Staphylococcus aureus Protein A Activates TNFR1 Signaling through Conserved IgG Binding Domains*

Received for publication, March 1, 2006, and in revised form, April 13, 2006 Published, JBC Papers in Press, May 18, 2006, DOI 10.1074/jbc.M601956200

Marisa I. Gómez1, Magnhus O’Seaghdha9, Mariah Magargee3, Timothy J. Foster1, and Alice S. Prince1,2

From the 1Departments of Pediatrics and Pharmacology, College of Physicians and Surgeons, Columbia University, New York, New York 10032 and the 3Department of Microbiology, Moyne Institute of Preventive Medicine, Trinity College, Dublin 2, Ireland

Staphylococcus aureus continues to be a major cause of infection in normal as well as immunocompromised hosts, and the increasing prevalence of highly virulent community-acquired methicillin-resistant strains is a public health concern. A highly expressed surface component of S. aureus, protein A (SpA), contributes to its success as a pathogen by both activating inflammation and by interfering with immune clearance. SpA is known to bind to IgG Fc, which impedes phagocytosis. SpA is also a potent activator of tumor necrosis factor α (TNF-α) receptor 1 (TNFR1) signaling, inducing both chemokine expression and TNF-converting enzyme-dependent soluble TNFR1 (sTNFR1) shedding, which has anti-inflammatory consequences, particularly in the lung. Using a collection of glutathione S-transf erase fusions to the intact IgG binding region of SpA and to each of the individual binding domains, we found that the SpA IgG binding domains also mediate binding to human airway cells. TNFR1-dependent CXCL8 production could be elicited by any one of the individual SpA IgG binding domains as efficiently as by either the entire SpA or the intact IgG binding region. SpA induction of sTNFR1 shedding required the entire IgG binding region and tolerated fewer substitutions in residues known to interact with IgG. Each of the repeated domains of the IgG binding domain can affect multiple immune responses independently, activating inflammation through TNFR1 and thwarting opsonization by trapping IgG Fc domains, while the intact IgG binding region can limit further signaling through sTNFR1 shedding.

Staphylococcus aureus is a common human pathogen associated with diverse types of infection, ranging from trivial pustules to overwhelming toxic shock and death (1). Much of its success as a pathogen can be attributed to the expression of surface proteins that enable the organism to bind efficiently to many host tissues (2). However, in contrast to other potential pathogens, staphylococci also have evolved numerous mechanisms to evade the immune responses that they evoke (3). The initial colonization of host tissues is mediated by the many proteins that promote adherence to extracellular matrix materials such as fibrinogen (clumping factors A and B, ClfA and ClfB), fibronectin (fibronectin-binding proteins, FnBPs), collagen (collagen-binding protein, Cna), and elastin (elastin-binding protein, EbpS) (4–7). These are not simply inert adhesins but often activate host responses. For example, FnBPs stimulate internalization of bacteria into epithelial and endothelial cells via a fibronectin bridge to the α5β1 integrin (8, 9). Along with ClfA the FnBPs also activate platelets (10, 11), likely to be important in endovascular infections such as endocarditis. Although many staphylococcal surface components are proinflammatory, activating chemokine expression and evoking the influx of leukocytes and especially phagocytic cells, the organism also has mechanisms to avoid phagocytic clearance. Protein A (SpA) expression has an important anti-phagocytic effect through its interactions with the Fc component of immunoglobulin. Although the interactions between SpA and human immunoglobulin chains have long been recognized, there is only recently a growing appreciation for the central importance of SpA in the pathogenesis of S. aureus infection.

