Human SAP Is a Novel Peptidoglycan Recognition Protein That Induces Complement-Independent Phagocytosis of Staphylococcus aureus

Jang-Hyun An, Kenji Kurokawa, Dong-Jun Jung, Min-Jung Kim, Chan-Hee Kim, Yukari Fujimoto, Koichi Fukase, K. Mark Coggeshall and Bok Luel Lee

J Immunol 2013; 191:3319-3327; Prepublished online 21 August 2013;
doi: 10.4049/jimmunol.1300940
http://www.jimmunol.org/content/191/6/3319

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/08/21/jimmunol.1300940.DC1

References
This article cites 51 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/191/6/3319.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Human SAP Is a Novel Peptidoglycan Recognition Protein That Induces Complement-Independent Phagocytosis of Staphylococcus aureus

Jang-Hyun An,* Kenji Kurokawa,* Dong-Jun Jung,* Min-Jung Kim,* Chan-Hee Kim,* Yukari Fujimoto,† Koichi Fukase,† K. Mark Coggeshall,‡ and Bok Luel Lee*

The human pathogen *Staphylococcus aureus* is responsible for many community-acquired and hospital-associated infections and is associated with high mortality. Concern over the emergence of multidrug-resistant strains has renewed interest in the elucidation of host mechanisms that defend against *S. aureus* infection. We recently demonstrated that human serum mannose-binding lectin binds to *S. aureus* wall teichoic acid (WTA), a cell wall glycopolymer—a discovery that prompted further screening to identify additional serum proteins that recognize *S. aureus* cell wall components. In this report, we incubated human serum with 10 different *S. aureus* mutants and determined that serum amyloid P component (SAP) bound specifically to a WTA-deficient *S. aureus* ΔtagO mutant, but not to tagO-complemented, WTA-expressing cells. Biochemical characterization revealed that SAP recognizes bacterial peptidoglycan as a ligand and that WTA inhibits this interaction. Although SAP binding to peptidoglycan was not observed to induce complement activation, SAP-bound ΔtagO cells were phagocytosed by human polymorphonuclear leukocytes in an FcγR-dependent manner. These results indicate that SAP functions as a host defense factor, similar to other peptidoglycan recognition proteins and nucleotide-binding oligomerization domain–like receptors. *The Journal of Immunology*, 2013, 191: 3319–3327.

Immunology constitutes the first line of host defense and recognizes evolutionarily conserved molecular patterns of pathogenic microbes using pattern recognition receptors (PRRs) (1). Based on their localization, PRRs are classified as either cell-associated receptors, including the Toll-like receptors (2) and scavenger receptors (3), or fluid-phase molecules (4). Fluid-phase molecules, such as collectins, ficolins, and pentraxins, constitute the humoral arm of the innate immune system and are generally believed to represent the functional ancestors of Abs (5).

The pentraxin family can be divided into two subclasses, the short-chain pentaxins, which include C-reactive protein (CRP) and serum amyloid P component (SAP), and the long-chain pentaxins, which contain an additional N-terminal domain. PTX3, a long-chain pentraxin, is produced by macrophages and may be involved in the response to proinflammatory stimuli (6–8). Whereas human CRP and mouse SAP are major acute-phase proteins (9), human SAP is a constitutive protein in blood (10). SAP, named for its universal presence in amyloid deposits, is the precursor of amyloid P component in tissue, where it may promote the development of pathogenic amyloid deposits and prevent their degradation. Both SAP and CRP have been reported to recognize numerous pathogenic bacteria and fungi and activate the classical complement pathway via C1q (8, 11). SAP is a conserved, circulating protein that exhibits calcium-dependent binding to various ligand molecules on the surface of microbial pathogens (4). Both CRP and SAP assemble into pentameric ring structures, which are arranged with the ridge helix of each subunit on one face and microbial ligand binding sites on the opposite side (12, 13). Previous reports found that members of the pentraxin family interact with cell-surface Fcγ receptors (FcγRs) and activate leukocyte-mediated phagocytosis (14, 15). More recently, the structural basis for the binding of pentraxins to FcγRs and the mechanism of activation of FcγR-mediated phagocytosis and cytokine secretion were reported (16). Notably, because pentraxins are broadly conserved, these proteins are thought to function as ancient mediators of immunity (17).

*Staphylococcus aureus* is a common human pathogen responsible for hospital-associated and community-acquired infections with complications such as wound infection, bacteremia, and sepsis. Recent studies have shown how this pathogen has evolved mechanisms to evade host innate immune responses and how it has acquired numerous virulence factors, which contribute to the diversity and severity of staphylococcal diseases (18). Any effort to respond to these challenges requires an examination of the molecular cross-talk between *S. aureus* and its host.

