Syndecan-1 Shedding Is Enhanced by LasA, a Secreted Virulence Factor of Pseudomonas aeruginosa*

Pyong Woo Park, Gerald B. Pier‡, Michael J. Preston‡, Olga Goldberger, Marilyn L. Fitzgerald, and Merton Bernfield§

From the Division of Newborn Medicine, Department of Medicine, Children's Hospital and §Channing Laboratory, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts 02115

Microbial pathogens frequently take advantage of host systems for their pathogenesis. Shedding of cell surface molecules as soluble extracellular domains (ectodomains) is one of the host responses activated during tissue injury. In this study, we examined whether pathogenic bacteria can modulate shedding of syndecan-1, the predominant syndecan of host epithelia. Our studies found that overnight culture supernatants of Pseudomonas aeruginosa and Staphylococcus aureus enhanced the shedding of syndecan-1 ectodomains, whereas culture supernatants of several other Gram-negative and Gram-positive bacteria had only low levels of activity. Because supernatants from all tested strains of P. aeruginosa (n = 9) enhanced syndecan-1 shedding by more than 4-fold above control levels, we focused our attention on this Gram-negative bacterium. Culture supernatants of P. aeruginosa increased shedding of syndecan-1 in both a concentration- and time-dependent manner, and augmented shedding by various host cells. A 20-kDa shedding enhancer was partially purified from the supernatant through ammonium sulfate precipitation and gel chromatography, and identified by N-terminal sequencing as LasA, a known P. aeruginosa virulence factor. LasA was subsequently determined to be a syndecan-1 shedding enhancer from the findings that (i) immunodepletion of LasA from the partially purified sample resulted in abrogation of its activity to enhance shedding and (ii) purified LasA increased shedding in a concentration-dependent manner. Our results also indicated that LasA enhances syndecan-1 shedding by activation of the host cell’s shedding mechanism and not by direct interaction with syndecan-1 ectodomains. Enhanced syndecan-1 shedding may be a means by which pathogenic bacteria take advantage of a host mechanism to promote their pathogenesis.

Shedding is a process in which cell surface proteins are cleaved by proteases known collectively as sheddases or secretases, and their ectodomains released from the surface as soluble effectors (1–3). It is an important biological mechanism of protein secretion and activation for approximately 1% of cell surface proteins. Numerous types of surface molecules are shed as soluble ectodomains and include cytokines, growth factors, and their receptors, and cell adhesion molecules such as selectins (4), CD14 (5), epidermal growth factor (6), TNF-α (7, 8) and its receptors (9, 10), IL-6 receptor (11), and transforming growth factor-α (12), to name a few. These shed ectodomains play pivotal roles in diverse pathophysiological events including septic shock, host defense, and wound healing. Furthermore, because shedding itself has been found to be controlled by various extracellular ligands (13–15) and intracellular signaling pathways (3, 12, 15, 16), it provides an additional level of regulation. Because protein kinase C agonists (phorbol ester) and peptide hydroxamates have been found to enhance and inhibit the shedding of most affected molecules, respectively, existence of a common shedding system has been proposed (3). However, shedding of some effectors is insensitive or only partially sensitive to hydroxamates (13, 17) and additional regulators of shedding have been identified (12, 16), suggesting that certain components may be unique to individual shedding systems.

The genetic variability that pathogenic microorganisms can generate have allowed variant pathogens to take advantage of the host environment for their growth and survival. For example, a diverse group of pathogens including Yersinia spp. (19), Bordetella pertussis (20, 21), and adenovirus (22) express RGD-containing cell surface ligands and use these “molecular mimics” to interact with host integral receptors for their colonization (23). Bacteria also produce molecules that can derange host homeostasis to their benefit. Several bacteria secrete toxins that can modify the host cell cytoskeleton (24) and secrete enzymes that can degrade extracellular matrix components, immunoglobulins and complement, either directly (25, 26) or indirectly by activating the matrix metalloproteinases in the host (27). Furthermore, lipopolysaccharide from Gram-negative bacteria, the causative agent of endotoxic shock, affects the expression of host defense effectors such as TNF-α and IL-1, -6, -8, and -10 (28).

Recent studies indicate that bacterial pathogens may also utilize the host cell’s shedding mechanism to enhance their virulence. For instance, the pore-forming toxins, streptolysin O and Escherichia coli hemolysin, trigger shedding of lipopolysaccharide (CD14) and IL-6 receptors (29). Culture supernatants from Pseudomonas aeruginosa, Staphylococcus aureus, Serratia marcescens, and Listeria monocytogenes can also augment shedding of the IL-6 receptor (30), and culture supernatants...

