Activation of Syndecan-1 Ectodomain Shedding by Staphylococcus aureus α-Toxin and β-Toxin*

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Exploitation of host components by microbes to promote their survival in the hostile host environment has been a recurring theme in recent years. Available data indicate that bacterial pathogens activate ectodomain shedding of host cell surface molecules to enhance their virulence. We reported previously that several major bacterial pathogens activate ectodomain shedding of syndecan-1, the major heparan sulfate proteoglycan of epithelial cells. Here we define the molecular basis of how Staphylococcus aureus activates syndecan-1 shedding. We screened mutant S. aureus strains devoid of various toxin and protease genes and found that only strains lacking both α-toxin and β-toxin genes do not stimulate shedding. Mutations in the agr global regulatory locus, which positively regulates expression of α- and β-toxins and other exoproteins, also abrogated the capacity to stimulate syndecan-1 shedding. Furthermore, purified S. aureus α- and β-toxins, but not enterotoxin A and toxic shock syndrome toxin-1, rapidly potentiated shedding in a concentration-dependent manner. These results establish that S. aureus activates syndecan-1 ectodomain shedding via its two virulence factors, α- and β-toxins. Toxin-activated shedding was also selectively inhibited by antagonists of the host cell shedding mechanism, indicating that α- and β-toxins shed syndecan-1 ectodomains through stimulation of the host cell’s shedding machinery. Interestingly, β-toxin, but not α-toxin, also enhanced ectodomain shedding of syndecan-4 and heparin-binding epidermal growth factor. Because shedding of these ectodomains has been implicated in promoting bacterial pathogenesis, activation of ectodomain shedding by α-toxin and β-toxin may be a previously unknown virulence mechanism of S. aureus.

Ectodomain shedding is a proteolytic mechanism of releasing the extracellular domains of cell surface proteins as soluble ectodomains (1–4). Approximately 2% of cell surface proteins are thought to be secreted into the extracellular environment by this mechanism. The diverse list of shed proteins includes cytokines, growth factors, and cell adhesion molecules, such as tumor necrosis factor α (TNFα), transforming growth factor α (TGFα), epidermal growth factors (EGFs),1 t-selectin, CD44, and syndecans, to name a few. Ectodomain shedding is an important regulatory mechanism since it rapidly changes the surface phenotype of affected cells and generates soluble, biologically active ectodomains that can function as paracrine or autocrine effectors. A growing body of evidence indicates that these cellular and cellular features enable ectodomain shedding to regulate many pathophysiological processes, such as microbial pathogenesis, inflammation, and tissue repair.

Ectodomain shedding is mediated by peptide hydroxamate-sensitive metalloproteinases, which are collectively called sheddases or secretases (1–4). Several ADAMs (a disintegrin and metalloproteinase) have been shown to function as cell surface-associated sheddases. Among these, TACE (TNFα converting enzyme, ADAM17) sheds a wide variety of surface proteins. In addition to TNFα (5, 6), TACE can shed TNFα receptors (7), various EGF family ligands such as heparin-binding EGF (HB-EGF) (8), TGFα (7), and amphiregulin (9), EGF receptor ErbB-4 (10), IL-6 receptor (IL-6R) (11), Notch (12), t-selectin (7), and β-amyloid precursor protein (13). However, HB-EGF, TGFα, and IL-6R ectodomains are still shed in TACE-deficient cells (8, 11), and available data indicate that ectodomain shedding of CD44 (14) and syndecan-1 and -4 (15) is TACE-independent. Consistent with these findings, other cell surface-associated and soluble sheddases have been described. Examples of other cell surface-associated sheddases include matrix metalloproteinase (MMP)-14 (MT1-MMP) (16) for CD44 shedding, and ADAM9 (17), ADAM10 (18), and ADAM12 (19) for HB-EGF ectodomain shedding. Among the soluble sheddases, MMP-3 has been shown to shed HB-EGF ectodomains (20) and MMP-7 has been found to shed TNFα (21), Fas ligand (22), E-cadherin (23), and syndecan-1 (24) ectodomains. These data indicate that certain shed proteins are substrates of more than one sheddase and suggest that different sheddases act in a tissue-specific manner, and also possibly in a disease-specific manner.

1 The abbreviations used are: EGFs, epidermal growth factors; ADAM, a disintegrin and metalloproteinase; HA, hemagglutinin; HB-EGF, heparin-binding epidermal growth factor; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; MAP kinase, mitogen-activated protein kinase; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; NMuMG, normal murine mammary gland; PKC, protein kinase C; PTK, protein-tyrosine kinase; SFA, staphylococcal enterotoxin A; TACE, TNFα-converting enzyme; TGFα, transforming growth factor α; TNFα, tumor necrosis factor α; TSB, tryptic soy broth; TSST-1, toxic shock syndrome toxin-1.