Like most Gram-positive bacteria, *S. aureus* incorporates peptidoglycan (PGN) and carbohydrate-based glycopolymers, such as wall teichoic acid (WTA) and lipoteichoic acid (LTA), into its cell envelope (19). PGN, an essential component of the bacterial cell wall, is composed of polymeric sugar chains with alternating 1,4-
β-linked N-acetylgalactosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues; the MutNAc residues within PGN are cross linked by short peptides (20). In *S. aureus*, WTA is also cross linked at its C6 position of the MurNAc unit of PGN. The disaccharide N-acetylmannosaminobiose-(1.3)-GlcNAc is connected to a polymer of 11–40 repeating units of ribitol phosphate via two glycerol phosphates. The hydroxyl groups on the ribitol phosphate repeats are modified with cationic D-alanine esters and to a polymer of 11–40 repeating units of ribitol phosphate via two cross linked at its C6 position of the MurNAc unit of PGN. The cross linked by short peptides (20).

In addition, we recently purified anti-WTA Ig from human i.v. *S. aureus* WTA and induces deposition of complement factor C4 (26). In contrast, in infants lacking a fully developed adaptive immunity, WTA is necessary for adherence of *S. aureus* Δspa, ΔtagO double mutant cells (1.3 × 10^10 CFU), and Δspa mutant cells (1.3 × 10^10 CFU), and bacteria were removed by centrifugation. The same absorption process was repeated three times for sufficient removal of *S. aureus*-recognizing serum factors, including SAP, MBL, and Abs, which were described in the legend to Supplemental Fig. 1C–E. Bacterial strains and mutants of deleted genes in mutant strains are summarized in Table I. *S. aureus* RN4220 is used as a parental strain. All of the bacterial strains were cultured with Luria–Bertani medium supplemented with antibiotics wherever required.

**Purification of insoluble and soluble PGNs from *S. aureus***

Insoluble PGNs (is-PGN) and soluble PGNs (s-PGNs) were purified as described previously (28, 29). The detailed method is described in the legend to Supplemental Fig. 2A.

**Flow cytometric measurements of C4 and C3 deposition on *S. aureus* cells**

Complement C4 and C3 deposition was measured as described previously (26). Briefly, 2.0 × 10^9 *S. aureus* cells were fixed with ethanol and incubated at 37°C for 1 h in 20 μl incubation buffer [10 mM Tris (pH 7.4), 140 mM NaCl, 1% human serum albumin (HSA), 2 mM CaCl_2, 1 mM MgCl_2] containing 10% human sera and purified anti-WTA Ig or SAP. After centrifugation, recovered cells were washed with washing buffer [10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 2 mM CaCl_2, 1 mM MgCl_2] and then were resuspended in 20 μl incubation buffer. For detection of bound C4b, mouse anti-human C4 mAb (diluted 1:500; BioPorto, Gentofte, Denmark) and FITC-conjugated goat F(ab’2) anti-mouse IgG mAb (diluted 1:200; Beckman Coulter, Indianapolis, IN) were used. For detection of bound C3b, FITC-conjugated mouse anti-human C3 IgG mAb (diluted 1:200) was used. Following the application of Abs, *S. aureus* cells were sonicated for 15 s to disperse clumped cells before measurement of fluorescence data using flow cytometry (Model FC500; Beckman Coulter).

**Determination of serum levels of SAP, MBL, and anti-Δ*spa* Ig**

Levels of human SAP, MBL, and anti-Δ*spa* Ig were determined by Western blot analysis, as described previously (26). The detailed methods of Western blot analyses are explained in the legends to Supplemental Fig. 1C and 1D. ELISA for human SAP and MBL was as described previously (26) and is explained in the legend to Supplemental Fig. 1E.

**PMN preparation**

PMNs were prepared as previously described (27), with some modification. The detailed method is described in the legend to Supplemental Fig. 2D. The FcγRII-expressing HEK293T cells were prepared as described previously (30). The detailed method is explained in the legend to Fig. 6.

**SAP-mediated phagocytosis assay**

The phagocytosis experiment was performed as previously described (27), with some modification. Briefly, Δ*spa* mutant and ΔtagO, Δ*spa* double mutant *S. aureus* cells grown in Luria–Bertani medium to postexponential phase were washed, killed with 70% ethanol, labeled with 0.02 mM FITC (Sigma-Aldrich) in 100 mM Na2CO3 buffer (pH 8.5) for 30 min at room temperature, and resuspended in HBSS. FITC-labeled bacteria (equivalent to 1.5 × 10^7 CFU) or FITC-labeled PGNs (40 μg) were incubated with...
10% depleted serum, with or without 1 μg SAP or CRP in 20 μl HBSS containing 2 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 150 mM NaCl, and 0.4% HSA for 30 min at 37°C with shaking. Then, the PMN preparation (1.5 × 10$^5$ cells in 35 μl) was added to 5 μl resuspended FITC-labeled bacteria (corresponding to 3.8 × 10$^7$ CFU, multiplicity of infection of ~25), and the mixture was incubated at 37°C for 60 min with shaking. Extracellular FITC-labeled S. aureus cells were quenched by 0.2% trypan blue. Finally, phagocytosed FITC-labeled S. aureus cells within the 100 PMNs or 100 FcγRII-expressing HEK293T cells were counted using fluorescence–phase contrast microscopy.