§ To whom correspondence and reprint requests should be addressed: Children’s Hospital, Harvard Medical School, 300 Longwood Ave., Enders-9, Boston, MA 02115. Tel.: 617-355-6366; Fax: 617-355-7677; E-mail: bernfield@al.tch.harvard.edu.

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1 The abbreviations used are: TNF-α, tumor necrosis factor α; IL-6, interleukin 6; PKC, protein kinase C; PTK, protein tyrosine kinase; PAGE, polyacrylamide gel electrophoresis; NMaMG, normal murine mammary gland; TPCR, 1,1-tosylamide-2-phenylethyl chloromethyl ketone; CAPS, 3-(cyclohexylaminopropanesulfonic acid.

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from *S. epidermidis* can activate shedding of TNF-α (31), although the responsible shedding enhancers were not defined in these studies. Furthermore, increased serum levels of soluble ectodomains of several surface effectors, such as CD14, TNF-α, and IL-4 receptors, have been documented during infection (32–34). These findings suggest that bacteria-enhanced shedding can modulate the activation and function of host effectors, and play a role in bacterial pathogenesis.

The syndecans are a family of cell surface heparan sulfate proteoglycans which, along with the glypicans, are the major source of cell surface heparan sulfate (35). There are currently four mammalian syndecans known, syndecan-1 through -4, each encoded by distinct genes. Syndecans can bind and modulate the activity of a diverse group of soluble and insoluble ligands, such as extracellular matrix components, growth factors, chemokines, cytokines, and proteases, through the action of their heparan sulfate chains. Syndecans have also been proposed to act as adhesion and internalization receptors for pathogenic microorganisms (36, 37).

The extracellular domains of syndecans can be shed as soluble, intact heparan sulfate proteoglycan ectodomains which, because they bind the same ligands as their precursor proteoglycans on the cell surface, can serve as soluble effectors. For example, shed syndecan-1 ectodomains have been found to regulate the proliferative response of cells to FGF-2 (38) and potentiate the activity of neutrophil enzymes, such as elastase and cathepsin G (39), by binding to the enzymes and protecting them from inhibition by their physiological inhibitors. All syndecans are shed constitutively as part of normal syndecan turnover, but available evidence also indicates that syndecan shedding is a regulated host response to tissue injury and that shed syndecan ectodomains are regulators of inflammation (15). Thus, regulation of syndecan shedding by pathogenic bacteria may play a role in pathogenesis through alteration of the host response to infection and/or the pathogen’s ability to colonize host tissue components.

We have studied whether pathogenic bacteria can modulate syndecan shedding and have found that culture supernatants from *S. aureus* and *P. aeruginosa*, but not from several other Gram-positive and Gram-negative bacteria, enhance shedding of syndecan-1 by host cells. Here we report the characterization of syndecan-1 shedding enhanced by *P. aeruginosa*. Syndecan-1 shedding augmented by overnight culture supernatants of *P. aeruginosa* is rapid, is seen with various types of host cells, and produces intact, soluble syndecan-1 ectodomains. A *P. aeruginosa* shedding enhancer has been purified from a clinical isolate and identified as the mature 20-kDa LasA protein, a known virulence factor of *P. aeruginosa* (40–42). LasA-enhanced shedding produces syndecan-1 ectodomain core proteins identical in size to ectodomains shed endogenously, and is inhibited by inhibitors of the host cell’s shedding mechanism. These results indicate that LasA enhances syndecan-1 shedding by activating the host cell’s shedding machinery. Enhancement of syndecan-1 shedding by LasA may be a mechanism by which *P. aeruginosa* parasitizes a host system to aid its pathogenesis.

**EXPERIMENTAL PROCEDURES**

*Materials—*Affi-Prep Hz Hydrazide affinity chromatography resins, Bio-Gel P-30 gel chromatography resins, Coomasie Brilliant Blue R-250, and pre-stained SDS-PAGE size standards were purchased from Bio-Rad. Bisindolylmaleimide I, genistein, and Tyrphostin A25 were from Calbiochem (La Jolla, CA). Heparan sulfate lyase (heparin lyase III, heparitinase) and chondroitin sulfate ABC lyase were obtained from Worthington (Freehold, NJ). 