Received for publication, August 4, 2003, and in revised form, October 16, 2003
Published, JBC Papers in Press, October 22, 2003, DOI 10.1074/jbc.M30587200

*This work was supported by Grants HL68905 from the National Institutes of Health and RG-054-N from the American Lung Association (to P. W. P.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Ectodomain shedding is highly regulated by various extracellular ligands and intracellular signaling pathways. Phorbol ester protein kinase C (PKC) agonists (i.e., PMA, TPA) enhance the shedding of most affected molecules (1). Protein-tyrosine kinase (PTK) activity is required for agonist-activated ectodomain shedding of syndecan-1 and -4 (15, 25) and the cell adhesion molecule L1 (26). Furthermore, specific antagonists of mitogen-activated protein (MAP) kinase pathways inhibit ectodomain shedding of syndecan-1 and -4 (15), HB-EGF (27), TGFβ (28), L1 (26), L-selectin (28), and TNFα (28) in an agonist-specific manner. At present, it is not clear if these signaling pathways are linked and a common signaling pathway exists to regulate ectodomain shedding. Nevertheless, available data clearly show that several signaling pathways can markedly affect the rate of ectodomain shedding at the cell surface, indicating that various pathways converge to modulate the cleavage event at the cell surface. The involvement of many signaling pathways in regulating ectodomain shedding may be one of the biological mechanisms that defines which cell surface protein gets shed in response to specific extracellular cues.

Recent studies have shown that microbial pathogens can stimulate ectodomain shedding by host cells. IL-6R shedding is activated by secreted products of *S. aureus*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, and *Serratia marcescens* (29). Similarly, TNFα shedding is provoked by culture supernatants of *S. epidermidis* (30). Although the physiological significance of pathogen-activated ectodomain shedding has yet to be clearly defined, several studies have shown that virulence factors enhance ectodomain shedding, suggesting that this is a pathogenic activity. For example, streptolysin O, a pore-forming toxin expressed by the majority of Group A *Streptococcus* clinical isolates, stimulates ectodomain shedding of L-selectin, IL-6R, and CD14 (31, 32). Another study recently demonstrated that lipotechoic acid released from the staphylococcal cell wall activates ADAM10-mediated shedding of HB-EGF ectodomains in epithelial cells via stimulation of the G protein-coupled platelet-activating factor receptor (18). Shed HB-EGF then activates the EGF receptor to induce mucin expression. This mechanism potentially increases the viscosity of mucus fluids, deters mucociliary clearance of pathogens, and promotes microbial pathogenesis.

We have shown that shedding of syndecan-1 ectodomains is triggered by culture supernatants of *P. aeruginosa* and *S. aureus* (25). Syndecan-1 is the major heparan sulfate proteoglycan (HSPG) of epithelial cells and it binds and regulates a wide variety of biological molecules through its heparan sulfate (HS) chains (33, 34). Because syndecan-1 ectodomains are replete with all of their HS chains, they can function as soluble regulators of various molecular interactions (33, 34). We have identified the *P. aeruginosa* shedding enhancer as LasA, a known virulence factor for *P. aeruginosa* lung infection (25), and results from our in vivo studies indicate that *P. aeruginosa* activates syndecan-1 shedding to enhance its virulence in a murine model of lung infection (35). It remains unclear, however, whether the pathogenic mechanism of *P. aeruginosa* that exploits syndecan-1 shedding to enhance its virulence is utilized by other pathogens.

