Several microbial pathogens stimulate the ectodomain shedding of host cell surface proteins to promote their pathogenesis. We reported previously that *Pseudomonas aeruginosa* and *Staphylococcus aureus* activate the ectodomain shedding of syndecan-1 and that syndecan-1 shedding promotes *P. aeruginosa* pathogenesis in mouse models of lung and burn skin infections. However, it remains to be determined whether activation of syndecan-1 shedding is a virulence mechanism broadly used by pathogens. Here we show that *Streptococcus pneumoniae* stimulates syndecan-1 shedding in cell culture-based assays. *S. pneumoniae*-induced syndecan-1 shedding was repressed by peptide hydroxamate inhibitors of metalloproteinases but not by inhibitors of intracellular signaling pathways previously found to be essential for syndecan-1 shedding caused by *P. aeruginosa*, *S. aureus*, or other shedding agonists. A 170-kDa protein fraction with a peptide hydroxamate-sensitive shedding activity was purified by ammonium sulfate precipitation, DEAE chromatography, and size exclusion chromatography. Mass spectrometry analyses revealed that the 170-kDa fraction is composed of ZmpB and ZmpC, two metalloproteinase virulence factors of *S. pneumoniae*. Both the purified 170-kDa ZmpB/ZmpC fraction and unfractonated *S. pneumoniae* culture supernatant generated syndecan-1 ectodomain shedding that are smaller than those released by endogenous shedding. Further, a mutant *S. pneumoniae* strain deficient in *zmpC*, but not *zmpB*, lost its capacity to stimulate syndecan-1 shedding. These data demonstrate that *S. pneumoniae* directly sheds syndecan-1 ectodomains through the action of ZmpC.

Numerous proteins can be released from host cell surfaces by a proteolytic cleavage mechanism known as ectodomain shedding (1–4). Approximately 2% of cell surface proteins are processed by this post-translational mechanism (1). Ectodomain shedding can rapidly down-regulate protein expression at the cell surface and also generate soluble ectodomains that can function in a paracrine or autocrine manner. Interestingly, the list of shed proteins includes many mediators of tissue injury and inflammation, such as tumor necrosis factor-α, epidermal growth factor, Fasl, L-selectin, and interleukin-6 receptor (1–4). Thus, ectodomain shedding is increasingly recognized as an important post-translational mechanism that regulates both infectious and noninfectious inflammatory processes.

Recent studies have demonstrated that microbial pathogens can stimulate the ectodomain shedding of several proteins from host cell surfaces to promote their pathogenesis (5–9). For example, lipoteichoic acid of *Staphylococcus aureus* binds to platelet-activating factor receptor and activates a signaling mechanism that results in a disintegrin and metalloproteinase-10-mediated shedding of heparin-binding epidermal growth factor in epithelial cells. Shed heparin-binding epidermal growth factor then activates the heparin-binding epidermal growth factor receptor to induce mucin overexpression, which promotes lung infection by obstructing airflow and inhibiting antibacterial agents (5). Extracellular proteinases secreted by *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Streptococcus pyogenes* shed dermatan sulfate proteoglycans, which bind to and inactivate neutrophil-derived α-defensins (6). Streptolysin O, a toxin virulence factor secreted by *S. pyogenes*, stimulates the shedding of L-selectin, interleukin-6 receptor, and CD14, and this is thought to dysregulate the host inflammatory response to promote streptococcal pathogenesis (8, 9). Similarly, secreted products of *S. aureus*, *P. aeruginosa*, *Listeria monocytogenes*, and *Serratia marcescens* activate interleukin-6 receptor shedding (7), and those of *S. epidermidis* stimulate tumor necrosis factor-α shedding (10). Collectively, these observations suggest that various microbial pathogens activate the ectodomain shedding of inflammatory factors to dysregulate the host response to infection and promote their pathogenesis.

Syndecan-1 is a type I transmembrane heparan sulfate proteoglycan predominantly expressed by epithelial cells and plasma cells, although it is also expressed by other cell types (e.g. endothelial cells, macrophages, fibroblasts) to a lesser degree (11–15). Several inflammatory mediators activate syndecan-1 shedding in vitro (16–19), and shedding is also activated under certain pathological conditions in vivo (20–23), suggesting that syndecan-1 shedding is one of the general host responses to tissue injury and inflammation (11, 14). We reported previously that *P. aeruginosa* (18) and *S. aureus* (17) specifically activate the ectodomain shedding of syndecan-1. *P. aeruginosa* stimulates syndecan-1 shedding through LasA, a virulence factor for
its lung infection, whereas \textit{S. aureus} augments shedding through $\alpha$- and $\beta$-toxins, two cytolytic toxins implicated in several staphylococcal infections. Interestingly, both \textit{P. aeruginosa} LasA and \textit{S. aureus} $\alpha$- and $\beta$-toxins do not directly cause syndecan-1 shedding. Instead, they activate a protein-tyrosine kinase (PTK)$^2$-dependent intracellular signaling mechanism that stimulates endogenous syndecan-1 shedding at the cell surface (14, 17).