**Syndecan-1 Shedding Assays—**Quantification of syndecan-1 shedding was performed as described previously (15). Briefly, confluent or 1-day post-confluent cultures of NMuMG, C127, NIH3T3, CT4 and NIH3T3 cells in 24-well plates were treated with their respective culture media, and various test samples diluted in culture media and supplements other than serum were from Mediatech (Herndon, VA); fetal calf and calf serum were from HyClone (Logan, UT) and tissue culture plastics were from Costar (Corning, NY). Enhanced chemiluminescence (ECL) Western blotting detection reagents DEAE ion exchange chromatography and further fractionation with a MWCO spin tubes were from Pall Filtron (Northborough, MA). TPCk-treated trypsin, soybean trypsin inhibitor, and all other materials were purchased from Sigma.

**Cells and Immunochromatographs—**Normal murine mammary gland (NMuMG), mouse lung adenoma (LA-4), and mouse mammary gland (CA6 and MMT-4) embryonic fibroblasts (NIH3T3) were from our culture collection, and cultured as described previously (43).

*P. aeruginosa* laboratory strains 7700 and 10145 were from the ATCC. The clinical *P. aeruginosa* isolates, BL1, BL2, CF1, CF2, and SP1, were from the Division of Infectious Diseases at Washington University School of Medicine (St. Louis, MO), CT4 was kindly provided by Dr. David Roberts at the NCI (Rockville, MD) (44) and PAO1 was from our culture collection (40). *S. aureus* laboratory strains 8095, 10832 (Woods), 12598 (Cowen), and 25904 (Newman) were from the ATCC. The clinical blood isolates of *S. aureus*, 070-0875, 093-0861, 108-0009, 111-0449, and 116-0031 were from the Division of Infectious Diseases at Washington University School of Medicine. The *Salmonella enteritidis* clinical isolate was kindly provided by Dr. Robert Lee of the Department of Vascular Surgery at Washington University School of Medicine. The laboratory strains of *Staphylococcus saprophyticus* (15305), *Staphylococcus xylosus* (29971), *S. enteritidis* (10376), *Salmonella typhimurium* (14028), *Streptococcus pneumoniae* (27336), and *Klebsiella pneumoniae* (27736) were from the ATCC. Frozen glycerol stocks of bacteria were grown overnight to stationary growth phase in tryptic soy broth at 37 °C with agitation.

The rat monoclonal anti-mouse syndecan-1 ectodomain antibody (281-2) was generated in our laboratory (45) and is now commercially available from Pharmingen (San Diego, CA). The purified LasA protein used for antisera production was prepared previously (40). Briefly, LasA was purified by ammonium sulfate precipitation (80%), DEAE ion exchange chromatography and further fractionation with a sulforoeprou unigril column. CS/Hen mice (Charles River Laboratories, Wilmington, MA) were immunized subcutaneously with 10 μg of purified LasA emulsified in complete Freund’s adjuvant. Mice were given a second injection of 5 μg of LasA emulsified in incomplete Freund’s adjuvant 2 weeks later. Two weeks after the second immunization, mice were bled via the dorsolateral tail vein and sera prepared. Non-immune sera were prepared by immunizing mice with complete Freund’s adjuvant 2 weeks later with incomplete Freund’s adjuvant as described above. Horseradish peroxidase-conjugated goat anti-rat secondary antibodies were obtained from either Jackson Immunoresearch Laboratories (West Grove, PA) or Cappel (Durham, NC).

**Syndecan-1 Shedding Assays—**Quantification of syndecan-1 shedding was performed as described previously (15). Briefly, confluent or 1-day post-confluent cultures of NMuMG and C127 cells in 96-well plates, 24-well plates, or 15 ml of NIH3T3 cells in 24-well plates were treated with their respective culture media, and various test samples diluted in culture media and supplements other than serum were from Mediatech (Herndon, VA), fetal calf and calf serum were from HyClone (Logan, UT) and tissue culture plastics were from Costar (Corning, NY). Enhanced chemiluminescence (ECL) Western blotting detection reagents DEAE ion exchange chromatography and further fractionation with a MWCO spin tubes were from Pall Filtron (Northborough, MA). TPCk-treated trypsin, soybean trypsin inhibitor, and all other materials were purchased from Sigma.

