean (SEM) from three independent experiments.

RESULTS

Serralysin induces IL-6 and IL-8 mRNA expression in EBC-1 cells. Proteases from house dust mites and fungi have been shown to induce IL-6 and IL-8 expression in respiratory cells (28, 34, 57, 60). To determine whether serralysin stimulation induces the augmentation of IL-6 and IL-8 mRNA expression in EBC-1 cells, EBC-1 cells were stimu-

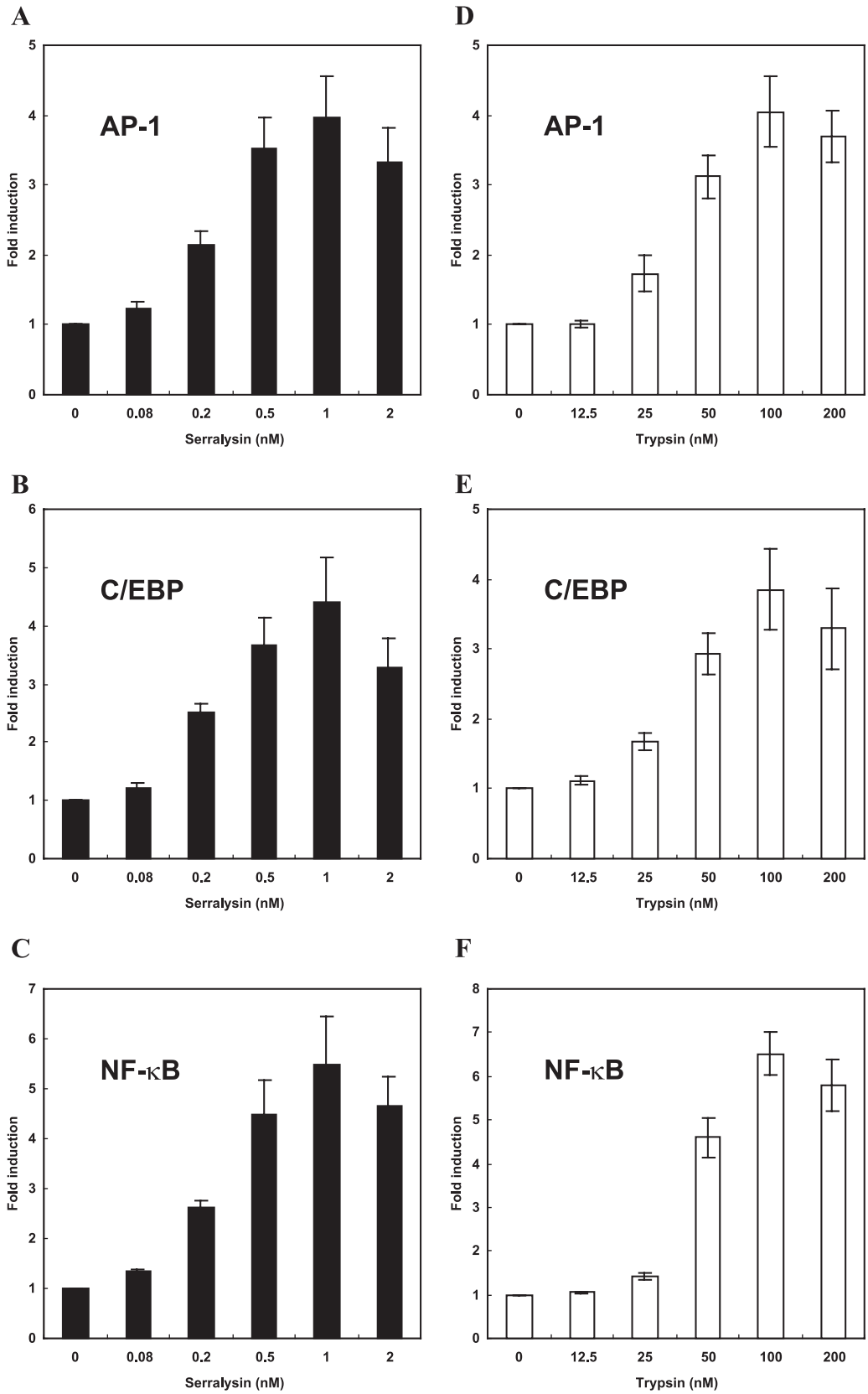


FIG. 3. Serralysin as well as trypsin activates AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells. EBC-1 cells were transfected with 95 ng of appropriate plasmids pAP-1-Luc (A and D), pC/EBP-Luc (B and E), and pNF-κB-Luc (C and F) as reporter plasmids and 5 ng of phRG-TK as an internal control plasmid. After 32 h, transfected cells were stimulated at the indicated concentrations of serralysin or trypsin. After a further 16 h of incubation, the cells were lysed and assayed for luciferase activity. The data are presented as the relative luciferase activity. The results for each set of transfections were normalized for *Renilla* luciferase activity and to the unstimulated samples. Values represent the mean ± the SEM from three independent experiments.