SpA is a 42-kDa protein covalently anchored in the staphylococcal cell wall through its carboxyl terminal end. The protein is comprised of five repeated domains (E, D, A, B, C) of ~58 residues each. The variable number of short 8-residue repeats (12, 13). The variability in the number and sequences of these repeated regions has been exploited as an epidemiological marker, although their significance is not established (14). Each SpA domain can bind with high affinity to the Fc region of immunoglobulin G and to the Fab region of immunoglobulin of the VH3 subclass (15–18). The interaction with IgG Fc hinders phagocytosis (3) because bacteria become coated with IgG in an inappropriate conformation not recognized by the Fc receptor on neutrophils. Moreover, SpA-bound IgG cannot stimulate complement fixation by the classical pathway. An additional consequence of the ability of SpA to bind to B lymphocytes displaying IgM bearing VH3 heavy chains is the induction of proliferation resulting in depletion of a significant part of the B cell repertoire (19, 20).

Both the SpA-Fc and SpA-Fab interactions have been analyzed at the molecular level with co-crystallized complexes (21–23). The SpA domains adopt three-helix bundles (23–25). One face comprising residues from helices I and II binds IgG Fc, whereas residues from helices II and III on the other face bind IgM. The residues from helix I that bind Fc are different from...
those that bind Fab, with the exception of a single glutamine (Gln-32 in SpA domain D) (21, 23).

SpA can also act directly as an immune effector itself through its ability to bind and activate tumor necrosis factor α (TNF-α)3 receptor 1 (TNFR1) (26). This interaction is particularly important at sites of infection where TNF-α signaling is critical, as in the lung. Protein A-TNFR1 interaction is essential for the pathogenesis of pneumonia as TNFR1 null mice are not susceptible to S. aureus pneumonia and SpA-defective mutants of S. aureus do not cause infection in wild-type animals. SpA activates proinflammatory signaling through binding to TNFR1 and activation of TRAF2, the p38/c-Jun NH2-terminal kinase MAPKs, and NF-κB (26). TNFR1 ectodomain shedding is induced by SpA (26), presumably by activating the TNF-converting enzyme (TACE or ADAM 17) through a signaling pathway not yet elucidated. As there is no apparent homology between the trimeric TNFR1 and IgG, both of which function as receptors for SpA, we were interested in defining the molecular basis for the SpA-TNFR1 interaction. In the experiments detailed in this report, we compared the binding of intact SpA and isolated regions of the protein expressed as glutathione S-transferase (GST) fusions to TNFR1 to determine whether the same domains activate TNFR1 signaling and shedding and to establish whether the known Ig G binding domains recognize multiple targets, including TNFR1.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions—**DNA cloning was performed in *Escherichia coli* XL1-Blue (Qiagen). *E. coli* BL21(DE3) (Invitrogen) was used for expression of recombinant proteins. *E. coli* was routinely grown in Luria Bertani broth at 37 °C with shaking at 200 rpm. Ampicillin (100 μg/ml) was incorporated into the medium where appropriate.

**DNA Constructions—**The full-length *spa*, the five- and single-domain repeats of *spa* encoding the extracellular immunoglobulin binding domains, and the fragment encoding the carboxyl-terminal region of SpA (Fig. 1) were cloned into plasmid pGEX-KG. Oligonucleotides were designed to allow amplification of each individual SpA repeated domain (Table 1). Cross-reaction of primers due to high sequence homology of the repeats was prevented by digestion of the *spa* template with unique restriction endonucleases prior to single-domain amplifications.

**Expression and Purification of Recombinant Proteins—**For expression of recombinant SpA truncates, pGEX-KG constructs were purified from *E. coli* XL1-Blue and transformed into *E. coli* BL21. Overnight cultures were inoculated into fresh medium and grown to an A600 of 0.5. Isopropyl β-D-thiogalactopyranoside was added to a concentration of 1 mM, and the culture was grown for a further 4 h. Cells were harvested by centrifugation at 7,000 rpm for 10 min in a Sorvall GS-3 rotor. The pellet was resuspended in phosphate-buffered saline (PBS) containing protease inhibitor (Roche Applied Science), lysozyme (200 μg/ml), and DNase I (3 μg/ml). Cells were lysed by repeated freeze/thaw cycles. Cell debris was removed by centrifugation. Recombinant proteins expressed from pGEX-KG contained an amino-terminal GST fusion of 26 kDa. The GST fusion proteins were purified using the MicroSpin GST purification module (Amersham Biosciences) and dialyzed against PBS. Recombinant five- and single-domain GST fusion proteins had approximate molecular masses of 59 and 32 kDa, respectively.