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Ammonium Sulfate Precipitation and Bio-Gel P-30 Gel Chromatography—Overnight culture supernatant (1 liter) of P. aeruginosa, strain BL2, was mixed overnight at 4 °C with ammonium sulfate at 80% saturation. The resulting precipitate was centrifuged at 15,000 rpm for 14–24 h, (iii) BL2OT for 30 min × 2 (wash), (iv) 1:8,000 dilution of horseradish peroxidase-conjugated goat anti-rat antibodies and (v) ECL development reagent. The developed blots were scanned and quantified using the public domain NIH Image (V. 1.60) software.

**RESULTS**

*Fig. 1. Culture supernatants of* P. aeruginosa *and S. aureus enhance shedding of syndecan-1 ectodomains by NMuMG cells.* Bacteria were grown overnight in tryptic soy broth at 37 °C to stationary growth phase and culture supernatants were collected. Fresh NMuMG culture media (media control) or filter-sterilized bacterial supernatants diluted to 20% (v/v) with NMuMG media were incubated with confluent NMuMG cells in 96-well plates for 14 h at 37 °C. At the end of incubation, conditioned media were collected, centrifuged to remove cells,acidified, and dot blotted onto cationic Immobilon N polyvinylidene difluoride membranes. Extent of syndecan-1 shedding was determined by the dot immunoblotting method using the anti-syndecan-1 ectodomain monoclonal antibody (281-2) as described under “Experimental Procedures.” Each data point represents the mean of duplicate or triplicate measurements, and results are presented as fold over media control. The number and horizontal bar in *P. aeruginosa* (PA) and *S. aureus* (StaphA) samples indicate mean values for these bacteria.

*P. aeruginosa* and *S. aureus* Secrete Soluble Enhancers of Syndecan-1 Shedding—Overnight culture supernatants from several Gram-negative and Gram-positive bacteria were screened for their ability to alter shedding of syndecan-1 ectodomains by NMuMG cells. NMuMG cells were chosen initially since they express syndecan-1 abundantly (43) and because the epithelium is the target cell type of many bacterial pathogens (47, 48). Overnight culture supernatants of bacteria were filter sterilized, diluted to 20% (v/v) with NMuMG culture media, and incubated with NMuMG cells for 14 h at 37 °C. As shown in Fig. 1, culture supernatants from all tested *P. aeruginosa* (7/7 clinical, 2/2 laboratory) and the majority of *S. aureus* (3/5 clinical, 3/4 laboratory) strains enhanced shedding of syndecan-1 by more than 4-fold over control levels, whereas strains from several other Gram-negative (*S. enteritidis, S. typhimurium, and K. pneumoniae*) and Gram-positive (*S. saprophyticus, S. xylosus, and S. pneumoniae*) bacteria did not. Cellular extracts of *P. aeruginosa* and *S. aureus* strains did not affect shedding (data not shown). These results indicate that *P. aeruginosa* and *S. aureus* secrete a soluble enhancer(s) of syndecan-1 ectodomain shedding, and suggest that this property may be specific for certain bacterial species.

Enhancement of Syndecan-1 Shedding by *P. aeruginosa* Is Rapid and Dose-dependent and Affects Various Host Cells—Because all tested strains of *P. aeruginosa* augmented syndecan-1 shedding, we focused our subsequent studies on this Gram-negative bacterium. The clinical blood isolate, BL2, showed the greatest activity (~14-fold enhancement, Fig. 1) and was therefore chosen for further studies. As shown in Fig. 2, stimulation of syndecan-1 shedding by BL2 culture supernatant was concentration-dependent (Fig. 2A), rapid (6-fold increase in 2 h, Fig. 2B), and extensive (11-fold increase by 20 h, Fig. 2B). In contrast to normal turnover (constitutive) shedding, during which constant levels of cell surface syndecan-1 are maintained, *P. aeruginosa*-enhanced shedding reduced the amount of cell surface syndecan-1 (90% reduction at 20% su-
pernatant, Fig. 2A). For at least 20 h of incubation, responding NMuMG epithelial cells remained morphologically normal by light microscopic examination and viable as measured by the tetrazolium salt conversion assay. However, viability and morphology of NMuMG cells were decreased and altered, respectively, when incubated for 35 h or longer with higher concentrations of the supernatant (>10%), suggesting that subtle morphological changes may not have been detected by light microscopy at earlier time points and at lower concentrations of the supernatant (data not shown).