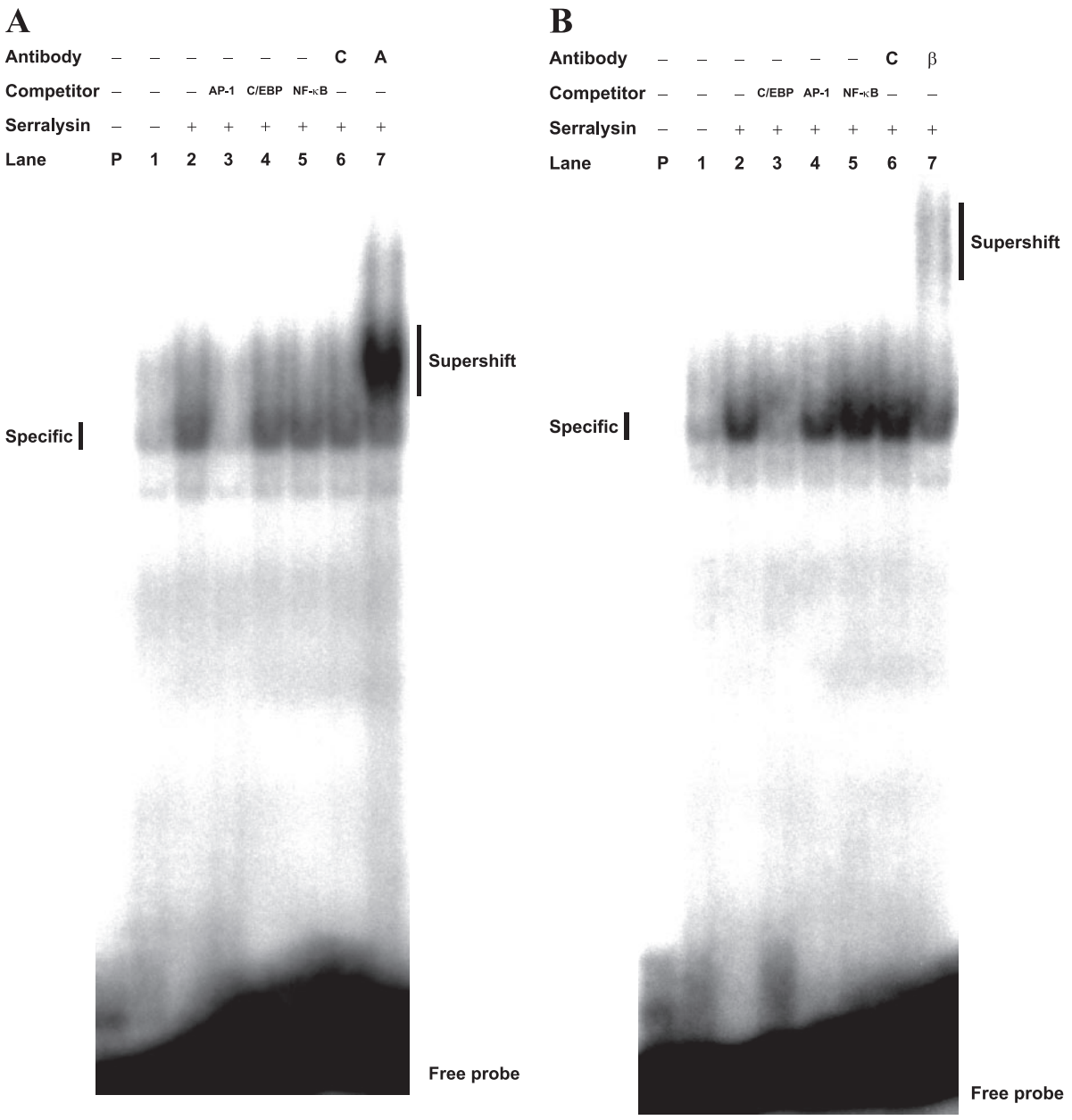


FIG. 4. EMSA of AP-1, C/EBPβ, and NF-κB in nuclear extract prepared from EBC-1 cells stimulated with serralysin. The following oligonucleotides were used: AP-1, 5′-GGATAGCCTGAGTCAGGGGATA-3′ (A); C/EBP, 5′-GGATAGGCTTGCGCAATGGATA-3′ (B); and NF-κB, 5′-GGATCCGGGGACTTTCCGCGGAT-3′ (C). The consensus sequences for the binding of these transcription factors are underlined. A ^{32}P-labeled, double-stranded oligonucleotide probe was incubated with nuclear extract from EBC-1 cells stimulated with or without 1 nM serralysin for 2 h as described in Materials and Methods. The specificity of the binding is shown by competition with unlabeled double-stranded oligonucleotides. The competitors were used in a 100-fold molar excess over labeled probes. Supershift assays were performed with 1 μg of the appropriate antibodies: rabbit anti-c-Jun/AP-1 antibody (lane A), rabbit anti-C/EBPβ antibody (lane β), rabbit anti-NF-κB p50 antibody (lane p50), and rabbit anti-NF-κB p65 antibody (lane p65), or normal rabbit IgG as a control (lanes C). Lane P indicates the probe-only control. The data are presented from a representative experiment, and similar results were obtained in three independent experiments.

lated with different concentrations of serralysin. After 2 h of incubation, total RNA from EBC-1 cells were prepared, and real-time PCR analysis was performed. As shown in Fig. 2, the levels of IL-6 and IL-8 mRNA expression were enhanced in response to stimulation with serralysin in a dose-dependent manner, with a plateau at the concentrations of 0.5 to 2 nM. Thus, these results indicate that IL-6 and IL-8 mRNA expression are induced in EBC-1 cells in response to stimulation with serralysin.

Serralysin activates AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells. Several studies provide evidence for the roles of AP-1, C/EBP, and NF-κB as the regulators in the inducible expression of IL-6 and IL-8 mRNA (1, 9, 48). We then determined whether AP-1-, C/EBP-, and NF-κB-driven