**Site-directed Mutagenesis—**Mutations were introduced into SpA domain D using a PCR-based mutagenesis strategy. Briefly, overlapping oligonucleotides carrying the desired mutation were combined with standard flanking primers to yield two overlapping mutant products. These were combined and amplified using the flanking primers alone to yield the mutant fusion product. In some cases, mutations were introduced using the QuikChange® method according to the manufacturer’s instructions (Stratagene). The following amino acid substitutions were constructed: F5A, F13A, Y14A, L17A, N21A, I31A, Q32A, and K35A. Oligonucleotides used to introduce mutations are listed in Table 2. Mutated domains were cloned directionally into pGEX-KG and expressed as described above. Each mutant protein was tested for binding to human IgG Fc domain and to human VH3 IgM. For each mutant that was defective in binding to IgG, normal levels of binding to IgM occurred (data not shown). This shows that the mutant proteins were likely to have taken up the proper conformation.

**Cell Lines—**1HAEo- (human airway epithelial) and 16HBE (human bronchoepithelial) cells (D. Gruenert, California Pacific Medical Center Research Institute, San Francisco, CA) were grown as previously detailed (27, 28). Primary airway epithelial cells isolated from human nasal polyps were grown on Transwell clear filters (Corning Costar) in M3 medium as previously described (29).

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3 The abbreviations used are: TNF-α, tumor necrosis factor α; TNFR1, TNF-α receptor 1; sTNFR1, soluble TNFR1; MAPK, mitogen-activated protein kinase; TACE, TNF-converting enzyme; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; PMN, polymorphonuclear; PBS, phosphate-buffered saline.
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*Protein A Binding to Epithelial Cells*—Cells were fixed with 1% paraformaldehyde for 10 min at 4°C and then incubated with increasing amounts of full-length SpA or the domain constructs for 1 h. Cells were washed three times and stained with anti-protein A monoclonal antibody (Sigma). Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) was used. An irrelevant isotype-matched antibody was used as a control, and the fluorescence intensity was subtracted from the fluorescence intensity obtained with the SpA-specific antibody. Cells were analyzed with a BD Biosciences FACSCalibur using CellQuest software. For the competition assays the cells were incubated with Alexa 488-labeled SpA and increasing amounts of unlabeled IgG binding domain (GST-E-C), full-length SpA (GST-SpA), or the carboxyl-terminal region of SpA (GST-X). Cells were washed, and fluorescence was determined by flow cytometry.

*Confocal Microscopy*—16HBE cells were grown on Transwell-Clear filters (Corning Costar) at an air-liquid interface to form polarized monolayers. Cells were fixed with 4% paraformaldehyde and incubated with SpA or the IgG binding domains (E-C) for 1 h. After washing, rabbit polyclonal anti-TNFRI antibody (Santa Cruz) and mouse monoclonal anti-protein A antibody were added for 1 h. Alexa Fluor 594-conjugated rabbit secondary antibody (Molecular Probes) and Alexa Fluor 488-conjugated mouse secondary antibody (Molecular Probes) were then added for 1 h. After washing, filters were removed from transwells and mounted with Vectashield (Vector Laboratories Inc.) onto glass slides.

*CXCL8 and sTNFR1 Detection*—1HAEo- cells, weaned from serum for 24 h, were exposed to the GST fusion proteins (2.5 μM) for 4 h. CXCL8 in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA) (BD Biosciences). Soluble TNFR1 (sTNFR1) in the supernatant was detected by using DuoSet ELISA for human sTNFR1 (R&D Systems).