There are several indications that syndecan-1 shedding enhances bacterial virulence. Syndecan-1 shedding occurs \textit{in vivo} when mice are infected with \textit{P. aeruginosa} (20, 22), and inhibition of syndecan-1 shedding with peptide hydroxamates reduces the virulence of \textit{P. aeruginosa} in lung infections in mice (22). Further, syndecan-1 null mice that are incapable of shedding their syndecan-1 ectodomains markedly resist \textit{P. aeruginosa} and \textit{S. aureus} lung infection relative to wild type mice (20, 22). How syndecan-1 shedding promotes bacterial pathogenesis is not completely understood, but syndecan-1 ectodomains bind to and inhibit various host defense factors, such as antimicrobial peptides, in a heparan sulfate (HS)-dependent manner (22). Moreover, because soluble HS can inhibit several cytokines (23–26), syndecan-1 ectodomains might similarly inhibit cytokines through their HS moiety. These observations suggest that activation of syndecan-1 shedding is a broadly used pathogenic strategy to enhance microbial virulence.

In this study, we investigated whether \textit{Streptococcus pneumoniae} enhances syndecan-1 shedding. \textit{S. pneumoniae}, a Gram-positive bacterium, is a major human pathogen that can cause both invasive and noninvasive infections, such as pneumonia, meningitis, sepsis, and otitis media (27, 28). Our results show that \textit{S. pneumoniae} can activate syndecan-1 shedding in a metalloproteinase-dependent but intracellular signaling-independent manner. Consistent with this activity, we also show that (i) biochemically purified fractions containing the \textit{S. pneumoniae} shedding activity are composed of zinc metalloproteinase B (ZmpB) and ZmpC, (ii) purified \textit{S. pneumoniae} zinc metalloproteinases directly shed syndecan-1 ectodomains, and (iii) \textit{S. pneumoniae} metalloproteinasises cleave syndecan-1 ectodomains at a site distinct from that cleaved by endogenous sheddases. Moreover, the deletion of \textit{zmpC} was found to abrogate syndecan-1 shedding activity, whereas deletion of \textit{zmpB} had no effect. These data indicate that \textit{S. pneumoniae} directly sheds syndecan-1 ectodomains through ZmpC in a manner distinct from previously described mechanisms of syndecan-1 shedding.

**EXPERIMENTAL PROCEDURES**

\textit{Materials}—Todd-Hewitt broth was purchased from Difco, and 5% sheep blood agar plates were from Remel (Lenexa, KS). Tissue culture medium and supplements were purchased from Mediatech (Herndon, VA). GM6001 and TAPI-1 were from Calbiochem. HiTrap DEAE FF and HiPrep 16/60 Sephacryl S-300 resins, prepacked PD-10 columns, and ECL Western blotting detection reagents were from Amersham Biosciences. Molecular weight cut-off spin tubes were from Pall Life Science (Northborough, MA). IODOGEN, Sulfolink coupling resin, and protein A- and protein G-agarose beads were purchased from Pierce. The cationic nylon membrane, Immobilon Ny$^+$, was from Millipore (Bedford, MA), and the ProBlot polyvinylidene difluoride membrane was from Applied Biosystems (Foster City, CA). Heparinase III and chondroitinase ABC were from Seikagaku (Cape Cod, MA). Normal murine mammary gland (NMuMG) epithelial cells were from our culture collection and cultured as described previously (17, 18). All other materials were purchased from VWR, Fisher, or Sigma.

\textit{Immunochromatography}—The rabbit monoclonal anti-mouse syndecan-1 ectodomain (281-2) and anti-mouse syndecan-4 ectodomain (Ky8.2) antibodies were purified from the conditioned medium of hybridoma cultures by protein G-agarose affinity chromatography. The rabbit anti-ZmpB antibody was generated by immunizing rabbits with the synthetic peptide C$^{1604}$KTLKTREDINRYM$^{1617}$K. A Cys residue was added to the N terminus of the ZmpB sequence for coupling to the Sulfolink resin. Affinity-purified anti-ZmpB antibodies were generated by consecutive protein A- and syndecan-4 peptide affinity chromatography. Horseradish peroxidase-conjugated donkey anti-rat and horseradish peroxidase-conjugated goat anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

\textit{Bacterial Strains and Growth Conditions}—\textit{S. pneumoniae} strains used in this study are listed in Table 1. Strains were grown on 5% sheep blood agar plates or in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY broth) at 37 °C without agitation. In addition to strains constructed specifically for this study, \textit{S. pneumoniae} mutant strains lacking pneumolysin (Ply$^-$), autolysin (Lyt$^-$), hyaluronidase (Hyl$^-$), neuraminidase (NanA$^-$), sortases (SrtB/CD$^-$), serine protease (SP0664- and SP2239-deficient), and adhesin PspC (PspC$^-$) were all screened after growth in THY broth supplemented with 0.2 $\mu$g/ml erythromycin (29, 30).

\textit{Syndecan Shedding Assays}—The cell culture-based syndecan shedding assay was performed as described previously (17, 18). Briefly, confluent NMuMG cells in 96-well plates were incubated with test samples diluted in the NMuMG culture medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 10 $\mu$g/ml insulin) for the indicated time periods at 37 °C. The conditioned medium was collected and acidified by the addition of NaOAc (pH 4.5), NaCl, and Tween 20 to final concentrations of 50 mm, 150 mm, and 0.1% (v/v), respectively. Various volumes of acidified samples were dot-blotted onto the Immobilon Ny$^+$ membrane, probed with 281-2 or Ky8.2 anti-syndecan-1 or -4 antibodies, and developed by ECL. Blots were scanned, and the intensity of dots was quantified by NIH Image software.