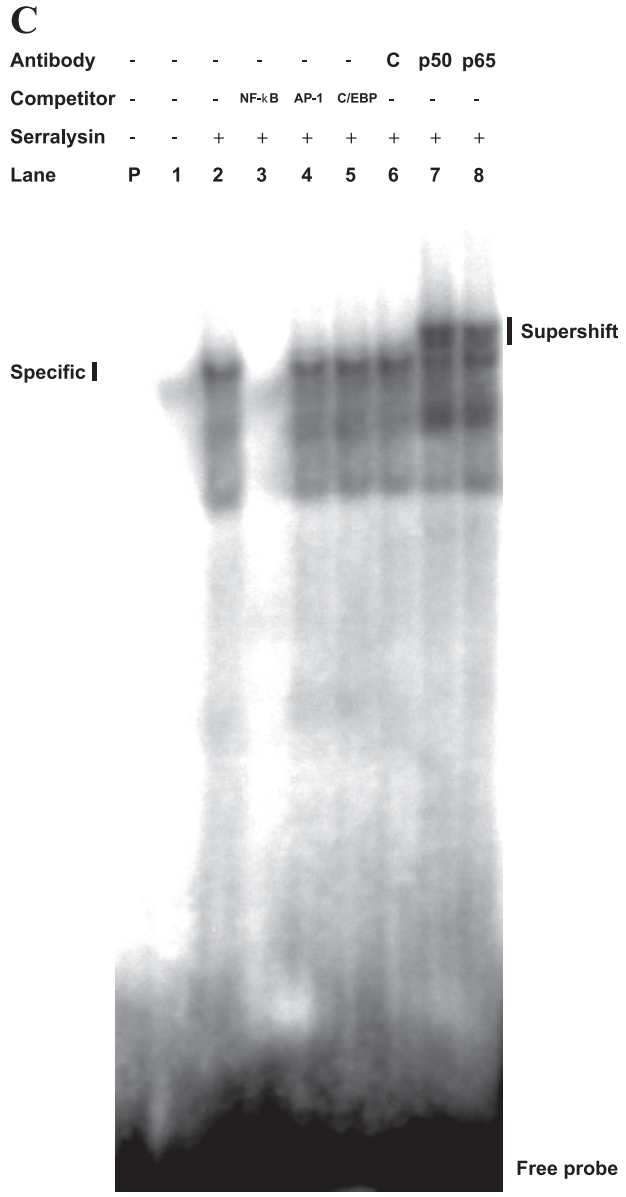


FIG. 4—*Continued.*

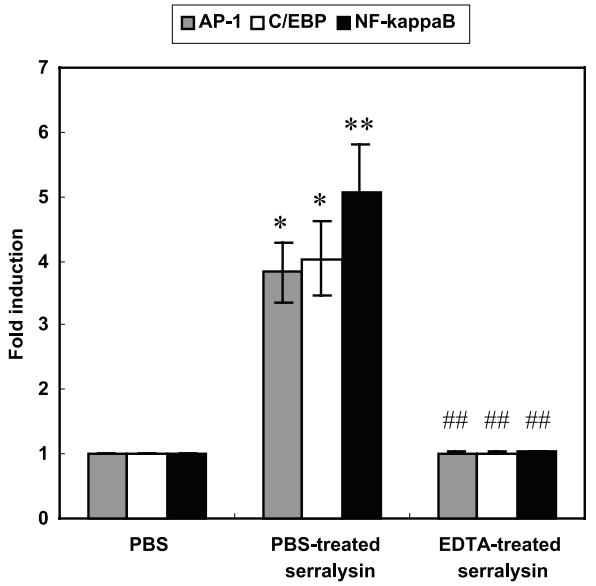
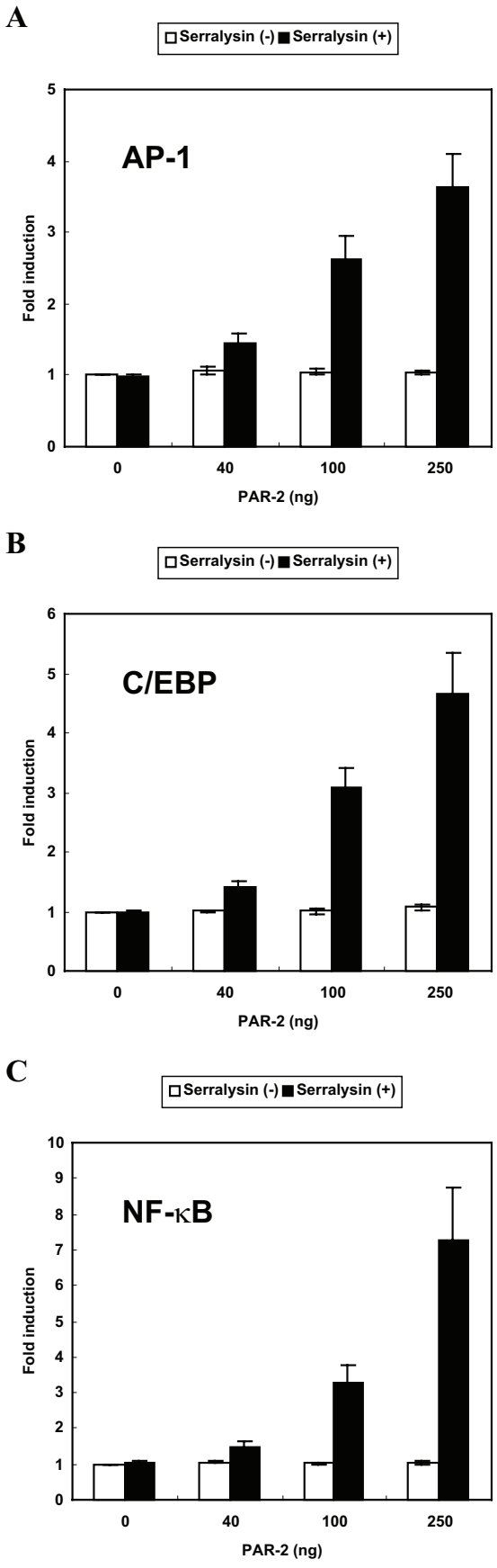


FIG. 5. Inactivation of serralysin leads to the abrogation of inducible AP-1-, C/EBP-, and NF-κB-driven promoters activity in response to simulation with serralysin in EBC-1 cells. EBC-1 cells were transfected with 95 ng of appropriate plasmids: pAP-1-Luc, pC/EBP-Luc, and pNF-κB-Luc as reporter plasmids and 5 ng of phRG-TK as an internal control plasmid. After 32 h, transfected cells were stimulated with EDTA- or PBS-treated serralysin at a final concentration of 1 nM. After a further 16 h of incubation, the cells were lysed and assayed for luciferase activity. The data are presented as in the legend to Fig. 2. *, $P < 0.05$; **, $P < 0.01$ (compared to unstimulated cells). ##, $P < 0.01$ (compared to PBS-treated serralysin-stimulated cells).

promoters are activated in the EBC-1 cells stimulated with serralysin. EBC-1 cells were transfected with the luciferase reporter plasmids and then were left untreated or were stimulated with different concentrations of serralysin. As shown in Fig. 3A to C, transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters was observed in response to stimulation with serralysin in a dose-dependent manner with a plateau at 0.5 to 2 nM, a finding consistent with the result shown in Fig. 2. Also, trypsin activated AP-1-, C/EBP-, and NF-κB-driven promoters in a dose-dependent manner with a plateau at 50 to 200 nM (Fig. 3D to F). Thus, these results indicate that serralysin, as well as trypsin, activates AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells.