*Mouse Studies*—C57BL/6 mice or C57BL/6-Tnfrsf1atm1Imx (TNFR1 null; Jackson Laboratories) (7–10 days old) were intranasally inoculated with 10 μl of *S. aureus* (10⁶ CFU), full-length SpA (GST-SpA, 50 μM), or PBS (control). For neutrophil detection in the lungs, cell suspensions were obtained from lung homogenates and red cells were lysed. Remaining cells were suspended in PBS containing 10% normal mouse serum and incubated for 30 min at 37°C. Cells were then double stained with phycoerythrin-labeled anti-CD45 and fluorescein isothiocyanate-labeled anti-Ly6G (BD Biosciences). Neutrophils were identified using the iScript synthesis kit (Bio-Rad). For quantitative real-time PCR, amplification was done in a Light Cycler using the DNA Master SYBR Green I kit (Roche Applied Science). Primers used for KC amplification were 5′-CCGCCCTATCGCACATGAGCTGCGCC-3′ and 5′-CTTGGGACACCTTTTAGCATCTTTTTGG-3′, and 35 cycles were run with denaturation at 95°C for 8 s, amplification at 56°C for 10 s, and extension at 72°C for 12 s. Actin was amplified on each individual sample and used as control for standardization. Primers used for actin amplification were 5′-GTGGGGGCGGCCAGGCACCA-3′ and 5′-CGGTGGGCGCTGTTGTCAGGGGGTCAAGGGGG-3′, and 35 cycles were run with denaturation at 95°C for 8 s, amplification at 63°C for 10 s, and extension at 72°C for 12 s. Soluble TNFR1 was assessed in bronchoalveolar lavage from adult mice intranasally inoculated with SpA domain D (GST-D, 50 μM), SpA domain D with Leu-17 replaced by alanine (GST-D, 50 μM), SpA domain D with Ile-31 replaced by alanine (GST-D, 50 μM), SpA domain D with Leu-17 replaced by alanine (GST-D, 50 μM), or PBS (control). Bronchoalveolar lavage was performed by instilling 1.5 ml of PBS into the lungs. The fluid recovered (~1 ml) was concentrated to 100 μl and stored at −80°C. Mice protocol number AAA1718 was approved by the Institutional Animal Care and Use Committee at Columbia University.

**RESULTS**

*The SpA IgG Binding Region Mediates the Biological Activity of SpA Binding to Human Airway Cells*—We postulated that multiple copies of the IgG binding region of SpA are highly conserved in clinical isolates of *S. aureus* because of their ability to recognize multiple eukaryotic receptors that are important in pathogenesis. To test this hypothesis, we compared the properties of intact SpA isolated as a GST fusion protein (GST-SpA), the complete IgG binding region (SpA GST-E-C), individual IgG binding domains (GST-E, GST-D, GST-A, GST-B, etc.), and the carboxyl-terminal X domain (Fig. 1). The intact SpA and the E-C domain displayed similar binding kinetics to 1HAEo- cells (Fig. 2A). Increasing amounts of the E-C fragment effectively competed with intact SpA for binding to airway cells (Fig. 2B). The interaction between the IgG binding region of SpA and TNFR1 on the surface of polarized airway cells was visualized by confocal imaging (Fig. 2C). Both the intact SpA and the E-C fragment co-localized with TNFR1 along the apical surface of the polarized airway cells. SpA-TNFR1 co-localization was confirmed using primary airway epithelial cells (Fig. 2D).
Each of the individual IgG binding domains was capable of binding to airway cells but with less affinity than the entire E-C domain, because 5–10-fold more protein was required to achieve saturation (Fig. 2). The binding of the isolated domains E and A was not saturable in contrast to that of the other IgG binding domains (D, B, and C).