To examine whether P. aeruginosa can enhance the shedding of syndecan-1 by other cell types, we tested the effects of BL2 culture supernatant (20%, v/v) on LA-4 lung and C127 mammary gland epithelia, and NIH3T3 fibroblasts. BL2 culture supernatants augmented syndecan-1 shedding by more than 5-fold during a 20-h incubation for all cell types tested. The extent of shedding stimulation was highest with NMuMG epithelia (13-fold), followed by C127 epithelia (10-fold), LA-4 epithelia (8-fold), and NIH3T3 fibroblasts (5-fold). These results demonstrate that although the epithelium, physiological target cell type of P. aeruginosa, respond most extensively to shedding enhancement by P. aeruginosa supernatant, other host cells such as fibroblasts also respond.

Identification of the Syndecan-1 Shedding Enhancer of P. aeruginosa—We next performed experiments to characterize the P. aeruginosa syndecan-1 shedding enhancer. We first examined whether the activity is susceptible to proteinase K treatment to determine whether the enhancer is a protein. BL2 supernatant was pretreated with 10 μg/ml proteinase K for 30 min at 37 °C, inactivated with 20 mm phenylmethylsulfonyl fluoride, and then tested for enhancement of syndecan-1 shedding. Proteinase K treatment abolished the activity of P. aeruginosa supernatant. We next fractionated the crude supernatant with molecular weight cutoff spin tubes to obtain a rough estimate of the enhancer's size. Using 3, 10, 30, and 100 kDa molecular mass cutoff tubes, we found that the size of the shedding enhancer is larger than 10 kDa but smaller than 30 kDa. These results suggest that the syndecan-1 shedding enhancer is a 10–30-kDa protein.

Based on these properties of the shedding enhancer, proteins in the BL2 supernatant were collected by 80% ammonium sulfate precipitation and fractionated by Bio-Gel P-30 (fractionation range = 2.5–40 kDa) gel chromatography in an effort to identify the enhancer. Fractions obtained from gel chromatography were assayed for their ability to enhance shedding of syndecan-1 ectodomains and analyzed by SDS-PAGE. As shown in Fig. 3, the shedding enhancing activity was isolated in one peak and two fractions, 12 and 13. Analysis of the active and inactive fractions by 12% SDS-PAGE and Coomassie staining revealed the presence of a single, major 20-kDa band in the active, but not in the inactive, fractions (Fig. 3, inset). To identify the putative 20-kDa shedding enhancer, N-terminal sequencing was performed. The first 10-amino acid sequence of the 20-kDa protein matched perfectly with mature LasA protein (Table I), a known virulence factor of P. aeruginosa (40–42).

The hypothesis that LasA is a syndecan-1 shedding enhancer of P. aeruginosa was tested by fractionating the partially purified active peak obtained from Bio-Gel P-30 gel filtration by immunoaffinity chromatography using mouse polyclonal anti-LasA IgGs covalently coupled to a cross-linked agarose resin. The rationale behind this experiment was that if LasA is the shedding enhancer, then the active component in the partially purified material will be bound to the affinity column, and shedding activity will be seen only with the specifically bound fractions and not with the flow-through or wash fractions. As
shown in Fig. 4, the specifically bound eluate (EL), but not the flow-through (FT) or wash (WSH) fractions, enhanced syndecan-1 shedding by NMuMG cells. The inactive flow-through fraction contained the contaminating smear seen in the active fractions partially purified by gel chromatography, and the eluate fraction contained the highly purified 20-kDa LasA protein (Fig. 4, inset). The purified LasA protein enhanced syndecan-1 shedding by various host cells (Fig. 5) and did not affect steady-state mRNA levels of syndecan-1 (data not shown). When culture supernatants from the P. aeruginosa strain lacking LasA (PAO-B1A1) (40) were subjected to the identical purification procedure, the resulting eluate fraction from anti-LasA immunoaffinity chromatography did not contain protein bands and did not enhance the shedding of syndecan-1 ectodomains from NMuMG cells (data not shown). Taken together, these results indicate that LasA is a syndecan-1 shedding enhancer of P. aeruginosa.

LasA Enhances Syndecan-1 Shedding by Stimulating the Shedding Mechanism of the Host Cell—To begin to elucidate the mechanism by which P. aeruginosa LasA enhances shedding of syndecan-1 ectodomains, we first examined the molecular size of shed syndecan-1 ectodomains and their core proteins. Conditioned media from NMuMG cells cultured to confluence (constitutively shed) and from NMuMG cells stimulated with purified LasA or crude P. aeruginosa supernatant were subjected to DEAE ion exchange chromatography to obtain partially purified samples of syndecan-1 ectodomain. These undigested samples were directly analyzed by Western immunoblotting (Fig. 6, lanes 1–3) or digested by heparitinase and chondroitin sulfate ABC lyase, and then analyzed by Western immunoblotting (Fig. 6, lanes 4–6) to determine the size of shed syndecan-1 ectodomain core proteins. Similar to the constitutively shed syndecan-1 ectodomain (lane 1), syndecan-1 ectodomains obtained from both purified LasA (lane 2) and crude supernatant (lane 3) conditioned media were intact proteoglycans decorated with glycosaminoglycans as evident from the smear of immunologically detected syndecan-1 ectodomain.