Here we report the characterization of *S. aureus* enhancers of syndecan-1 ectodomain shedding. *S. aureus* is a common and clinically important Gram-positive bacterial pathogen that is responsible for causing a wide variety of life-threatening diseases, such as pneumonia, toxic shock syndrome, osteomyelitis, endocarditis, and sepsis (36, 37). The impressive pathogenic capabilities of *S. aureus* are mediated by the large number of virulence factors that the bacterium elaborates, including many enzymes, adhesins, and toxins. *S. aureus* is also one of the leading causes of nosocomial infections and the current emergence of multidrug resistant strains adds to the threat of staphylococcal infections. It is therefore imperative to define the molecular mechanisms involved in *S. aureus* virulence. We initially screened the effects of specific mutations of *S. aureus* virulence genes on syndecan-1 ectodomain shedding, and determined that mutant strains deficient in both α- and β-toxin genes could not stimulate shedding. Consistent with these results, we also found that purified α- and β-toxins augment syndecan-1 shedding. Surprisingly, the stimulatory effects of the *S. aureus* toxins on syndecan-1 shedding were sensitive to antagonists of the host cell shedding mechanism. Furthermore, β-toxin, but not α-toxin, stimulated ectodomain shedding of syndecan-4 and HB-EGF in a host sheddase-dependent manner. These results indicate that α- and β-toxins stimulate syndecan-1 shedding via activation of the host cell shedding machinery and that β-toxin also enhances shedding of other cell surface proteins. Because ectodomain shedding of syndecan-1 and HB-EGF has been implicated in bacterial pathogenesis, these results suggest that activation of ectodomain shedding may be one of the mechanisms by which *S. aureus* α- and β-toxins function as virulence factors.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bisindolylmaleimide I, PD98059, SB203580, tyrphostin A25, herbimycin A, PP2, AG490, piceatannol, GM6001, TAPI-1, and PMA were from Calbiochem (La Jolla, CA). Tryptic soy agar were purchased from Remel (Lenexa, KS). Purified *S. aureus* α-toxin, β-toxin, enterotoxin A (SEA), and toxic shock syndrome toxin-1 (TSST-1) were from Toxin Technology (Sarasota, FL) or Sigma. The cationic nylon membrane, Immobilon Ny+, was from Millipore (Bedford, MA). Heparinase II and chondroitin sulfate ABC lyase were purchased from Seikagaku (Cape Cod, MA). Tissue culture media and supplements were from Mediatech (Herndon, VA). Enhanced chemiluminescence (ECL) Western blotting detection reagents and DEAE Sepharose Fast Flow were from Amersham Biosciences. All other materials were purchased from VWR (Houston, TX), Fisher (Houston, TX), or Sigma.

**Immunochromatography**—The rat monoclonal anti-mouse syndecan-1 (251–2) and syndecan-4 (Kv8.2) antibodies (25) were purified from either ascites fluids or conditioned media of hybridoma cultures by protein G affinity chromatography. Rabbit anti-mouse syndecan-1 cytoplasmic domain antibody was generated by immunizing rabbits with the synthetic peptide CNGGAYQKPTKQEEFYA. Affinity-purified antibody was prepared by protein A affinity chromatography followed by synthetic peptide affinity chromatography. The affinity-purified antibody detects detergent-extracted transmembrane syndecan-1 but does not react with purified syndecan-1 ectodomains. The anti-hemagglutinin (HA) antibody 9E10 was purchased from Sigma and HRP-conjugated donkey anti-rat, -mouse, or -rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

**S. aureus Strains and Culture Conditions**—The *S. aureus* strains used in this study are described in Table I. Glyceraldehyde stocks of each strain were made from cultures grown in TSB with erythromycin (10 μg/ml) or tetracycline (2 μg/ml) where appropriate. Culture supernatants were prepared by growing glycerol stocks in TSB overnight without antibiotics at 37°C with agitation and filter sterilization. The culture supernatants were tested at 5, 10, or 20% (v/v) for their ability to activate syndecan-1 shedding via activation of the host cell shedding machinery and that β-toxin also enhances shedding of other cell surface proteins. Because ectodomain shedding of syndecan-1 and HB-EGF has been implicated in bacterial pathogenesis, these results suggest that activation of ectodomain shedding may be one of the mechanisms by which *S. aureus* α- and β-toxins function as virulence factors.
\textbf{Table I: \textit{S. aureus} strains used in this study}

| Strain   | Genotype          | Representative features                                      | Source or Ref. |
|----------|-------------------|-------------------------------------------------------------|----------------|
| 8325-4   | rsbU              | Parent strain for mutants; NCTC 8325 Cured of prophages \(\phi11, \phi12, \phi13\); Defective RsB and SigB       | (43,56)        |
| DU1090   | hla::ermC         | \(\alpha\)-Toxin deficient mutant of 8325-4; Erythromycin resistant | O'Reilly (57)  |
| DU5719   | hlb::orf2E        | \(\beta\)-Toxin-deficient mutant of 8325-4 caused by lysogenization by phage 42E | Patel (58)     |
| Not assigned | hlg::tetK     | \(\gamma\)-Toxin deficient mutant of 8325-4; Tetracycline resistant | Nilsson (60)   |
| DU5720   | hla::ermC hlb::orf2E | \(\alpha, \beta, \gamma\)-Toxin deficient mutant of 8325-4 | Patel (58)     |
| DU5983   | hla::ermC hlb::orf2E | \(\alpha, \beta\)-Toxin-deficient mutant of 8325-4 | Patel (58)     |
| DU5996   | aur::lacZ ermC    | Metalloprotease ( aureolysin)-deficient mutant of 8325-4 caused by integration of \(pZ\)106 | (61)           |
| RN6911   | agr::tmn          | Agr-deficient mutant of 8325-4 Tetracycline-resistant        | Novick (62)    |