\textit{Purification of the S. pneumoniae Syndecan-1 Shedding Enhancer}—The TIGR4 strain was grown in 4 liters of THY broth for 8 h at 37 °C to late log growth phase, and the culture supernatant was subjected to 40% ammonium sulfate precipitation. The precipitate was resuspended in 50 ml of autoclaved deionized H$_2$O$_2$, dialyzed three times against 4 liters of autoclaved deionized H$_2$O, and lyophilized. The lyophilized

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\^{2}The abbreviations used are: PTK, protein-tyrosine kinase; HS, heparan sulfate; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; NMuMG, normal murine mammary gland; TAPI, tumor necrosis factor-$\alpha$ protease inhibitor.
sample was resuspended in 10 ml of DEAE binding buffer (20 mM Tris, pH 7.4, 20 mM NaCl) and applied to a HiTrap DEAE FF 5-ml column pre-equilibrated with DEAE binding buffer. The applied sample was eluted with a 0.1–1.0 M NaCl gradient, and fractions were dialyzed against autoclaved deionized H₂O and then tested for their capacity to shed syndecan-1 ectodomains by the cell culture-based shedding assay. Active fractions that eluted at ~0.3 M NaCl were pooled, lyophilized, resuspended in 2 ml of TBS (50 mM Tris, pH 7.4, 150 mM NaCl), and fractionated by Sephacryl S-300 size exclusion chromatography. TBS was applied to the column at a flow rate of 0.4 ml/min, and 10-min fractions (4 ml) were collected. Aliquots (200 μl) of each fraction were Speed Vac-dried and resuspended in NMuMG culture medium, filter-sterilized, and tested for their syndecan-1 shedding activity. This purification scheme typically yielded 0.8–1 mg of the purified 170-kDa syndecan-1 shedding enhancer from 4 liters of bacterial culture supernatant.

Radioiodination and Autoradiography — The purified 170-kDa syndecan-1 shedding-enhancing protein (10 μg) was incubated with 20 μg of immobilized IODOGEN and 500 μCi of Na¹²⁵I in 50 μl of TBS for 10 min at room temperature. Free Na¹²⁵I was separated from iodinated protein by PD-10 chromatography. To estimate the purity of the 170-kDa fraction, 3 ng of the radioiodinated sample was separated by 12% SDS-PAGE. The gel was vacuum-dried and exposed to Eastman Kodak Co. BioMax film with an intensifying screen at −80 °C. The film was scanned, and the intensity of radioactive protein bands was quantified using the NIH Image software.

Mass Spectrometry and N-terminal Microsequencing — The purified 170-kDa protein fraction containing the syndecan-1 shedding activity was subjected to in-gel trypsin digestion after reduction with dithiothreitol and alkylation with iodoacetamide. Tryptic digests were analyzed by MALDI-TOF mass spectrometry (MS) (ABI 4700 MALDI-TOF system) at the Baylor College of Medicine Protein Chemistry Core Laboratory (Houston, TX). MALDI-TOF MS peptide fingerprints were searched against public domain databases using MS-FIT software. One of the peptides with an m/z value of 1128.6 was further analyzed by MS/MS (ABI 4000 Q-TRAP LC MS/MS system). For N-terminal sequencing, the 170-kDa protein was transferred to a polyvinylidene difluoride membrane, excised, and analyzed by the ABI 477A peptide sequencer. Alternatively, the 170-kDa protein was digested with trypsin, fractionated by SDS-PAGE, and transferred to polyvinylidene difluoride, and the 50- and 30-kDa tryptic fragments were excised from the membrane and loaded onto the ABI 477A peptide sequencer. The deduced N-terminal sequence of the 50-kDa tryptic digest was searched by BLAST.

Generation of Zinc Metalloproteinase Mutant Strains — Mutant strains devoid of the three zinc metalloproteinases were generated in the TIGR4 strain (Table 1). The strains were made by PCR ligation mutagenesis, creating gene replacements.