Because the similarity in molecular size of the shed syndecan-1 ectodomain suggested that LasA enhances syndecan-1 shedding by a mechanism similar to that of the host cell’s shedding mechanism, the effects of a hydroxamate derivative (BB1101), protein kinase C antagonist (bisindolylmaleimide I), and protein tyrosine kinase (PTK) inhibitors (genistein, Tyrphostin A25) were tested. Genistein (49) and Tyrphostin A25 (50) inhibit PTKs by competing for binding with ATP and tyrosine residues to PTKs, respectively. These general PTK inhibitors inhibit syndecan-1 and -4 shedding stimulated by all known agonists such as epidermal growth factor, thrombin, sphingomyelinase, ceramide, and stress conditions (e.g. heat, hyperosmolarity), whereas the antagonistic effect of the protein kinase C inhibitor, bisindolylmaleimide I, is restricted to syndecan-1 and -4 shedding induced by hyperosmolarity, ceramide, and phorbol esters (15). Hydroxamate derivatives inhibit the activity of the putative cleaving enzyme by chelating its active site zinc atom (51). Thus, general PTK inhibitors are inhibitors of regulated syndecan shedding whereas hydroxamate derivatives are inhibitors of both regulated and constitutive shedding. As shown in Table II, when co-incubated, BB1101 and Tyrphostin A25 inhibited both purified LasA- and P. aeruginosa supernatant-enhanced syndecan-1 shedding by more than 70 and 60%, respectively, at the highest concentra-
performed to verify this hypothesis since the number of strains
previously, a larger sampling of bacterial pathogens needs to be
Gram-negative and Gram-positive bacteria failed to do so. Ob-
shedding (p cells, none of them significantly inhibited enhanced syndecan-1
and BB1101 were preincubated with purified LasA and re-
the PTK inhibitors and BB1101 are acting on the host cell's
when inhibiting LasA-enhanced shedding, and that LasA en-
the PTK inhibitors and BB1101 are acting on the host cell
host effector shedding may be one such target of the pathogenesis cascade. Many bacterial patho-
gens as diverse as P. aeruginosa, S. aureus, S. epidermidis, E.
coli, S. marcescens, and L. monocytogenes have the ability to
ability to enhance shedding of host surface effectors, such as CD14,
aeruginosa
Sephacel for 2 h at 4 °C, and bound materials were eluted with 2 M
Warning: Contaminated media from unstimulated NMuMG cells (lanes 1 and 4) and from NMuMG cells stimulated with 5 μg/ml purified LasA (lanes 2 and 5) or 20% (v/v) crude P. aeruginosa supernatant (lanes 3 and 6) were incubated with DEAE-Sephaloc for 2 h at 4 °C, and bound materials were eluted with 2 M NaCl. Undigested samples were analyzed by 3.5–10% gradient SDS-PAGE and Western immunoblotting using the 281-2 anti-syndecan-1 ectodomain monoclonal antibody (undigested, lanes 1–3) or samples were digested with 10 milliunits/ml heparitinase and 20 milliunits/ml chondroitin sulfate ABC lyase and then analyzed by SDS-PAGE and Western immunoblotting (digested, lanes 4–6). Molecular masses of the immunoreactive proteins were approximated from the migration pattern of pre-stained size standards.

| 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|
| 200 kDa | 70 kDa |

**DISCUSSION**

We report here that the major opportunistic bacterial patho-
gens, P. aeruginosa and S. aureus, secrete potent enhancers of
syndecan-1 shedding. Although we have not yet identified the
S. aureus shedding enhancer, we have found that the synde-
can-1 shedding enhancer of P. aeruginosa is the 20-kDa LasA
protein, a virulence factor in animal models of corneal (40) and
lung (41, 42) infections. LasA is secreted as a precursor protein
of approximately 40 kDa, which is then processed to the mature
20-kDa form by unknown mechanisms (52, 53). Mature LasA is
a zinc metalloendopeptidase with strong staphylocytic and
weak elastolytic activities (54, 55). The alternative name of
LasA, staphylolysin, is derived from its ability to lyse staphy-
lococcal cells, and because of its elastolytic activity, LasA was
first thought of as P. aeruginosa elastase. It is now known that
the role of LasA in elastolysis is to render the insoluble elastin
substrate more susceptible to cleavage by the true P. aerugi-
nosa elastase and other elastolytic enzymes (52, 56).