**RESULTS**

\textit{S. aureus} Mutant Strains Lacking \(\alpha\)-Toxin and \(\beta\)-Toxin Do Not Shed Syndecan-1 Ectodomains—We reported previously that the majority of \textit{S. aureus} strains secrete factors that stimulate syndecan-1 ectodomain shedding by cultured epithelial cells (25). To identify the \textit{S. aureus} shedding enhancers, we initially screened a panel of \textit{S. aureus} strains with specific mutations in their toxin, enzyme, or regulatory genes (Table I) for their ability to activate syndecan-1 shedding. Confluent cultures of NMuMG epithelia were incubated with 20\% (vol/vol) TSB (media), culture supernatants obtained from the parent \textit{S. aureus} 8325-4 strain (WT), or supernatants from mutant \textit{S. aureus} 8325-4 strains lacking \(\alpha\)-toxin (H\(\alpha\)\(^{-}\)), \(\beta\)-toxin (H\(\beta\)\(^{-}\)), \(\gamma\)-toxin (H\(\gamma\)\(^{-}\)), \(\alpha, \beta\)-toxins (H\(\alpha\)\(^{-}\)H\(\beta\)\(^{-}\)H\(\gamma\)\(^{-}\)), metalloprotease (Aur\(^{-}\)), or the Agr regulatory locus (Agr\(^{-}\)) (Fig. 1). \textit{S. aureus} \(\alpha\)-toxin is a pore-forming cytolytic toxin and it is one of the most potent bacterial toxins known (38). \textit{S. aureus} \(\alpha\)-toxin is secreted as a water soluble monomer, binds to target membranes as a soluble monomer, and then forms heptameric transmembrane pores in target cell membranes (39–41). \textit{S. aureus} \(\gamma\)-toxin is also a pore-forming toxin (38). In contrast, \(\beta\)-toxin exerts its cytotoxic effects through its neutral sphingomyelinase activity (38). The role of the staphylococcal metalloprotease in pathogenesis is not fully understood, but it can digest both host and bacterial factors (42). The Agr locus specifies a density-dependent regulatory system that stimulates transcription of genes encoding exoproteins in the stationary phase of growth. Agr\(^{-}\) mutants express significantly reduced levels of \textit{S. aureus} exoproteins, including \(\alpha, \beta\)-toxins (43).
Syndecan-1 shedding activity was partially reduced (~40%) in the α-toxin and β-toxin single mutant strains relative to the WT strain and was completely absent in the α- and β-toxin double and α-, β- and γ-toxin triple mutant strains (Fig. 1). In contrast, shedding activity was not affected in the γ-toxin and metalloprotease single mutant strains. Furthermore, consistent with the role of Agr in positively regulating the expression of exoproteins, the Agr7 mutant strain also did not stimulate syndecan-1 ectodomain shedding (Fig. 1). Cell viability was not significantly different among epithelial cells incubated with different S. aureus supernatants (~85–95%) and syndecan-1 was not detected in the conditioned media of S. aureus supernatant-treated cells when probed with an affinity-purified antibody directed against the syndecan-1 cytoplasmic domain (data not shown), verifying that the ectodomains were shed and not released intact from damaged cells. These results suggest that S. aureus α- and β-toxins are the enhancers of syndecan-1 shedding.

Purified S. aureus α-Toxin and β-Toxin Stimulate Syndecan-1 Ectodomain Shedding—To determine whether α- and β-toxins are indeed the S. aureus shedding enhancers, we tested the effects of several purified S. aureus toxins on syndecan-1 shedding. NMuMG epithelia were incubated with various concentrations of purified α-toxin, β-toxin, toxic shock syndrome toxin-1 (TSST-1), or staphylococcal enterotoxin A (SEA) and the extent of syndecan-1 ectodomain shedding was measured (Fig. 2A). SEA is the major cause of staphylococcal food poisoning and TSST-1 is one of the causative toxins of staphylococcal toxic shock syndrome (38). Both SEA and TSST-1 can function as superantigens by binding to class II major histocompatibility complexes (MHC) and stimulating the class II MHC-restricted immune response. Because of these functional similarities, SEA, and TSST-1 are classified in the family of staphylococcal pyrogenic superantigen toxins, along with other staphylococcal enterotoxins.