**Biacore analysis**

The two types of PGN fragments used in this study [monomeric PGN (MurNAc-t-Ala-d-isoGln-GlcNAc) and dimeric PGN (MurNAc-t- Ala-d-isoGln-t-Lys-t-Ala-GlcNAc$_2$)] were synthesized based on the methods described previously (31). For determination of the dissociation constants (K$_D$) for SAP binding to the different forms of PGN, biotinylated SAP was immobilized onto a streptavidin-coated biosensor chip (SA chip; Biacore, Neuchâtel, Switzerland). Subsequently, the different concentrations of PGN fragments [10–40 μM in 10 mM HEPES (pH 7.4), 150 mM NaCl] were passed over the surface of the sensor chip at a flow rate of 25 μl/min. The change in surface plasmon resonance (SPR) at 25°C was measured using Biacore 2000 software (Biacore). After 300 s of monitoring, buffer without PGN was passed over the chip to initiate dissociation. At the end of each cycle, regeneration of the chip was accomplished by washing away the bound PGN fragment using 12.5 μl 10 mM EDTA solution. Both the association rate constant (k$_a$) and the dissociation rate constant (k$_d$) were determined using the SPR binding data with BIAevalulation software (version 3.2; Biacore) and used to calculate the K$_D$ (defined as k$_d$/k$_a$).

**Data processing and statistical analysis**

Results from quantitative analysis of the data are expressed as the mean ± SD from at least three independent experiments, unless otherwise stated. Other data are representative of at least three independent experiments that yielded similar results. Statistical analysis was performed using the Student t test, and p < 0.05 was considered significant.

**Results**

**SAP binds specifically to WTA-deficient S. aureus ΔtagO mutant cells**

To identify serum factors capable of binding to S. aureus cell-surface components, we used nine different S. aureus gene mutations (Table I). Human serum was incubated with each mutant, and serum proteins bound to the bacteria were eluted with 0.1 M glycine (pH 2.5) and analyzed by SDS-PAGE under nonreducing conditions. As shown in Fig. 1A, the WTA-deficient ΔtagO mutant bound strongly to a 25-kDa human serum protein (lane 5); however, when the ΔtagO mutant was complemented with a tagO::erm mutant cell surface may serve as a ligand of SAP. To examine this possibility, we used 10 preparations of insoluble bacterial cell wall components, each one depleted for a different set of PGN-associated surface proteins or WTA through gene mutation or treatment with trypsin or TCA (Fig. 2A). For use as a control, intact PGN nondepleted for WTA and surface proteins was obtained from the parental S. aureus RN4220 strain without treatment of trypsin or TCA (Fig. 2A, lane 1). WTA and surface proteins were removed from the crude cell wall, using both trypsin and TCA treatments (lane 4). When SAP was incubated with the 10 different S. aureus cell wall preparations, SAP bound only to WTA-depleted S. aureus PGNs (Fig. 2A, lanes 4, 5, 6, 8, and 10) and not to WTA-containing PGNs (lanes 1, 3, 7, and 9). In addition, surface proteins were apparently capable of blocking the interaction between SAP and the cell wall (lane 2). The strA gene encoding sortase A—an enzyme that covalently attaches surface proteins, including protein A to PGN—seems to be involved in this surface protein-mediated inhibition. To investigate this observation, we measured the binding between FITC-labeled SAP and WTA-linked or WTA-depleted PGNs, using flow cytometry (Fig. 2B). SAP was confirmed to bind specifically and with high affinity to WTA-depleted insoluble bacterial PGN, but not to WTA-containing PGNs (Fig. 2B). These results suggest that SAP can recognize bacterial PGN, unless prevented from doing so by WTA.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN4220</td>
<td>Parental strain</td>
<td>Parental strain</td>
<td>(46)</td>
</tr>
<tr>
<td>M0107</td>
<td>RN4220 ΔtagO::phleo</td>
<td>Protein A depleted</td>
<td>(47)</td>
</tr>
<tr>
<td>T775</td>
<td>M0107 ΔtagS::erm/pM101</td>
<td>Protein A/LTA depleted</td>
<td>(27)</td>
</tr>
<tr>
<td>T777</td>
<td>M0107 ΔtagS::erm/pM101-tasS</td>
<td>Protein A depleted/tasS complement</td>
<td>(27)</td>
</tr>
<tr>
<td>T258</td>
<td>M0107 ΔtagO::erm</td>
<td>Protein A/WTA depleted</td>
<td>(26)</td>
</tr>
<tr>
<td>T358</td>
<td>M0107 ΔtagO::erm/pStagO</td>
<td>Protein A depleted/ermO complement</td>
<td>(26)</td>
</tr>
<tr>
<td>T174</td>
<td>RN4220 ΔtagO::erm</td>
<td>WTA depleted</td>
<td>(48)</td>
</tr>
<tr>
<td>T002</td>
<td>RN4220 ΔsortA::erm</td>
<td>PGN O-acetyltransferase depleted</td>
<td>(26)</td>
</tr>
<tr>
<td>T013</td>
<td>RN4220 ΔtagF::erm</td>
<td>Lipoxygen lipidation depleted</td>
<td>(49)</td>
</tr>
<tr>
<td>N1</td>
<td>RN4220 ΔmprF::erm</td>
<td>Lysyl-phosphatidylglycerol depleted</td>
<td>(50)</td>
</tr>
<tr>
<td>M0875</td>
<td>RN4220 ΔacylF::erm</td>
<td>Glycolipids depleted</td>
<td>(48)</td>
</tr>
<tr>
<td>M0793</td>
<td>RN4220 ΔdictA::erm</td>
<td>D-Ala of both WTA and LTA depleted</td>
<td>(48)</td>
</tr>
<tr>
<td>T2316</td>
<td>RN4220 ΔsortA::erm</td>
<td>Sortase A depleted</td>
<td>(51)</td>
</tr>
</tbody>
</table>