Serralysin induces the binding of AP-1, C/EBPβ, and NF-κB to each consensus sequence in EBC-1 cells. To further examine the activation of AP-1, C/EBP, and NF-κB in EBC-1 cells stimulated with serralysin, EMSAs were performed. Double-strand oligonucleotides containing the AP-1, C/EBP, and NF-κB binding site of the luciferase reporter gene were used as probes to detect a specific protein binding in the nuclear extract from EBC-1 cells stimulated with serralysin. As shown in Fig. 4A, nuclear extract prepared from the EBC-1 cells generated a specific binding to the probe encompassing an AP-1 binding site (lane 2), whereas unstimulated nuclear extract from the cells failed to do so (lane 1). In a competition assay, the presence of excess unlabeled AP-1 probe efficiently outcompeted the retarded band (lane 3), whereas the nonspecific DNA probes containing a C/EBP or NF-κB binding site failed to do so (lanes 4 and 5). To identify the AP-1 protein, the nuclear extract from stimulated EBC-1 cells was preincubated with c-Jun/AP-1-specific antibody before the addition of a radiolabeled probe. The specific retarded band was shifted in the presence of anti-c-Jun/AP-1 antibody (lane 7). However, in the presence of normal rabbit IgG, the specific retarded band was not shifted (lane 6). These results show that AP-1 binds to the AP-1 binding site in EBC-1 cells stimulated with serralysin.

Figure 4B shows that serralysin induces the binding of C/EBP to a probe containing a C/EBP binding site (lane 2). The binding competed with excess amounts of unlabeled C/EBP probe (lane 3) but not with unrelated probes containing an AP-1 or an NF-κB binding site (lanes 4 and 5). C/EBPβ is a member of the C/EBP family of transcription factors, including C/EBPα, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ (50).



Although the C/EBP molecules other than C/EBPβ can also bind to the C/EBP binding site, C/EBPβ is especially involved in the inducible expression of several genes that are important for inflammatory and immune responses (31, 32, 49, 50). Therefore, to identify the C/EBPβ protein, the nuclear extract from the stimulated EBC-1 cells was preincubated with C/EBPβ-specific antibody before the addition of a radiolabeled probe. The specific retarded band was shifted in the presence of C/EBPβ antibody (lane 7). However, in the presence of normal rabbit IgG, the specific retarded band was not shifted (lane 6). These results indicate that C/EBPβ binds to the C/EBP binding site in EBC-1 cells stimulated with serralysin.

Figure 4C shows that serralysin induces the binding of NF-κB to a probe containing an NF-κB binding site (lane 2). The binding competed with excess amounts of unlabeled NF-κB probe (lane 3) but not with unrelated probes containing an AP-1 or a C/EBP binding site (lane 4 to 5). Although NF-κB exists as homo- or heterodimers with distinct DNA-binding specificities, a heterodimer composed of p50 and p65 subunits is the most common dimer (6, 56, 59). To identify the NF-κB protein, the nuclear extract from stimulated EBC-1 cells was preincubated with NF-κB p50- or p65-specific antibody before the addition of a radiolabeled probe. Both antibodies could shift the specific retarded bands (lane 7 to 8). However, in the presence of normal rabbit IgG, the specific band was not shifted (lane 6). These results show that NF-κB binds to the NF-κB binding site in EBC-1 cells stimulated with serralysin.

Proteolytic activity of serralysin participates in serralysin-induced transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells. We next determined whether inactivation of serralysin leads to the failure of activation of AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells. To this aim, serralysin was treated with EDTA, a metalloprotease inhibitor, as previously described by Maeda et al. (39). Figure 5 shows that EDTA-treated serralysin abrogated the activation of AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells. In contrast, PBS-treated serralysin activated the AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells. Therefore, these results indicate that the proteolytic activity of serralysin is required for inducible AP-1-, C/EBP-, and NF-κB-driven promoters activity.

Serralysin activates AP-1-, C/EBP-, and NF-κB-driven promoters via human PAR-2 in HeLa cells. To examine the mechanism of serralysin-induced transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters, we tested whether PAR-2 participates in the activation. To address this question, HeLa cells

FIG. 6. Serralysin activates AP-1-, C/EBP-, and NF-κB-driven promoters in HeLa cells transfected with human PAR-2. HeLa cells were transfected with 95 ng of appropriate plasmids: pAP-1-Luc (A), pC/EBP-Luc (B), and pNF-κB-Luc (C) as reporter plasmids and 5 ng of phRG-TK as an internal control plasmid, in addition to human PAR-2 expression plasmid. The pcDNA3.1 was used to ensure that all transfection mixtures had a total of 350 ng of DNA. After 48 h, transfected cells were stimulated with or without 1 nM serralysin. After a further 6 h of incubation, the cells were lysed and assayed for luciferase activity. The data are presented as in the legend to Fig. 2.