As shown in Fig. 2A, syndecan-1 shedding was increased ~4-fold when epithelial cells were incubated with 1 μg/ml purified α- or β-toxin for 4 h at 37 °C. Maximum stimulation was reached at a concentration of 5 μg/ml for both α-toxin (~8-fold increase) and β-toxin (~6-fold increase). In contrast, both SEA and TSST-1 failed to activate shedding at all concentrations tested. Furthermore, shedding activation was rapid (>2-fold increase by 30 min) and saturable (by 2 h) when incubated with 5 μg/ml α- or β-toxin (Fig. 2B). At the range of concentrations tested in our shedding assays, both α-toxin (44) and β-toxin (31) have been shown to have minimal toxic effects on host cells. Consistent with these findings, we found that NMuMG epithelial cells were >95% viable after a 4-h incubation with 5 μg/ml α- or β-toxin (data not shown). These results establish that S. aureus activates syndecan-1 ectodomain shedding via α- and β-toxins.

S. aureus α-Toxin and β-Toxin Activate Syndecan-1 Shedding through Stimulation of the Host Cell’s Shedding Mechanism—We next analyzed the size of the syndecan-1 ectodomains shed by S. aureus culture supernatants and α- and β-toxins, and found that both intact ectodomains (Fig. 3A) and heparinase II- and chondroitinase ABC-digested core proteins...
inhibitors of PKC (bisindolylmaleimide I) and the ERK (PD98059) and p38 (SB203580) MAP kinase pathways, and found that these do not significantly inhibit shedding activated by \textit{S. aureus} factors (Table II). We also tested the effects of Tyrophostin A25, a general inhibitor of PTKs that interferes with binding of PTKs to target Tyr residues, because it has been shown to inhibit syndecan-1 shedding activated by various agonists (15, 25). As shown in Table II, Tyrophostin A25 significantly inhibited syndecan-1 shedding activated by \textit{S. aureus} culture supernatant and purified toxins, indicating that PTK activity is essential for \textit{S. aureus}-activated shedding. Taken together, these results indicate that \textit{S. aureus} exploits a PTK-dependent shedding mechanism of host cells to enhance syndecan-1 ectodomain shedding.

To further study the role of PTKs in \textit{S. aureus}-activated syndecan-1 shedding, we next tested the effects of the following specific PTK inhibitors: herbimycin A, an inhibitor of Src family PTKs; PP2, an inhibitor of Lck, Fyn, and Hck PTKs belonging to the Src family PTKs; AG490, an inhibitor of JAK family PTKs; and piceatannol, an inhibitor of Syk family PTKs (45). As shown in Table II, piceatannol abrogated syndecan-1 ectodomain shedding augmented by \textit{S. aureus} culture supernatant and purified \(\alpha\)- and \(\beta\)-toxins. Interestingly, AG490-inhibited \(\beta\)-toxin-activated, but not \(\alpha\)-toxin-activated, syndecan-1 shedding. Herbimycin A and PP2 had no significant effect. Furthermore, AG490 and piceatannol did not inhibit shedding if they were preincubated with \textit{S. aureus} toxins and removed prior to incubation with cells, indicating that the effects of AG490 and piceatannol were on the host cell and not on the toxins. The differential effects of specific PTK inhibitors on shedding suggest that \(\alpha\)- and \(\beta\)-toxins stimulate related, yet distinct, signaling pathways that converge to activate a common cleavage mechanism at the cell surface.

\textit{S. aureus} \(\beta\)-Toxin, but Not \(\alpha\)-Toxin, Activates Ectodomain Shedding of Syndecan-4 and HB-EGF—Ectodomain shedding of affected cell surface molecules is mediated by metalloproteinase sheddases (1). Our results indicate that \textit{S. aureus}-activated syndecan-1 shedding is also mediated by the host cell sheddase, suggesting that \textit{S. aureus} toxins may stimulate shedding of other host cell surface molecules. To test this hypothesis, we examined whether \textit{S. aureus} \(\alpha\)- and \(\beta\)-toxins activate shedding of syndecan-4 and HB-EGF ectodomains. HB-EGF is a member of the EGF ligand family and binds to the EGF receptors ErbB-1 and -4 (46, 47). Ectodomain shedding of HB-EGF has been implicated in various pathophysiological processes, such as wound repair (48), cardiac hypertrophy (19), and staphylococcal infections (18). Syndecan-4 is a member of the syndecan family of HSPGs and is expressed in most adult tissues, including epithelia, albeit at a lower level than that of syndecan-1 (33, 34). The physiological function of syndecan-4 ectodomain shedding is not known. However, functions of syndecan-1 ectodomains are mediated by their HS chains (33, 34), and HS chains of syndecan-1 and syndecan-4 obtained from the same cell type have been shown to be structurally similar (49). Thus, we speculate that syndecan-4 ectodomains may possess functions similar to that of syndecan-1 ectodomains.