### Table 1

**S. pneumoniae strains and primers used**

| Strain/Primer | Description or sequence* | Resistance/RE | Source/Reference |
|--------------|--------------------------|---------------|-----------------|
| TIGR4        | Wild type, capsule type 4 | None          | Ref. 35         |
| MA-M11       | Wild type, capsule type 14| XbaI          | Upstream flank-iga |
| MB-M41       | Wild type, capsule type 23F| BamHI         | Downstream flank-iga |
| MJ-V-012     | Wild type, capsule type 35| None          | Upstream flank-zmpB |
| MK-V-142     | Wild type, capsule type 22| XbaI          | Downstream flank-zmpC |
| L82016       | Wild type, capsule type 6B| BamHI         | Downstream flank-iga |
| EF3030       | Wild type, capsule type 19F| None          | Upstream flank-zmpC |
| D39          | Wild type, capsule type 2 | None          | Downstream flank-iga |
| WU2          | Wild type, capsule type 3 | XbaI          | Upstream flank-zmpB |
| Δiga         | TIGR4 Δiga:Janus, lacking IgA1 protease| Kan*           | This study       |
| ΔzmpB        | TIGR4 ΔzmpB:Janus, lacking ZmpB| Kan*           | This study       |
| ΔzmpC        | TIGR4 ΔzmpC:Janus, lacking ZmpC| Kan*           | This study       |
| TIGR4 strBCD  | strBCD KO mutant in strain TIGR4| Erm*          | Refs. 44 and 45 |
| PLN-A        | pLy KO mutant in strain D39| Erm*           | Refs. 43 and 45 |
| AL-2         | LyA KO mutant in strain D39| Erm*           | Refs. 44 and 45 |
| L82016 nanA  | nanA KO mutant in strain L82016| Erm*          | Ref. 43         |
| L82016 lyl   | lyl KO mutant in strain L82016| Erm*           | Ref. 43         |
| WU2 SP2339   | SP2339 KO mutant in strain WU2| Erm*           | Ref. 43         |
| WU2 SP0641   | SP0641 KO mutant in strain WU2| Erm*           | Ref. 43         |
| EF3030 SP0641| SP0641 KO mutant in strain EF3030| Erm*          | Ref. 43         |
| EF3030 pspC   | pspC KO mutant in strain EF3030| Erm*           | Ref. 43         |

* Primers were based on complete genome sequence of *S. pneumoniae* TIGR4 (35). Lowercase letters represent mismatches used to incorporate restriction enzyme sites.

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**Syndecan-1 Shedding by Pneumococcus**

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**TABLE 1**

*S. pneumoniae* strains and primers used

| Strain/Primer | Description or sequence* | Resistance/RE | Source/Reference |
|--------------|--------------------------|---------------|-----------------|
| TIGR4        | Wild type, capsule type 4 | None          | Ref. 35         |
| MA-M11       | Wild type, capsule type 14| XbaI          | Upstream flank-iga |
| MB-M41       | Wild type, capsule type 23F| BamHI         | Downstream flank-iga |
| MJ-V-012     | Wild type, capsule type 35| None          | Upstream flank-zmpB |
| MK-V-142     | Wild type, capsule type 22| XbaI          | Downstream flank-zmpC |
| L82016       | Wild type, capsule type 6B| BamHI         | Downstream flank-iga |
| EF3030       | Wild type, capsule type 19F| None          | Upstream flank-zmpC |
| D39          | Wild type, capsule type 2 | None          | Downstream flank-iga |
| WU2          | Wild type, capsule type 3 | XbaI          | Upstream flank-zmpB |
| Δiga         | TIGR4 Δiga:Janus, lacking IgA1 protease| Kan*           | This study       |
| ΔzmpB        | TIGR4 ΔzmpB:Janus, lacking ZmpB| Kan*           | This study       |
| ΔzmpC        | TIGR4 ΔzmpC:Janus, lacking ZmpC| Kan*           | This study       |
| TIGR4 strBCD  | strBCD KO mutant in strain TIGR4| Erm*          | Refs. 44 and 45 |
| PLN-A        | pLy KO mutant in strain D39| Erm*           | Refs. 43 and 45 |
| AL-2         | LyA KO mutant in strain D39| Erm*           | Refs. 44 and 45 |
| L82016 nanA  | nanA KO mutant in strain L82016| Erm*          | Ref. 43         |
| L82016 lyl   | lyl KO mutant in strain L82016| Erm*           | Ref. 43         |
| WU2 SP2339   | SP2339 KO mutant in strain WU2| Erm*           | Ref. 43         |
| WU2 SP0641   | SP0641 KO mutant in strain WU2| Erm*           | Ref. 43         |
| EF3030 SP0641| SP0641 KO mutant in strain EF3030| Erm*          | Ref. 43         |
| EF3030 pspC   | pspC KO mutant in strain EF3030| Erm*           | Ref. 43         |

* Primers were based on complete genome sequence of *S. pneumoniae* TIGR4 (35). Lowercase letters represent mismatches used to incorporate restriction enzyme sites.
of the metalloproteinase genes with the Janus cassette containing the selectable marker aphIII, encoding kanamycin resistance (31, 32). Primers were designed to amplify the 5'-' and 3'-flanking regions of target genes zmpC, zmpB, and iga with genomic DNA from strain TIGR4 as template. Flanking amplicons were 500–1200 bp in length. Primers AEB-35F and AEB-31R were also used to amplify the 2903-bp Janus cassette. Restriction sites were integrated into the two primers nearest each target gene and into the Janus primers to allow for directional ligation as described (31). The expected replacement of each target gene with the aphIII gene by reciprocal recombination was confirmed in kanamycin-resistant transformants by PCR.

Statistical Analysis—Data are expressed as mean ± S.D. Statistical analyses were performed using STATVIEW 4.51 software (Abacus Concepts, Berkeley, CA). Differences between experimental groups and respective controls were examined by Student’s t test. p values of <0.05 were deemed statistically significant.