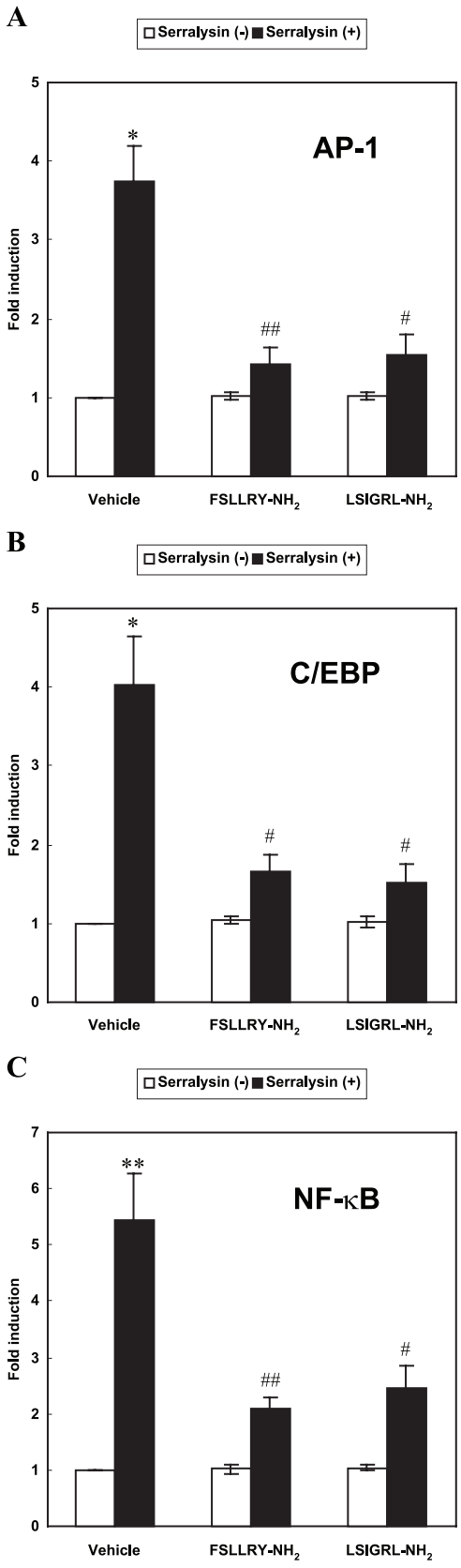


FIG. 7. Human PAR-2 antagonist peptides reduce serralysin-induced transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters. EBC-1 cells were transfected with 95 ng of the appropriate plasmids: pAP-1-Luc (A), pC/EBP-Luc (B), and pNF-κB-Luc (C) as reporter plasmids and 5 ng of phRG-TK as an internal control plasmid. After 32 h, transfected cells were incubated with the indicated antagonists at a final concentration of 200 μM for 1 h prior to stimulation with or without 1 nM serralysin. After a further 16 h of incubation, the cells were lysed and assayed for luciferase acitivity. The data are presented as in the legend to Fig. 2. *, $P < 0.05$; **, $P < 0.01$ (compared to unstimulated cells). #, $P < 0.05$; ##, $P < 0.01$ (compared to stimulated cells in the absence of the antagonists).

without functional PAR-2 were used for transfection experiments (52). As shown in Fig. 6, cotransfection of HeLa cells with increasing amounts of a human PAR-2 expression plasmid enhanced serralysin-induced AP-1-, C/EBP-, and NF-κB-driven promoters activity in a dose-dependent manner. In contrast, serralysin failed to activate AP-1-, C/EBP-, and NF-κB-driven promoters in HeLa cells cotransfected with a mock plasmid. Like serralysin, trypsin and the human PAR-2 agonist peptide SLIGKV-NH_2, known as the activators of PAR-2, were able to activate AP-1-, C/EBP-, and NF-κB-driven promoters in PAR-2-transfected HeLa cells (data not shown). Therefore, these results suggest that serralysin can activate AP-1-, C/EBP-, and NF-κB-driven promoters via PAR-2 in human cells.

Human PAR-2 antagonist peptides reduce serralysin-induced transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters. In the next experiment, we examined the inhibitory effect of PAR-2 antagonist peptides on serralysin-induced transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells. To antagonize PAR-2, human PAR-2 antagonist peptides were added to the culture medium at 1 h prior to stimulation with or without serralysin. As shown in Fig. 7, pretreatment with PAR-2 antagonist peptides, FSLLRY-NH_2 and LSIGRL-NH_2, significantly reduced the inducible AP-1-, C/EBP-, and NF-κB-driven promoters activity in response to stimulation with serralysin. The peptides have been shown not to inhibit the proteolytic activity of trypsin but to block trypsin-induced the activation of PAR-2 by a mechanism that they possibly interact with a tethered ligand receptor-docking site (2). We observed that neither FSLLRY-NH_2 nor LSIGRL-NH_2 inhibits the proteolytic action of serralysin in the protease activity assay (data not shown). Thus, these results indicate that PAR-2 plays an important role for serralysin-induced transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters.

DISCUSSION

In the present study, we elucidated whether *S. marcescens*-derived protease serralysin activates inflammatory responses via PAR-2. Our results indicated that serralysin induces IL-6 and IL-8 mRNA expression in a respiratory cell line, EBC-1. Moreover, we demonstrated that serralysin activates the critical transcription factors AP-1, C/EBPβ, and NF-κB for host inflammatory and immune responses via PAR-2.

Proteases from house dust mites and fungi have been shown to induce IL-6 and IL-8 expression in respiratory cells (28, 34, 57, 60). Several studies provide evidence for the roles of AP-1, C/EBPβ, and NF-κB as the regulators in the inducible expres-

sion of IL-6 and IL-8 mRNA in response to stimulation with proteases (1, 9, 48). Thus, these findings support our data that serralysin and proteases from house dust mite and fungi can activate host inflammatory responses via the transcription factors AP-1, C/EBPβ, and NF-κB.

Inactivation of serralysin by EDTA treatment failed to induce transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters (Fig. 5). The results indicate that proteolytic activity of serralysin is necessary for the transactivation. *S. marcescens* produces three, or perhaps four, discrete proteases, of which serralysin is the most abundant (40). The molecular mass of serralysin is 50,600 Da (470 amino acid residues), and its amino acid sequence contains the consensus sequence HEXXHXUGUXH (in which X represents an arbitrary amino acid and U is a bulky hydrophobic residue) that is responsible for zinc binding (39). Serralysin can efficiently cleave peptide bonds after arginine in synthetic substrates such as *tert*-butyloxycarbonyl-FSR-4-methylcoumaryl-7-amide and benzoyl-R-4-methylcoumaryl-7-amide, which are the optimal substrates for trypsin (41). As mentioned above, PAR-2 is activated by trypsin and also several other trypsin-like serine proteases (47, 51). Therefore, it is conceivable that serralysin would cleave and subsequently activate PAR-2, leading to activation of AP-1, C/EBPβ, and NF-κB. In contrast, *Pseudomonas aeruginosa*-derived elastolytic metalloprotease has been shown to disarm PAR-2 in respiratory epithelial cells (16). Intriguingly, a neutrophil elastase, a member of neutrophil-derived serine proteases, has been shown not to disarm but to activate PAR-2 expressed by nonepithelial cells (61). A possible explanation for the opposite functions is that the susceptibility of PAR-2 to cleavage and/or activation by protease is dependent on the glycosylation pattern of its amino-terminal exodomain (13, 14). The pattern of glycosylation of PAR-2 is different by cell type. However, the involvement of serralysin in disarming of PAR-2 remains to be elucidated.