To determine the effects of \textit{S. aureus} toxins on syndecan-4 shedding, confluent cultures of NMuMG cells were incubated with various concentrations of \textit{S. aureus} 8325–4 culture supernatant, purified \(\alpha\)-toxin, or purified \(\beta\)-toxin and shed syndecan-4 was quantified by immunoblotting using the Ky8.2 antibody. Syndecan-4 was quantified by immunoblotting using the Ky8.2 antibody (Fig. 4). In contrast, purified
α-toxin did not augment syndecan-4 shedding at the three concentrations shown to enhance syndecan-1 shedding (Fig. 4). The effect of \textit{S. aureus} toxins on ectodomain shedding of HB-EGF was assessed by incubating CHO-K1 cells stably expressing an HA-tagged transmembrane construct of HB-EGF (proHB-EGF) with purified α- and β-toxins (both at 10 μg/ml). The extent of shedding was visualized by detecting HB-EGF ectodomains in the conditioned media and transmembrane proHB-EGF in cell lysates by Western immunoblotting using an anti-HA antibody. As shown in Fig. 5, the intensity of the bands corresponding to HB-EGF ectodomains (21–25 kDa) and proHB-EGF (25–32 kDa) were increased and decreased, respectively, in CHO-K1 cells incubated with 1 μM PMA (positive control) or purified β-toxin. These results indicate that β-toxin stimulates HB-EGF ectodomain shedding. Furthermore, β-toxin- and PMA-activated HB-EGF shedding was inhibited by the peptide hydroxamate sheddase inhibitor. However, similar to its effect on syndecan-4 shedding, purified α-toxin did not stimulate HB-EGF ectodomain shedding. These findings indicate that α-toxin is a specific enhancer of syndecan-1 ectodomain shedding, whereas β-toxin can potentiate ectodomain shedding of several cell surface proteins.

**DISCUSSION**

Our study defines the molecular basis of how \textit{S. aureus} activates syndecan-1 ectodomain shedding. We have shown previously that the majority of \textit{S. aureus} strains secrete factors that stimulate syndecan-1 shedding (25). Several independent criteria provided by this study establish that \textit{S. aureus} α- and β-toxins are the secreted enhancers of syndecan-1 shedding. First, analyses of several \textit{S. aureus} mutant strains showed that the capacity to stimulate syndecan-1 shedding is absent in strains with mutations that eliminate both α- and β-toxins and partially reduced in single α-toxin and β-toxin-deficient mutants. Second, inactivation of the Agr global regulatory locus, which positively regulates expression of α- and β-toxin genes along with those encoding other exoproteins, abrogated the ability to stimulate syndecan-1 ectodomain shedding. Third, purified α- and β-toxins rapidly activated ectodomain shedding in a concentration-dependent manner. Furthermore, activation of syndecan-1 shedding by both α and β-toxins was inhibited by specific antagonists of the host cell shedding mechanism and the effects of these inhibitors were determined to be on the host cell and not on the staphylococcal toxins. These findings demonstrate that \textit{S. aureus} α- and β-toxins activate syndecan-1 ectodomain shedding via stimulation of the host cell shedding machinery. \textit{S. aureus} α-toxin is a major virulence factor of this bacterium. It exerts its cytotoxic effects by spontaneously forming heptameric pores on target cell membranes (39–41). When α-toxin pore formation is extensive, cells are killed by loss of ATP and an imbalance of critical ions. Our results demonstrate that syndecan-1 ectodomain shedding is activated at α-toxin concentrations that are non-cytotoxic for epithelial cells, indicating that extensive pore formation is not required for shedding activation. Furthermore, pore formation alone is not a signal for syndecan-1 ectodomain shedding since our results suggest that γ-toxin and other non-α pore forming toxins of \textit{S. aureus Virulence Factors Enhance Ectodomain Shedding**