RESULTS

S. pneumoniae Activates Syndecan-1 Shedding—To determine whether S. pneumoniae strains possess the capacity to activate syndecan-1 shedding, nine S. pneumoniae strains with different capsular serotypes were examined for their shedding activity. Various concentrations of 8-h culture supernatants collected from S. pneumoniae strains were incubated with confluent NMuMG epithelial cells for 5 h at 37 °C. Incubation with fresh NMuMG culture medium served as the background control. Syndecan-1 and -4 shedding was assessed by measuring the concentration of shed ectodomains in the conditioned medium by a dot immunoblot assay. Culture supernatants from all nine S. pneumoniae strains enhanced syndecan-1 shedding 4–10-fold over control (Fig. 1) in a concentration- and time-dependent manner (data not shown). Interestingly, S. pneumoniae did not stimulate syndecan-4 shedding, suggesting that the activation of ectodomain shedding is specific to syndecan-1. These data indicate that several S. pneumoniae strains possess the capacity to specifically activate syndecan-1 shedding.

S. pneumoniae-activated Syndecan-1 Shedding Is Specifically Inhibited by Peptide Hydroxamate Inhibitors of Zinc Metalloproteinases—To define how S. pneumoniae stimulates syndecan-1 shedding, we first examined the effects of several protease and signaling inhibitors on shedding stimulated by the TIGR4 strain that exhibited the highest shedding activity. Activation of syndecan-1 shedding by chemical agonists, such as phorbol 12-myristate 13-acetate, requires protein kinase C, PTK, extracellular signal-regulated kinase mitogen-activated protein kinase, and metalloproteinase activities (16, 19). Similarly, P. aeruginosa (18) and S. aureus (17) activate syndecan-1 shedding by stimulating a host cell mechanism dependent on PTK and metalloproteinase activities. Thus, syndecan-1 shedding enhanced by chemical agonists, P. aeruginosa, or S. aureus is inhibited by PTK and metalloproteinase inhibitors, such as tyrphostin 25 and peptide hydroxamates, respectively (16–19).

We screened the effects of various metalloproteinase (GM6001, TAPI-1), PTK (tyrphostin, herbimycin, PP2, AG490, piceatanol), protein kinase C (bisindolylmaleimide (BIM); 0.1 and 1 μM), extracellular signal-regulated kinase mitogen-activated protein kinase (PD98059; 1 and 10 μM), p38 mitogen-activated protein kinase (SB203580; 1 and 10 μM), PTK (tyrphostin 25; 3 and 30 μM), Src PTK (herbimycin and PP2; 1 and 10 μM), JAK2 PTK (AG 490; 0.5 and 5 μM), or Syk PTK (piceatanol; 0.5 and 5 μM). Results are shown as mean percentage of shed syndecan-1 of control ± S.D. of triplicate measurements, where control is the level of syndecan-1 ectodomains in the conditioned medium of cells incubated with 20% (v/v) of TIGR4 culture supernatant alone.

We confirmed that S. pneumoniae-activated syndecan-1 shedding is specifically inhibited by peptide hydroxamate inhibitors of zinc metalloproteinases. Fresh NMuMG culture medium (media) or 8-h bacterial culture supernatants diluted to 20% (v/v) with culture medium was incubated with confluent NMuMG cells in 96-well plates for 5 h at 37 °C. Conditioned media were collected, centrifuged to remove cells, acidified, and dot-blotted onto Immobilon Ny membranes. Shed syndecans were quantified by dot immunoblotting using the rat monoclonal 281-2 anti-mouse syndecan-1 ectodomain or Kyb.2 anti-mouse syndecan-4 ectodomain antibodies. The concentration of shed syndecan ectodomains was determined using syndecan-1 or -4 ectodomains purified from NMuMG cells as standards. Results represent mean ± S.D. of triplicate measurements.
Syndecan-1 Shedding by Pneumococcus

FIGURE 3. *S. pneumoniae* enhances syndecan-1 shedding through a peptide hydroxamate-sensitive 170-kDa protein. Supernatants from an 8-h culture of *S. pneumoniae* TIGR4 were subjected to consecutive 40% ammonium sulfate precipitation, DEAE ion exchange chromatography, and Sephacryl S-300 size exclusion chromatography, and the syndecan-1 shedding activity was identified after each purification step by the cell culture-based assay as described under "Experimental Procedures." A, results from the final size exclusion chromatography step are shown as mean -fold increase over control of duplicate measurements, where control is the level of shed syndecan-1 in cells incubated with fresh NMuMG culture medium. B, fractions with shedding activity (4–6) and inactive fractions in the vicinity (1–3, 7–9) were analyzed by 12% SDS-PAGE and Coomassie staining. The purity of fraction 5 was assessed by radioiodination and autoradiography. C, NMuMG cells were incubated with 2 μg/ml fraction 5 in the absence or presence of GM6001 or TAPI-1, and syndecan-1 ectodomains in the conditioned medium were quantified. Data points represent mean -fold increase over control ± S.D. (n = 3).