SpA IgG Binding Domains Are Sufficient to Activate CXCL8 Production—In response to ligation of TNFR1 by SpA a proinflammatory signaling cascade is activated (26). To determine whether the IgG binding domains of SpA are sufficient to mediate this response, we incubated intact SpA, the complete IgG binding domain (GST-E-C), or each individual IgG binding domain (GST-A, GST-B, GST-C, etc.) with 1HAEo- cells or primary airway epithelial cells and monitored CXCL8 production. The entire E-C domain as well as each of the individual IgG binding domains was comparable with the intact SpA in activating CXCL8 expression. Neither the COOH-terminal X domain nor the GST tag had immunostimulatory activity.

SpA IgG Binding Domains Activate sTNFR1 Shedding—SpA ligation of TNFR1 initiates shedding of the soluble portion of TNFR1 from the surface of airway cells by activating the TNF-α-converting enzyme (TACE or ADAM 17) (26). We compared the ability of intact SpA, the IgG binding domains, and the carboxyl-terminal X region to induce sTNFR1 shedding (Fig. 3). The IgG binding region E-C was equivalent to the intact SpA in activating sTNFR1 shedding. As with CXCL8 induction, the COOH-terminal X domain was inactive. However, individual IgG domains were substantially less potent than the intact E-C fragment in stimulating sTNFR1 shedding (Fig. 3). The isolated A and E domains were less potent than the other individual IgG binding domains. TNFR1 shedding in response to the IgG binding domain D was confirmed in primary airway epithelial cells (Fig. 3). Thus, two consequences of SpA-TNFR1 binding are differentially activated by the same IgG binding region of SpA, with the TNFR1-CXCL8 pathway apparently less constrained by ligand-receptor affinities than the TNFR1-TACE-dependent cascade.

SpA Amino Acids That Interact with IgG Are Involved in TNFR1 Recognition—Having established that the SpA IgG binding domains are responsible for the TNFR1 recognition, we then tested whether the same amino acids that mediate SpA binding to the IgG Fcγ fragment also bind to TNFR1. A collection of domain D alanine substitution mutants, each with a
substitution affecting an amino acid previously demonstrated to be involved in IgG recognition (Fig. 4A), was tested for binding to human airway cells, for CXCL8 induction, and for the ability to stimulate sTNFR1 shedding (Fig. 4). A substitution in asparagine 21 (not involved in IgG binding) was used as control.

Alanine substitutions in phenylalanine 13 and tyrosine 14 almost completely abolished binding to airway epithelial cells (Fig. 4B) as well as CXCL8 production (Fig. 4C) and sTNFR1 shedding (Fig. 4D). A second group of mutants (phenylalanine 5, leucine 17, isoleucine 31, and lysine 35) resulted in decreased levels of binding when compared with unmodified domain D (Fig. 4B). Despite the decrease in binding, only the substitution in isoleucine 31 impaired CXCL8 production, whereas shedding of TNFR1 was completely abolished by all the substitutions (Fig. 4C). The replacement of leucine 17 by alanine did not affect CXCL8 induction as compared with the unmodified domain D but totally inhibited sTNFR1 shedding (Fig. 4D). These results indicate that the SpA residues that bind to TNFR1 form a cluster of mainly hydrophobic residues between helices I and II (Fig. 4E).

FIGURE 4. Interaction of SpA domain D with epithelial cells. A, alignment of the five domains of SpA with residues involved in Fcγ (gray) highlighted. Residues substituted by alanine are indicated (bold). B, epithelial cells were incubated with SpA domain D or the different domain D variants, and binding to the surface was quantified by flow cytometry. MFI, mean fluorescence intensity. One representative experiment of three is shown. C and D, CXCL8 (C) and sTNFR1 (D) were assayed by ELISA after exposure of 1HAEo- cells to PBS (control, C), the IgG binding domain D (D), or the variants of domain D (alanine substitutions). Data represent the mean and S.D. from sextuplicate wells. *, p <0.001 compared with CXCL8 or sTNFR1 induced by domain D, Student’s t Test. E, SpA domain D viewed from above (left) and from the side (right). Its three-helix bundle structure allows SpA to bind Fcγ through residues in helices I and II and VH3 Fab through helices II and III. Residues shown to be important in TNFR1 binding are highlighted in red. Structures are visualized using The PyMOL Molecular Graphics System (2002) (DeLano Scientific, San Carlos, CA).