Serralysin activated AP-1-, C/EBP-, and NF-κB-driven promoters via PAR-2 in HeLa cells (Fig. 6). In addition, PAR-2 antagonist peptides decreased serralysin-induced transactivation of AP-1-, C/EBP-, and NF-κB-driven promoters in EBC-1 cells (Fig. 7). The results suggest that serralysin requires PAR-2 to activate host inflammatory responses. Activation of PAR-2 is achieved when the extracellular amino terminus of the receptor is cleaved to expose a tethered ligand sequence that binds to the extracellular body of the receptor, leading to the G-protein-coupled signal transduction (e.g., activation of phospholipase C, generation of inositol 1,4,5-triphosphate, and diacylglycerol, increased intracellular Ca^{2+}, and activation of protein kinase C [PKC]) (36, 47). Activation of phospholipase C leads to generation of 1,4,5-triphosphate, which increases intracellular Ca^{2+} and diacylglycerol, which in turn activates PKC. The increment of intracellular Ca^{2+} leads to activation of PKC and calmodulin-dependent protein kinases. The PKC activation leads to activation of mitogen-activated kinase cascades (extracellular signal-regulated kinase 1/2 pathway, c-Jun N-terminal kinase pathway, and p38 kinase pathway), which activates AP-1 and C/EBPβ. Alternatively, the PKC has been shown to lead to the activation of NF-κB (27). In our experimental systems, however, a specific pathway or a combination of pathways that serralysin would take in inducing the transcription factors related to inflammatory and immune responses via PAR-2 is at present unclear.

In the present study, we propose a new function of serralysin, which activates host inflammatory responses via PAR-2. Although *S. marcescens* produces various proteases, serralysin is particularly produced in the largest amounts (40) and is considered to play a critical role as a virulence factor of *S. marcescens* (37, 38, 41). Microbial proteases have been also shown to degrade and inactivate various antimicrobial proteins and peptides (7, 54). Indeed, we previously reported that antimicrobial activity against *S. marcescens* of an antimicrobial peptide composed of L-amino acids is decreased compared to that of the peptide with D-amino acid substitutions resistant to proteolysis (19). The finding suggests that *S. marcescens* proteases such as serralysin would participate in lowering the antimicrobial activity of antimicrobial peptides. Moreover, *S. marcescens* infection in a compromised host is reported to occasionally cause endotoxin shock (3, 21). Nevertheless, inflammation seems to often remain even though the procedure of adsorption of endotoxin (lipopolysaccharide) is performed (43). The findings indicate that PAR-2-mediated inflammatory response might be involved in the pathogenesis of such shock. Thus, it is possible that pathogenic microorganism proteases may function not only as an escape mechanism from the host defense mechanisms against pathogens but also as a modulator of host inflammatory responses.

In summary, we investigated whether serralysin activates host inflammatory responses via PAR-2. Our results indicated that serralysin induces IL-6 and IL-8 mRNA expression in a respiratory cell line. Furthermore, we demonstrated that serralysin requires PAR-2 to activate the critical transcription factors AP-1, C/EBPβ, and NF-κB for host inflammatory and immune responses. Thus, these results suggest that bacterial proteases such as serralysin would require PARs to modulate various host responses against bacterial infection.

ACKNOWLEDGMENT

This study was partly supported by grant from the Ishibashi Foundation for the Promotion of Science.

REFERENCES

1. **Adam, E., K. K. Hansen, O. F. Astudillo, L. Coulon, F. Bex, X. Duhant, E. Jaumotte, M. D. Hollenberg, and A. Jacquet.** 2006. The house dust mite allergen Der p 1, unlike Der p 3, stimulates the expression of interleukin-8 in human airway epithelial cells via a proteinase-activated receptor-2-independent mechanism. J. Biol. Chem. **281:**6910–6923.
2. **Al-Ani, B., M. Saifeddine, S. J. Wijesuriya, and M. D. Hollenberg.** 2002. Modified proteinase-activated receptor-1 and -2 derived peptides inhibit proteinase-activated receptor-2 activation by trypsin. J. Pharmacol. Exp. Ther. **300:**702–708.
3. **Albers, M. J., J. W. Mouton, and D. Tibboel.** 2001. Colonization and infection by *Serratia* species in a paediatric surgical intensive care unit. J. Hosp. Infect. **48:**7–12.
4. **Asokananthan, N., P. T. Graham, D. J. Stewart, A. J. Bakker, K. A. Eidne, P. J. Thompson, and G. A. Stewart.** 2002. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. J. Immunol. **169:**4572–4578.
5. **Aucken, H. M., and T. L. Pitt.** 1998. Antibiotic resistance and putative virulence factors of *Serratia marcescens* with respect to O and K serotypes. J. Med. Microbiol. **47:**1105–1113.
6. **Baeuerle, P. A., and D. Baltimore.** 1996. NF-κB: ten years after. Cell **87:**13–20.
7. **Belas, R., J. Manos, and R. Suvanasuthi.** 2004. *Proteus mirabilis* ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides. Infect. Immun. **72:**5159–5167.
8. **Bohm, S. K., W. Kong, D. Bromme, S. P. Smeekens, D. C. Anderson, A.**

Connolly, M. Kahn, N. A. Nelken, S. R. Coughlin, D. G. Payan, and N. W. Bunnett. 1996. Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. Biochem. J. **314**(Pt. 3)**:**1009–1016.