Thus, we adopted a biochemical purification approach where we monitored the shedding activity of fractions obtained from TIGR4 supernatants subjected to consecutive 40% ammonium sulfate precipitation, DEAE ion exchange chromatography, and Sephacryl S-300 size exclusion chromatography. The shedding activity was ultimately isolated into three size exclusion chromatography fractions (Fig. 3A), which primarily contained a major 170-kDa protein band that was absent from neighboring inactive fractions (Fig. 3B). The purity of the 170-kDa protein band was determined to be ~95% by radioiodination and subsequent autoradiography of the active fraction (Fig. 3B). Further, similar to *S. pneumoniae* culture supernatants, the purified 170-kDa protein fraction activated syndecan-1 shedding in a dose-dependent (Fig. 3C), syndecan-1-specific (Fig. 3C), and peptide hydroxamate-sensitive (Fig. 3D) manner. These data suggest that the 170-kDa protein fraction contains a peptide hydroxamate-sensitive *S. pneumoniae* factor that specifically activates syndecan-1 shedding.

To determine the identity of the 170-kDa protein, we first performed N-terminal microsequencing and found that the N terminus was blocked. Thus, we subjected the 170-kDa protein to MALDI-TOF MS analysis. The MS-FIT search showed that 51/87 and 20/87 of the generated peptides matched *S. pneumoniae* ZmpB and ZmpC, respectively (Fig. 4A). ZmpB, ZmpC, and IgA1 protease are the three zinc metalloproteinases expressed by *S. pneumoniae* TIGR4 (33–35). To verify the MALDI-TOF MS results, we digested the 170-kDa protein with trypsin and subjected the 50- and 30-kDa tryptic fragments to N-terminal microsequencing. The N-terminal sequence of the 50-kDa tryptic fragment was LVELSEKPIILT, corresponding to amino acid residues 519–529 of ZmpB (Fig. 4B). The N terminus of the 30-kDa tryptic fragment was blocked. To confirm the presence of ZmpC, we subjected the peptide with a molecular mass of 1128.6 from the MALDI-TOF MS to MS/MS sequencing and found that the deduced sequence, LHTYNPVER, completely matches amino acid residues 1560–1568 of ZmpC (Fig. 4C). These data indicate that *S. pneumoniae* enhances syndecan-1 shedding through a 170-kDa zinc metalloproteinase fraction that contains both ZmpB and ZmpC.

*S. pneumoniae* Metalloproteinases Directly Cleave Syndecan-1 Ectodomains—To understand how *S. pneumoniae* zinc metalloproteinases activate syndecan-1 shedding, we compared the structure of syndecan-1 ectodomains generated by *S. pneumoniae* activation and those generated by endogenous constitutive shedding. Syndecan-1 ectodomains shed by *S.
syndecan-1 core proteins between 45 and 56 kDa (Fig. 5D). More important, the purified 170-kDa protein was digested with trypsin, and a 50-kDa tryptic fragment was subjected to N-terminal sequencing. A BLAST search of the deduced sequence revealed that it corresponds to amino acid residues 159–329 of ZmpB, C, the 1128.6 (m/z) peptide from MALDI-TOF MS was analyzed by MS/MS sequencing. The deduced sequence completely matched amino acid residues 1560–1568 of ZmpC.

**FIGURE 4.** The *S. pneumoniae* 170-kDa protein with syndecan-1 shedding activity is composed of ZmpB and ZmpC. A, the purified 170-kDa protein was subjected to in-gel reduction, alkylation, and trypsin digestion, and resulting peptides were analyzed by MALDI-TOF MS. MS-FIT analysis determined that 51 of 87 and 20 of 87 of the generated peptides correspond to *S. pneumoniae* ZmpB and ZmpC, respectively. B, the 170-kDa protein was digested with trypsin, and a 50-kDa tryptic fragment was subjected to N-terminal sequencing. A BLAST search of the deduced sequence revealed that it corresponds to amino acid residues 159–329 of ZmpB, C, the 1128.6 (m/z) peptide from MALDI-TOF MS was analyzed by MS/MS sequencing. The deduced sequence completely matched amino acid residues 1560–1568 of ZmpC.

**TABLE 2.**

| Mass (m/z) | MS/MS analysis |
|-----------|----------------|
| 1128.6    | LHTYNPVER      |
| ZmpC      | 1560 LHTYNPVER 1568 |

**DISCUSSION**

Our present study defines how *S. pneumoniae* stimulates syndecan-1 ectodomain shedding. Several independent criteria not further separate these metalloproteinases by additional biochemical fractionation approaches (not shown), most likely due to the similar size, composition of hydrophobic residues (30% versus 31%), and pI (5.1 versus 5.4) of these two metalloproteinases. Thus, to determine whether both ZmpB and ZmpC shed syndecan-1 ectodomains under biological conditions, we generated mutant *S. pneumoniae* strains lacking genes for zmpB, zmpC, or igA1 protease in the TIGR4 background and tested their capacity to activate syndecan-1 shedding. Consistent with previous studies showing that these zinc metalloproteinases are not essential for *in vitro* growth (34, 36), we found that the growth rate was similar among the three mutant strains (not shown). Varying concentrations of culture supernatants obtained from the igA1 protease, zmpB, or zmpC mutant strains were incubated with confluent NMuMG epithelial cells, and the concentration of syndecan-1 ectodomains in the conditioned medium was measured. Deletion of IgA1 or ZmpB did not affect the ability to activate syndecan-1 shedding because igA1 and zmpB mutant strains retained their capacity to significantly shed syndecan-1 ectodomains in a dose-dependent manner (Fig. 6A). However, deletion of ZmpC abrogated the capacity of this mutant strain to shed syndecan-1 ectodomains at all concentrations tested (Fig. 6A).