| Inhibitors | Shed syndecan-1 (mean ± % of control ± S.E.) |
|------------|-----------------------------------------------|
| None (control) | S. aureus supernatant | α-Toxin | β-Toxin |
| Shedase Inhibitors | 100.0 ± 10.9 | 100.0 ± 5.3 | 100.0 ± 8.9 |
| GM6001 | | | |
| 5 μM | 22.7 ± 11.0 | 27.5 ± 5.7 | 19.3 ± 15.3 |
| 5 μM (Pre)* | ND | 98.2 ± 8.4 | 94.1 ± 5.6 |
| 0.5 μM TAPI-1 | 32.1 ± 10.4 | 21.1 ± 14.8 | 36.9 ± 5.2 |
| 5 μM | 30.0 ± 5.7 | 12.8 ± 16.1 | 24.1 ± 12.4 |
| 0.5 μM | 45.0 ± 6.5 | 32.0 ± 15.1 | 24.2 ± 12.4 |
| PKC Inhibitor | | | |
| Bisindolylmaleimide I | 87.4 ± 7.4 | 78.9 ± 12.3 | 92.3 ± 16.1 |
| 1 μM | 91.3 ± 4.5 | 81.3 ± 8.7 | 90.5 ± 1.5 |
| MAP Kinase Inhibitors | | | |
| PD98059 (ERK) | 10 μM | 69.2 ± 3.7 | 85.7 ± 18.0 | 81.9 ± 12.8 |
| 1 μM | 73.9 ± 1.7 | 90.1 ± 5.3 | 82.0 ± 11.2 |
| SB203580 (p38) | 10 μM | 71.3 ± 5.0 | 73.0 ± 7.3 | 81.0 ± 13.9 |
| 5 μM | 93.0 ± 4.0 | 86.9 ± 17.2 | 90.1 ± 2.1 |
| General PTK Inhibitor | | | |
| Tyrophostin A25 | 30 μM | 25.8 ± 5.7 | 35.6 ± 8.9 | 26.0 ± 12.6 |
| 3 μM | 38.3 ± 6.5 | 36.5 ± 11.6 | 39.2 ± 6.8 |
| Specific PTK Inhibitors | | | |
| Herbimycin A (Src) | 10 μM | 87.4 ± 7.4 | 105.1 ± 7.0 | 88.9 ± 10.6 |
| PP2 (Src, Lck, Fyn, Hck) | 10 μM | 95.4 ± 1.1 | 97.6 ± 17.7 | 96.9 ± 8.2 |
| AG490 (JAK2) | 0.5 μM | 35.7 ± 12.4 | 104.0 ± 3.5 | 9.5 ± 18.3 |
| 0.5 μM (Pre)* | ND | 99.0 ± 10.4 | 101.9 ± 1.5 |
| Piceatannol (Syk) | 5 μM | 16.8 ± 14.8 | 8.1 ± 23.4 | 9.0 ± 7.6 |
| 5 μM (Pre)* | ND | 100.5 ± 6.7 | 96.4 ± 11.1 |

* Pre, purified toxins were pretreated with the indicated concentration of inhibitors for 1 h at room temperature and the inhibitors were removed by centrifugation against 10 kDa MWCO spin tubes prior to incubation with NMuMG cells.

* ND, not determined.
**S. aureus Virulence Factors Enhance Ectodomain Shedding**

**Fig. 4.** Syndecan-4 ectodomain shedding is enhanced by *S. aureus* β-toxin, but not α-toxin. NMuMG cells were incubated with *S. aureus* 8325-4 supernatant at 5, 10, or 20% (v/v) or 10% supernatant plus 5 μM GM6001 or purified α- or β-toxin at 5, 10, or 20 μg/ml or 10 μg/ml plus 5 μM GM6001 for 4 h at 37 °C. Conditioned media were harvested and the relative amount of syndecan-4 ectodomains was measured using the Ky8.2 anti-syndecan-4 ectodomain antibody. Syndecan-4 ectodomains, shown as absorbance units (AU), were quantified by densitometric scanning of dot blots using the NIH Image software as described. Each bar represents the mean ± S.E. of triplicate determinations.

**Fig. 5.** *S. aureus* β-toxin, but not α-toxin, stimulates the ectodomain shedding of HB-EGF. CHO-K1 cells stably expressing an HA-tagged transmembrane construct of HB-EGF (proHB-EGF) were pretreated with or without the KB-R8301 hydroxamate shedding inhibitor and then incubated with media (+), PMA (1 μM), α-toxin (10 μg/ml), or β-toxin (10 μg/ml) for 30 min at 37 °C. Conditioned media were collected and cells were lysed after extensive washes. HB-EGF ectodomains and proHB-EGF in total cell lysates were fractionated by 15% SDS-PAGE and detected by Western immunoblotting using the 9E10 anti-HA antibody.

*aureus* do not stimulate shedding. At low concentrations, α-toxin forms discrete pores in target cell membranes (44) so it is possible that formation of small, discrete pores signals to trigger syndecan-1 shedding. At non-cytotoxic concentrations, α-toxin stimulates phosphatidylinositol hydrolysis to generate inositol phosphate signaling mediators in A549 lung epithelial cells (44). Our results show that α-toxin-induced syndecan-1 shedding involves PTKs, possibly Syk, but not PKC and MAP kinase signaling pathways. Interestingly, streptolysin O has been shown to stimulate L-selectin shedding via discrete pore formation and this activity was not affected by PKC inhibitors (31). These findings suggest that, similar to streptolysin O, α-toxin activates syndecan-1 ectodomain shedding via small, discrete pore formation. Alternatively, pore formation may not be required and binding of α-toxin to a putative α-toxin receptor may trigger signals essential for syndecan-1 shedding. Low and high affinity binding sites on host cells have been described for α-toxin and the high affinity binding site is thought to be an α-toxin receptor protein (38). Therefore, an important issue of future studies will be to test these possibilities using mutant α-toxin constructs that lack the pore forming activity but retain the binding function or one that lacks binding to the high affinity site.