9. **Borger, P., G. H. Koeter, J. A. Timmerman, E. Vellenga, J. F. Tomee, and H. F. Kauffman.** 1999. Proteases from *Aspergillus fumigatus* induce interleukin (IL)-6 and IL-8 production in airway epithelial cell lines by transcriptional mechanisms. J. Infect. Dis. **180:**1267–1274.
10. **Bosi, C., A. Davin-Regli, R. Charrel, B. Rocca, D. Monnet, and C. Bollet.** 1996. *Serratia marcescens* nosocomial outbreak due to contamination of hexetidine solution. J. Hosp. Infect. **33:**217–224.
11. **Cenac, N., A. M. Coelho, C. Nguyen, S. Compton, P. Andrade-Gordon, W. K. MacNaughton, J. L. Wallace, M. D. Hollenberg, N. W. Bunnett, R. Garcia-Villar, L. Bueno, and N. Vergnolle.** 2002. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. Am. J. Pathol. **161:**1903–1915.
12. **Chung, W. O., S. R. Hansen, D. Rao, and B. A. Dale.** 2004. Protease-activated receptor signaling increases epithelial antimicrobial peptide expression. J. Immunol. **173:**5165–5170.
13. **Compton, S. J., B. Renaux, S. J. Wijesuriya, and M. D. Hollenberg.** 2001. Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. Br. J. Pharmacol. **134:**705–718.
14. **Compton, S. J., S. Sandhu, S. J. Wijesuriya, and M. D. Hollenberg.** 2002. Glycosylation of human proteinase-activated receptor-2 (hPAR2): role in cell surface expression and signalling. Biochem. J. **368:**495–505.
15. **Dery, O., C. U. Corvera, M. Steinhoff, and N. W. Bunnett.** 1998. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. Am. J. Physiol. **274:**C1429–C1452.
16. **Dulon, S., D. Leduc, G. S. Cottrell, J. D'Alayer, K. K. Hansen, N. W. Bunnett, M. D. Hollenberg, D. Pidard, and M. Chignard.** 2005. *Pseudomonas aeruginosa* elastase disables proteinase-activated receptor 2 in respiratory epithelial cells. Am. J. Respir. Cell Mol. Biol. **32:**411–419.
17. **Ferrell, W. R., J. C. Lockhart, E. B. Kelso, L. Dunning, R. Plevin, S. E. Meek, A. J. Smith, G. D. Hunter, J. S. McLean, F. McGarry, R. Ramage, L. Jiang, T. Kanke, and J. Kawagoe.** 2003. Essential role for proteinase-activated receptor-2 in arthritis. J. Clin. Investig. **111:**35–41.
18. **Goullet, P., and B. Picard.** 1997. An epidemiological study of *Serratia marcescens* isolates from nosocomial infections by enzyme electrophoresis. J. Med. Microbiol. **46:**1019–1028.
19. **Hamamoto, K., Y. Kida, Y. Zhang, T. Shimizu, and K. Kuwano.** 2002. Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions. Microbiol. Immunol. **46:**741–749.
20. **Hansen, K. K., P. M. Sherman, L. Cellars, P. Andrade-Gordon, Z. Pan, A. Baruch, J. L. Wallace, M. D. Hollenberg, and N. Vergnolle.** 2005. A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. Proc. Natl. Acad. Sci. USA **102:**8363–8368.
21. **Hejazi, A., and F. R. Falkiner.** 1997. *Serratia marcescens*. J. Med. Microbiol. **46:**903–912.
22. **Hines, D. A., P. N. Saurugger, G. M. Ihler, and M. J. Benedik.** 1988. Genetic analysis of extracellular proteins of *Serratia marcescens*. J. Bacteriol. **170:** 4141–4146.
23. **Hollenberg, M. D., and S. J. Compton.** 2002. International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol. Rev. **54:**203–217.
24. **Hou, L., G. L. Howells, S. Kapas, and M. G. Macey.** 1998. The protease-activated receptors and their cellular expression and function in blood-related cells. Br. J. Haematol. **101:**1–9.
25. **Howells, G. L., M. G. Macey, C. Chinni, L. Hou, M. T. Fox, P. Harriott, and S. R. Stone.** 1997. Proteinase-activated receptor-2: expression by human neutrophils. J. Cell Sci. **110**(Pt. 7)**:**881–887.
26. **Kamata, R., K. Matsumoto, R. Okamura, T. Yamamoto, and H. Maeda.** 1985. The serratial 56K protease as a major pathogenic factor in serratial keratitis. Clinical and experimental study. Ophthalmology **92:**1452–1459.
27. **Kanke, T., S. R. Macfarlane, M. J. Seatter, E. Davenport, A. Paul, R. C. McKenzie, and R. Plevin.** 2001. Proteinase-activated receptor-2-mediated activation of stress-activated protein kinases and inhibitory κB kinases in NCTC 2544 keratinocytes. J. Biol. Chem. **276:**31657–31666.
28. **Kauffman, H. F., J. F. Tomee, M. A. van de Riet, A. J. Timmerman, and P. Borger.** 2000. Protease-dependent activation of epithelial cells by fungal allergens leads to morphologic changes and cytokine production. J. Allergy Clin. Immunol. **105:**1185–1193.
29. **Kawagoe, J., T. Takizawa, J. Matsumoto, M. Tamiya, S. E. Meek, A. J. Smith, G. D. Hunter, R. Plevin, N. Saito, T. Kanke, M. Fujii, and Y. Wada.** 2002. Effect of protease-activated receptor-2 deficiency on allergic dermatitis in the mouse ear. Jpn. J. Pharmacol. **88:**77–84.
30. **Kida, Y., K. Kuwano, Y. Zhang, and S. Arai.** 2001. *Acholeplasma laidlawii* up-regulates granulysin gene expression via transcription factor activator protein-1 in a human monocytic cell line, THP-1. Immunology **104:**324–332.
31. **Kida, Y., T. Shimizu, and K. Kuwano.** 2005. Conditional expression of liver-enriched transcriptional activator protein augments *Acholeplasma laidlawii*-induced granulysin gene expression in a human monocytic cell line, THP-1. Immunology **114:**121–132.
32. **Kida, Y., T. Shimizu, and K. Kuwano.** 2002. Opposing roles of activator protein-1 and CCAAT/enhancer binding protein beta in the regulation of inducible granulysin gene expression in a human monocytic cell line, THP-1. Immunology **107:**507–516.
33. **Kida, Y., T. Shimizu, and K. Kuwano.** 2006. Sodium butyrate up-regulates cathelicidin gene expression via activator protein-1 and histone acetylation at the promoter region in a human lung epithelial cell line, EBC-1. Mol. Immunol. **43:**1972–1981.
34. **King, C., S. Brennan, P. J. Thompson, and G. A. Stewart.** 1998. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. J. Immunol. **161:**3645–3651.
35. **Lindner, J. R., M. L. Kahn, S. R. Coughlin, G. R. Sambrano, E. Schauble, D. Bernstein, D. Foy, A. Hafezi-Moghadam, and K. Ley.** 2000. Delayed onset of inflammation in protease-activated receptor-2-deficient mice. J. Immunol. **165:**6504–6510.
36. **Macfarlane, S. R., M. J. Seatter, T. Kanke, G. D. Hunter, and R. Plevin.** 2001. Proteinase-activated receptors. Pharmacol. Rev. **53:**245–282.
37. **Maeda, H.** 1996. Role of microbial proteases in pathogenesis. Microbiol. Immunol. **40:**685–699.
38. **Maeda, H., and A. Molla.** 1989. Pathogenic potentials of bacterial proteases. Clin. Chim. Acta **185:**357–367.
39. **Maeda, H., and K. Morihara.** 1995. Serralysin and related bacterial proteinases. Methods Enzymol. **248:**395–413.
40. **Matsumoto, K., H. Maeda, K. Takata, R. Kamata, and R. Okamura.** 1984. Purification and characterization of four proteases from a clinical isolate of *Serratia marcescens* kums 3958. J. Bacteriol. **157:**225–232.
41. **Matsumoto, K., T. Yamamoto, R. Kamata, and H. Maeda.** 1984. Pathogenesis of serratial infection: activation of the Hageman factor-prekallikrein cascade by serratial protease. J. Biochem. **96:**739–749.
42. **Molla, A., K. Matsumoto, I. Oyamada, T. Katsuki, and H. Maeda.** 1986. Degradation of protease inhibitors, immunoglobulins, and other serum proteins by *Serratia* protease and its toxicity to fibroblast in culture. Infect. Immun. **53:**522–529.
43. **Naka, T., M. Shinozaki, T. Akizawa, Y. Shima, H. Takaesu, and H. Nasu.** 2006. The effect of continuous veno-venous hemofiltration or direct hemoperfusion with polymyxin B-immobilized fiber on neutrophil respiratory oxidative burst in patients with sepsis and septic shock. Ther. Apher. Dial. **10:**7–11.
44. **Nakahama, K., K. Yoshimura, R. Marumoto, M. Kikuchi, I. S. Lee, T. Hase, and H. Matsubara.** 1986. Cloning and sequencing of *Serratia* protease gene. Nucleic Acids Res. **14:**5843–5855.
45. **Nystedt, S., K. Emilsson, A. K. Larsson, B. Strombeck, and J. Sundelin.** 1995. Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. Eur. J. Biochem. **232:**84–89.
46. **Nystedt, S., V. Ramakrishnan, and J. Sundelin.** 1996. The proteinase-activated receptor 2 is induced by inflammatory mediators in human endothelial cells: comparison with the thrombin receptor. J. Biol. Chem. **271:**14910–14915.
47. **Ossovskaya, V. S., and N. W. Bunnett.** 2004. Protease-activated receptors: contribution to physiology and disease. Physiol. Rev. **84:**579–621.
48. **Page, K., V. S. Hughes, K. K. Odoms, K. E. Dunsmore, and M. B. Hershenson.** 2005. German cockroach proteases regulate interleukin-8 expression via nuclear factor for interleukin-6 in human bronchial epithelial cells. Am. J. Respir. Cell Mol. Biol. **32:**225–231.
49. **Poli, V.** 1998. The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J. Biol. Chem. **273:**29279–29282.
50. **Ramji, D. P., and P. Foka.** 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem. J. **365:**561–575.
51. **Reed, C. E., and H. Kita.** 2004. The role of protease activation of inflammation in allergic respiratory diseases. J. Allergy Clin. Immunol. **114:**997–1008.
52. **Riewald, M., V. V. Kravchenko, R. J. Petrovan, P. J. O'Brien, L. F. Brass, R. J. Ulevitch, and W. Ruf.** 2001. Gene induction by coagulation factor Xa is mediated by activation of protease-activated receptor 1. Blood **97:**3109–3116.
53. **Schmidlin, F., S. Amadesi, K. Dabbagh, D. E. Lewis, P. Knott, N. W. Bunnett, P. R. Gater, P. Geppetti, C. Bertrand, and M. E. Stevens.** 2002. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. J. Immunol. **169:**5315–5321.
54. **Schmidtchen, A., I. M. Frick, E. Andersson, H. Tapper, and L. Bjorck.** 2002. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. Mol. Microbiol. **46:**157–168.
55. **Seeliger, S., C. K. Derian, N. Vergnolle, N. W. Bunnett, R. Nawroth, M. Schmelz, P. Y. Von Der Weid, J. Buddenkotte, C. Sunderkotter, D. Metze, P. Andrade-Gordon, E. Harms, D. Vestweber, T. A. Luger, and M. Steinhoff.** 2003. Proinflammatory role of proteinase-activated receptor-2 in humans and mice during cutaneous inflammation in vivo. FASEB J. **17:**1871–1885.
56. **Siebenlist, U., G. Franzoso, and K. Brown.** 1994. Structure, regulation and function of NF-κB. Annu. Rev. Cell Biol. **10:**405–455.
57. **Stacey, M. A., G. Sun, G. Vassalli, M. Marini, A. Bellini, and S. Mattoli.** 1997. The allergen Der p1 induces NF-κB activation through interference with IκB α function in asthmatic bronchial epithelial cells. Biochem. Biophys. Res. Commun. **236:**522–526.