Next, we isolated the 170-kDa protein fraction from culture supernatants of zmpB and zmpC mutant strains by ammonium sulfate precipitation, DEAE ion exchange chromatography, and Sephacryl S-300 size exclusion chromatography and verified the presence of ZmpB in the zmpC mutant strain by Western immunoblotting with an affinity-purified rabbit anti-ZmpB antibody (Fig. 6B). Because an antibody against ZmpC was not available, the presence of ZmpC in the zmpB mutant strain was verified by MALDI-TOF MS analysis, in which we found that 35 of 43 peptide sequences matched those of ZmpC, indicating that the 170-kDa protein purified from the zmpB mutant is ZmpC. We then tested the capacity of the 170-kDa protein isolated from the parent TIGR4, zmpB mutant, or zmpC mutant strain and found that the 170-kDa ZmpC purified from the zmpB mutant strain activates syndecan-1 shedding, but the 170-kDa ZmpB purified from the zmpC mutant strain does not (Fig. 6C). Collectively, these results indicate that ZmpC is the dominant syndecan-1 sheddase of *S. pneumoniae* TIGR4.
Syndecan-1 Shedding by Pneumococcus

Figure 5. *S. pneumoniae* metalloproteinases directly cleave syndecan-1 ectodomains. A, NMuMG cells were incubated with or without 20% (v/v) of TIGR4 culture supernatant for 5 h at 37 °C, and the conditioned medium was collected. Syndecan-1 ectodomains were partially purified by DEAE chromatography, and samples containing ~75-ng ectodomains were analyzed by 7% SDS-PAGE, Western blotting onto an Immobilon Ny membrane, and immunoblotting using the 281-2 anti-syndecan-1 ectodomain antibody. Lane 1, samples obtained from constitutively shed syndecan-1 ectodomains; lane 2, samples from TIGR4 supernatant-shed syndecan-1 ectodomains. B, partially purified constitutively shed (lane 1) and TIGR4 supernatant-shed (lane 2) syndecan-1 ectodomains (100 ng) were digested with heparinase III and chondroitinase ABC and analyzed by 7% SDS-PAGE, Western blotting onto a nitrocellulose membrane, and immunoblotting with the 281-2 antibody. C, purified syndecan-1 ectodomains (0.1 μg) were incubated with 0, 0.1, 0.5, or 1 μg of the purified 170-kDa protein fraction for 5 h at 37 °C and analyzed by SDS-PAGE and Western immunoblotting as described in A. D, purified syndecan-1 ectodomains (0.1 μg) were incubated with or without 1.6 μg of the purified 170-kDa protein. The resulting mixtures were digested with heparinase III and chondroitinase ABC and analyzed by SDS-PAGE and Western immunoblotting as described in B.

Figure 6. ZmpC is the dominant syndecan-1 shedding enhancer of *S. pneumoniae*. A, culture supernatants from *S. pneumoniae* TIGR4 mutant strains devoid of IgA1 protease (IgA1<sup>-</sup>), ZmpB (ZmpB<sup>-</sup>), or ZmpC (ZmpC<sup>-</sup>) were collected, and varying concentrations (5–20%) were incubated with NMuMG cells in 96-well plates for 5 h at 37 °C. Conditioned media were collected, and levels of syndecan-1 ectodomains were quantified. Results are shown as mean-fold increase over control ± S.D. (n = 6), where control is the level of syndecan-1 ectodomains in the conditioned medium of cells incubated with fresh NMuMG culture medium. The inset shows the actual dot immunoblot from cells incubated with 10% bacterial culture supernatants. B, culture supernatants from the parent TIGR4 (T), ZmpB<sup>-</sup> mutant (B), or ZmpC<sup>-</sup> mutant (C) mutant strain were subjected to 40% ammonium sulfate precipitation, DEAE ion exchange chromatography, and Sephacryl S-300 size exclusion chromatography to isolate the 170-kDa protein fraction. SDS-PAGE and Coomassie staining show that a 170-kDa protein is isolated from all three strains (Zmp<sup>C</sup> mutant strain <i>arrowhead</i>). C, the 170-kDa protein (4 μg/ml) isolated from the parent TIGR4 (T), ZmpB<sup>-</sup> mutant (B), or ZmpC<sup>-</sup> mutant (C) mutant strain was incubated with confluent NMuMG cells, and the extent of syndecan-1 shedding was assessed by dot immunoblotting. Results are shown as mean-fold increase over control ± S.D. (n = 3).