The ability to enhance shedding of syndecan-1 appears to be
specific for P. aeruginosa and S. aureus since several other
Gram-negative and Gram-positive bacteria failed to do so. Ob-
viously, a larger sampling of bacterial pathogens needs to be
performed to verify this hypothesis since the number of strains
examined for the inactive bacteria was minimal in this study.

Nevertheless, the finding that evolutionarily diverse bacteria,
such as P. aeruginosa and S. aureus, can enhance syndecan-1
shedding suggests that this activity may augment their patho-
genesis at target host sites common to both. In this regard, it is
interesting to note that P. aeruginosa and S. aureus are the
dominant pathogens in cystic fibrosis and burn patients, and
that syndecan-1 is the major syndecan of target cell types at
these tissue sites, the lung epithelia and epidermal keratin-
cytes, respectively.

**Host Effector Shedding by Pathogenic Bacteria**—The current
emergence of antibiotic-resistant strains has been driven
mainly by overuse of antibiotic agents aimed at inhibiting
essential aspects of bacterial metabolism, such as cell wall and
protein synthesis, thereby placing selective pressure on bacte-
ria to become rapidly resistant to these agents for their sur-
vival. Thus, to prevent development of resistance, it may be
ideal to develop prophylactic and therapeutic agents that tar-
gel specific host-pathogen interactions involved in bacterial
pathogenesis. Enhanced host effector shedding may be one
such target of the pathogenesis cascade. Many bacterial patho-
gen such as P. aeruginosa, S. aureus, S. epidermidis, E.
coli, S. marcescens, and L. monocytogenes have the ability to
ability to enhance shedding of host surface effectors, such as CD14,
TNF-α, and IL-6 receptor (29–31). These bacteria are not only
distinguished by their cell wall characteristics and sites of
colonization, but also by their arsenal of genetically distinct
virulence factors. Thus, the shared ability to enhance shedding
of host molecules indicates functional convergence and sug-
gests that bacterial stimulation of shedding may play a role in
pathogenesis.

**Mechanism of Syndecan-1 Shedding Enhancement by
LasA**—The capacity of LasA to hydrolyze protein substrates
such as elastin, albeit weak, suggests that LasA may enhance
syndecan-1 shedding by direct cleavage of the proteoglycan.
However, several lines of evidence indicate that this is not the
mechanism of syndecan-1 shedding enhanced by LasA. First,
the proteolytic specificity of LasA is rather restricted in that
potential substrates are those with G glyc in the P1 and P2 posi-
tions, Gly, Ala, or Phe at P1’ and apolar residues in the flank-
ing sequences (54). This stringent requirement is exemplified
by the fact that elastin and the cell wall peptidoglycan of S.
aureus with these motifs are susceptible, but casein without
these motifs is not hydrolyzed by LasA (54). Syndecan-1 also
does not contain these LasA-susceptible motifs. Second, our
results show that the size of the core protein shed by LasA and
endogenous host cell mechanisms is identical by SDS-PAGE
analysis, and that PTK and hydroxamate inhibitors of LasA-
mediated syndecan-1 shedding inhibit shedding by acting on
the responding host cells and not on LasA. The PTK inhibitors
and the hydroxamate derivative (BB1101) inhibit shedding
only when LasA, the reagents and host cells are co-incubated,
and not when the inhibitors are preincubated with LasA and
removed prior to incubation of the pretreated LasA with host
cells. Furthermore, hydroxamate inhibitors are thought to be
specific for the HEXXH zinc-binding catalytic domain of met-
alloendopeptidases, such as the matrix metalloproteinases
(51), but the zinc-binding motif of LasA is HXH (55). Taken
altogether, these findings indicate that LasA augments shedding
of syndecan-1 ectodomains by activating the host cell's shed-
ding mechanism.