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SpA is a pluripotent virulence factor capable of several important interactions with host components. In infection of the airways where serum components are lacking, *S. aureus* potently activates epithelial signaling through TNFR1 activation, induction of chemokine expression, and recruitment of neutrophils, a hallmark of staphylococcal infection (26). The SpA-TNFR1 interaction initially provokes a proinflammatory response eliciting CXCL8 expression immediately after binding. The resulting tissue damage at the site of infection and extravasation of PMNs and serum components provide IgG and complement to promote phagocytosis and clearance of bacteria. However, SpA-IgG interactions act to impede phagocytosis and foster bacterial persistence (3). In addition, SpA ligation of TNFR1 initiates TACE-dependent cleavage of the receptor and release of the soluble form of TNFR1 from the cell surface (26). The induction of TNFR1 shedding has anti-inflammatory consequences as it prevents further signaling through the membrane-bound portion of the receptor and neutralizes free TNF-α in the airway, limiting the potential for further TNF-dependent signaling.

By analyzing a series of amino acid substitutions in the SpA D domain, it became apparent that the residues important in the interaction between SpA D and the Fc region of IgG are also involved in binding to and activating TNFR1. SpA residues that are on the opposite face of the protein that are involved in IgM Fab binding are not involved in the interaction with TNFR1. As shown both in vitro and in vivo, the activation of sTNFR1 shedding is eliminated by the F5A and L17A substitutions, changes that have little effect on CXCL8 or KC activation. While phenylalanine 13, tyrosine 14, and isoleucine 31 are the most critical residues for CXCL8 production, the induction of sTNFR1 shedding appears to require either a larger ligand domain or perhaps a higher affinity interaction between SpA and TNFR1 than the induction of CXCL8 involves. TNFR1 shedding involves the stimulation of TACE, which occurs through an anti-inflammatory pathway that is entirely distinct from that involved in the canonical proinflammatory cascade initiated by TNFR1-TRAF2-MAPK/NF-κB and exhibits different kinetics. Thus, differences in the conformation of the ligand-receptor complex that activate two discrete responses are not anticipated. Moreover, it is likely that the recruitment of additional co-receptors or kinases may also affect the stoichiometry of the binding complex. These questions can be resolved by solving the structure of the SpA-TNFR1 complex, as was done for SpA-Fc, and by defining the components of the TACE induction cascade. As the structural requirements for proinflammatory activation of TNFR1 as well as IgG binding appear to be less stringent than for the activation of TACE and the anti-inflammatory effects of sTNFR1 shedding, identification of SpA variants with activity skewed toward receptor shedding would be particularly interesting.

Epidemiological studies show that SpA is expressed by the majority of *S. aureus* commensal and clinical isolates (30) and at especially high levels in strains causing pneumonia (31). The...
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prevalence of infection in healthy individuals caused by community-acquired methicillin-resistant *S. aureus* strains is increasing and is predominant at several major medical centers (32). These strains can cause a severe form of necrotizing pneumonia (33) that might indicate an important role for protein A in pathogenesis. The structural organization of SpA, as well as other staphylococcal surface proteins with tandem arrays of repeated sequences, suggests environmental pressure for the conservation of these genes although they are not essential for the growth of the organism *in vitro* (34). Their multiplicity ensures that random mutation does not eliminate their function. As there has been great interest in developing therapeutic agents that target the TNF-α signaling cascade, it may also be possible to target the SpA-TNFR1 interaction to prevent or treat *S. aureus* pneumonia.

Acknowledgment—Confocal microscopy was done at the Herbert Irving Optical Microscopy facility at Columbia University.

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