indicate that *S. pneumoniae* activates syndecan-1 shedding through a mechanism distinct from those that have been described previously. First, *S. pneumoniae*-activated syndecan-1 shedding was inhibited by peptide hydroxamate inhibitors of metalloproteinases but not by signaling inhibitors that have been shown to inhibit syndecan-1 shedding stimulated by chemical agonists, *P. aeruginosa*, or *S. aureus*. These results indicate that unlike *P. aeruginosa* and *S. aureus*, *S. pneumoniae* does not require stimulation of intracellular signaling pathways to enhance shedding. Second, the size of syndecan-1 ectodomains shed by *S. pneumoniae* was smaller than ectodomains shed by the endogenous mechanism, suggesting that *S. pneumoniae* sheds ectodomains by cleaving a different site in the syndecan-1 core protein. Third, a 170-kDa protein fraction with shedding activity was purified and determined to contain ZmpB and ZmpC. The purified 170-kDa zinc metalloproteinase fraction activated syndecan-1 shedding in a peptide hydroxamate-sensitive manner and directly cleaved purified syndecan-1 ectodomains. Further, an *S. pneumoniae* mutant strain deficient in zmpC, but not zmpB or IgA1 protease, lost its capacity to shed syndecan-1 ectodomains, indicating that ZmpC is the dominant *S. pneumoniae* syndecan-1 sheddase. These data indicate that, unlike other agonists that activate syndecan-1 shedding through stimulation of the endogenous shedding machinery, *S. pneumoniae* directly sheds syndecan-1 ectodomains through ZmpC.

The zmpC gene in the TIGR4 strain is 5.57 kb and encodes for a full-length ZmpC protein of 1856 amino acids with a molecular mass of 207 kDa. Similarly, the 5.65-kb zmpB gene encodes for a full-length ZmpB protein of 1881 amino acids with a molecular mass of 210 kDa. All three zinc metalloproteinases contain the highly conserved LPXTG motif of Gram-
positive cell surface proteins, and prototypical signal sequence and propeptide motifs near the N terminus of the protein. For IgA1 protease, processing at the N terminus releases 155 amino acids (37). Similar signal and sorting sequences for ZmpB and ZmpC are predicted to be cleaved by signal peptidase and sortase to release mature ZmpB and ZmpC proteins of ~191–200 kDa. However, the size of the purified ZmpB and ZmpC was estimated to be ~170 kDa in our study. The cause of this discrepancy is not known. Western immunoblotting of the unfractonated TIGR4 supernatant with the anti-ZmpB antibody also detected a 170-kDa ZmpB protein (not shown), indicating that protein degradation during purification was not the cause of the size discrepancy. Under laboratory growth conditions, ZmpB and ZmpC are found on the cell surface and readily detected in the culture supernatant in our study and others (34, 36). Thus, it is plausible that additional processing during their release from the cell surface reduces the size of ZmpB and ZmpC to 170 kDa. Alternatively, because both ZmpB and ZmpC are acidic proteins, SDS may not have uniformly coated these proteins, causing their aberrant migration in SDS-PAGE. The observation that both ZmpB and ZmpC eluted in very early size exclusion chromatography fractions, estimated to contain 200–250-kDa proteins, lends further support to the possibility that SDS-PAGE analysis underestimated the size of these zinc metalloproteinases.

Our data indicate that S. pneumoniae TIGR4 directly sheds syndecan-1 ectodomains through ZmpC. However, because not all strains express ZmpC (34, 38), it remains possible that strains other than TIGR4 may shed syndecan-1 by an alternative mechanism. How ZmpC functions as a specific syndecan-1 sheddase is not fully understood. Despite the fact that ZmpC, ZmpB, and IgA1 protease all contain the HEXXH zinc-binding catalytic motif (34, 36), our results show that ZmpC is the dominant syndecan-1 sheddase in the TIGR4 strain. Further, the expression of ZmpB and IgA1 protease was not altered in the zmpC mutant strain that lost its shedding activity, indicating that ZmpB and IgA1 protease do not shed syndecan-1 ectodomains. Biological substrates of ZmpB have not been identified, but human IgA has been shown to be a substrate of ZmpC. ZmpC has been shown to cleave human IgA (34), showing that ZmpC hydrolyzes elastin in addition to syndecan-1. Thus, ZmpC may be the dominant syndecan-1 sheddase because it shows higher proteolytic activities against host extracellular matrix components in general.

Alternatively, ZmpC-specific motifs may enable this metalloproteinase to function specifically as a syndecan-1 sheddase. In contrast to the majority of S. pneumoniae gene products that show high homology among different strains, S. pneumoniae zinc metalloproteinases only share 26–37% identity at the protein level (34). In addition, both ZmpB and ZmpC do not cleave human IgA (34), showing that S. pneumoniae metalloproteinases are highly specific and that specificity is likely to be mediated by domains other than the common zinc-binding catalytic domain.

The physiological function of syndecan-1 shedding in S. pneumoniae pathogenesis has yet to be defined. Several studies showed that ZmpC is an important virulence factor in mouse models of S. pneumoniae pneumonia (34, 38, 42). Chiavolini et al. (38) found that relative to ZmpB or IgA protease, ZmpC has a lower, yet significant, influence on lethality in an intranasal infection model, but the role of these metalloproteinases in lung colonization was not assessed. Further, syndecan-1 shedding was found to promote P. aeruginosa pathogenesis in the lung and burned skin (20, 22). Collectively, these data suggest that syndecan-1 shedding by ZmpC is an important virulence activity of S. pneumoniae. In addition, because S. pneumoniae, S. aureus, and P. aeruginosa enhance syndecan-1 shedding through distinct molecular mechanisms, the activation of syndecan-1 shedding might be yet another example of an important virulence mechanism shared by several bacterial pathogens.

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Syndecan-1 Shedding by Pneumococcus

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