*S. aureus* β-toxin exerts its cytotoxic effects through its neutral sphingomyelinase activity. Hydrolysis of membrane sphingomyelin by β-toxin generates ceramide, a potent lipid second messenger, that regulates many signaling pathways (50). Results from this study show that β-toxin-activated syndecan-1 shedding is PTK- and sheddase-dependent. Importantly, we have previously shown that incubation of epithelial cells with membrane-permeable C8-ceramide also activates syndecan-1 shedding in a PTK- and sheddase-dependent manner (15). Thus, it is likely that β-toxin activates syndecan-1 ectodomain shedding via hydrolysis of membrane sphingomyelin and subsequent generation of ceramide.

Our results indicate that β-toxin also activates syndecan-4 and HB-EGF ectodomain shedding. Walev et al. (31) have shown that β-toxin stimulates L-selectin shedding via its sphingomyelinase activity. These observations indicate that β-toxin, via ceramide generation, likely enhances ectodomain shedding of a wide variety of cell surface proteins. However, how ceramide activates ectodomain shedding in a sheddase-dependent manner is not clear. Although available results indicate that TACE is not the syndecan sheddase, TACE can shed HB-EGF (8) and L-selectin (7), and the cytoplasmic domain of TACE can be Ser-phosphorylated (51). Because ceramide activates a Ser/Thr kinase (50), Ser phosphorylation of TACE by this kinase may activate TACE-mediated shedding of HB-EGF and L-selectin ectodomains. Another possibility is stabilization of the putative syndecan sheddase and TACE since ceramide has been shown to stabilize β-secretase and augment its ability to shed β-amyloid precursor protein (52). Alternatively, the ability of ceramide to re-organize the cell membrane into a signaling platform and to cluster cell surface proteins (53) may augment shedding by increasing the physical proximity of cell
surface proteins and their sheddases. 

Stimulation of syndecan-1 ectodomain shedding by β-toxin is regulated by PTKs, possibly JAK2 and Syk, based on our inhibitor studies. In fact, available data indicate that PTK activity is essential for all known agonists of syndecan-1 shedding (15, 25). How PTKs regulate syndecan-1 shedding is not clear. However, all four mammalian syndecans are shed and contain three tandemly repeated thioredoxin-like repeats in their cytoplasmic domain (33), and so far, Tyr phosphorylation of the cytoplasmic domains of syndecan-1 and -3 have been documented (54, 55). It is not known whether syndecan shedding can be regulated by Tyr phosphorylation of its cytoplasmic domain, but ectodomain shedding of L1 has been shown to be regulated by Tyr phosphorylation of its cytoplasmic tail (26). These findings suggest that regulation of syndecan shedding by Tyr phosphorylation of their cytoplasmic domain warrants further investigation.

In summary, we have determined that S. aureus activates syndecan-1 ectodomain shedding via its two virulence factors, α- and β-toxins. Both toxins activate shedding by exploiting the host cell’s shedding machinery. Our results also revealed that α-toxin specifically activates syndecan-1 shedding, whereas β-toxin enhances ectodomain shedding of not only syndecan-1, but also of syndecan-4 and HB-EGF. The significance of these to the pathophysiology of S. aureus infection remains largely undefined. If S. aureus uses cell surface HSpgs as its attachment receptor, syndecan shedding may inhibit pathogenesis by interfering with its adhesion to host tissue components. However, available data indicate that syndecan-1 ectodomains facilitate P. aeruginosa lung infection by inhibiting various host defense factors (e.g. antimicrobials) through their HS chains (35), suggesting that syndecan-1 and -4 ectodomains may function similarly in S. aureus pathogenesis. Activation of HB-EGF ectodomain shedding has been suggested to contribute to the generation of viscous mucus fluids (18), which is a major determinant to mucociliary clearance of microbial pathogens. These observations suggest that S. aureus activates ectodomain shedding of host components to promote its pathogenesis.

Acknowledgments—We thank Gordon Leung for excellent technical support and Dr. Paul Kincade, Oklahoma Medical Research Foundation, for providing the K882 rat-anti mouse syndecan-4 monoclonal antibody.

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