How PTK activities are involved in syndecan-1 shedding
enhanced by LasA is not understood. In fact, the role which
PTK activities play in stimulation of syndecan shedding by all
other known physiological agonists (e.g., epidermal growth fac-
tor, thrombin, stress-related agents) is not known. PTKs may
activate shedding by affecting the syndecan substrate, the
Enhancement of Syndecan-1 Shedding by P. aeruginosa

TABLE II

| Inhibitors                  | Syndecan-1 ectodomain shed | P. aeruginosa supernatant |
|-----------------------------|-----------------------------|---------------------------|
|                             | Purified LasA               | mean % of control ± S.D.  |
|                             | Co-incubated                |                           |
| None                        | 100.0 ± 9.5                 | 100.0 ± 8.7               |
| BB1101 (2 μM)               | 28.5 ± 2.7                  | 22.1 ± 10.3               |
| Bisindolylmaleimide (1 μM)  | 81.8 ± 4.4                  | 88.7 ± 1.8                |
| Bisindolylmaleimide (0.2 μM)| 85.0 ± 18.8                 | 111.6 ± 13.4              |
| Genistein (10 μg/ml)        | 54.4 ± 22.7                 | 55.6 ± 14.1               |
| Tyrphostin (10 μg/ml)       | 36.5 ± 5.3                  | 31.6 ± 11.6               |
| Pretreated                  |                            |                           |
| None                        | 100.0 ± 9.5                 | ND*                       |
| BB1101 (2 μM)               | 89.6 ± 11.6                 | ND                        |
| Bisindolylmaleimide (1 μM)  | 81.5 ± 15.5                 | ND                        |
| Genistein (10 μg/ml)        | 98.2 ± 1.3                  | ND                        |
| Tyrphostin (10 μg/ml)       | 105.5 ± 9.3                 | ND                        |

* ND, not determined.

Implications of LasA-enhanced Syndecan-1 Shedding in P. aeruginosa Pathogenesis—At present, we do not know whether enhanced syndecan-1 shedding by LasA is beneficial for the bacteria or the host. Based on the finding that LasA is a virulence factor in animal models of corneal (40) and lung infections (41, 42), two tissue sites where syndecan-1 is the predominant syndecan on target epithelia, we hypothesize that LasA-enhanced shedding of syndecan-1 ectodomains promotes bacterial pathogenesis. There are several ways by which syndecan-1 shedding can contribute to P. aeruginosa pathogenesis. First, our results show that enhancement of syndecan-1 shedding by P. aeruginosa not only dramatically increases the amount of soluble ectodomains, but is also accompanied by a significant decrease in the level of cell surface syndecan-1. This property may be pathologically significant since in a previous study, we have found that antisense induced depletion of cell surface syndecan-1 altered cell morphology and organization, expression of other adhesion molecules like E-cadherin and β1-integrins, and anchorage-dependent growth characteristics in NMuMG cells (18). Because highly polarized epithelia are thought of as an effective barrier against microbial colonization (47, 48), the concomitant decrease in cell surface syndecan-1 levels observed during LasA-enhanced shedding could enhance P. aeruginosa colonization by altering the morphology of target epithelia, disrupting the epithelial barrier, and exposing intercellular, basolateral, and subepithelial adhesive components.

Syndecan-1 shedding enhanced by LasA may also contribute to P. aeruginosa pathogenesis by interfering with host defense mechanisms. Syndecan shedding is a host response activated during tissue injury, and is thought of as a defense mechanism against insults including wounding and cellular stress (e.g. hyperosmolarity, mechanical shear, heat). In this mechanism, excess shed syndecan-1 ectodomains, via their heparan sulfate chains, bind and neutralize the activity of potentially deleterious proinflammatory mediators such as proteases, chemokines, and cytokines (35). Thus, enhancement of syndecan-1 shedding by some bacterial pathogens, such as P. aeruginosa and S. aureus, may be a pathogenetic mechanism that takes advantage of a normal defense mechanism to promote their survival in the host environment. In support of this hypothesis, we have found in a separate study that shed syndecan-1 ectodomains can inhibit the antibacterial activity of Prf/Arg-rich antimicrobial peptides by binding to the peptides and preventing them from interacting with target bacterial cells. These findings suggest that shed syndecan-1 ectodomains are a host-derived component of the virulence mechanism mediated by LasA. To decipher the role of enhanced shedding in bacterial pathogenesis further, we are currently evaluating the role of syndecan-1 shedding in murine models of bacterial infection using specific agonists and antagonists of shedding, and also using mice lacking syndecan-1 or overexpressing a constitutively shed form of syndecan-1.

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3 P. W. Park and M. Bernfield, unpublished results.
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Fitzgerald and Merton Bernfield

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