58. **Sun, G., M. A. Stacey, M. Schmidt, L. Mori, and S. Mattoli.** 2001. Interaction of mite allergens Der p3 and Der p9 with protease-activated receptor-2 expressed by lung epithelial cells. J. Immunol. **167:**1014–1021.
59. **Thanos, D., and T. Maniatis.** 1995. NF-κB: a lesson in family values. Cell **80:**529–532.
60. **Tomee, J. F., A. T. Wierenga, P. S. Hiemstra, and H. K. Kauffman.** 1997. Proteases from *Aspergillus fumigatus* induce release of proinflammatory cytokines and cell detachment in airway epithelial cell lines. J. Infect. Dis. **176:**300–303.
61. **Uehara, A., K. Muramoto, H. Takada, and S. Sugawara.** 2003. Neutrophil serine proteinases activate human nonepithelial cells to produce inflammatory cytokines through protease-activated receptor 2. J. Immunol. **170:**5690–5696.
62. **van Ogtrop, M. L., D. van Zoeren-Grobben, E. M. Verbakel-Salomons, and C. P. van Boven.** 1997. *Serratia marcescens* infections in neonatal departments: description of an outbreak and review of the literature. J. Hosp. Infect. **36:**95–103.
63. **Yu, V. L.** 1979. *Serratia marcescens*: historical perspective and clinical review. N. Engl. J. Med. **300:**887–893.

Editor: W. A. Petri, Jr.