**Biocore analysis**

Bacterial PGNs are covalently modified with WTA via a phosphodiester linkage (19). Because of previous data indicating that staphylococcal WTA blocks PGN recognition protein-SA (PGPR-SA) from binding to PGN during induction of the innate immune system in insects (32, 33), together with the inability of ΔtagO mutant cells to synthesize WTA, we hypothesized that S. aureus PGN exposed on the ΔtagO mutant cell surface may serve as a ligand of SAP. To examine this possibility, we used 10 preparations of insoluble bacterial cell wall components, each one depleted for a different set of PGN-associated surface proteins or WTA through gene mutation or treatment with trypsin or TCA (Fig. 2A). For use as a control, intact PGN nondepleted for WTA and surface proteins was obtained from the parental S. aureus RN4220 strain without treatment of trypsin or TCA (Fig. 2A, lane 1). WTA and surface proteins were removed from the crude cell wall, using both trypsin and TCA treatments (lane 4). When SAP was incubated with the 10 different S. aureus cell wall preparations, SAP bound only to WTA-depleted S. aureus PGNs (Fig. 2A, lanes 4, 5, 6, 8, and 10) and not to WTA-containing PGNs (lanes 1, 3, 7, and 9). In addition, surface proteins were apparently capable of blocking the interaction between SAP and the cell wall (lane 2). The strA gene encoding sortase A—an enzyme that covalently attaches surface proteins, including protein A to PGN—seems to be involved in this surface protein-mediated inhibition. To investigate this observation, we measured the binding between FITC-labeled SAP and WTA-linked or WTA-depleted PGNs, using flow cytometry (Fig. 2B). SAP was confirmed to bind specifically and with high affinity to WTA-depleted insoluble bacterial PGN, but not to WTA-containing PGNs (Fig. 2B). These results suggest that SAP can recognize bacterial PGN, unless prevented from doing so by WTA.

To characterize the specificity of SAP binding to WTA-depleted PGN, we performed a competitive inhibition assay using s-PGN (Fig. 2C). The s-PGN was prepared from WTA-depleted is-PGN by digestion with lysostaphin followed by size exclusion column.

**Table I. Bacterial strains used in this study**

The Journal of Immunology 3321
FIGURE 1. Biochemical characterization of a ΔtagO mutant–binding 25-kDa human serum protein. (A) Screening of human serum for factors that bind S. aureus cell wall mutants. Twelve ethanol-fixed S. aureus cells (1.0 × 10^9 cells) were incubated with 1 ml human serum. Cells were recovered by centrifugation and washed. Bound proteins were eluted with 0.1 M glycine (pH 2.5) and analyzed by SDS-PAGE on a 15% gel under nonreducing conditions. Protein bands were stained with Coomassie brilliant blue. The S. aureus mutants are described in Table I. Lane 1, parental S. aureus; lane 2, Δspa; lane 3, ΔltaS; Δspa/pM101; lane 4, ΔltaS; Δspa/pM101-ΔltaS; lane 5, ΔtagO; Δspa; lane 6, ΔtagO, Δspa/ΔtagO; lane 7, Δspa; lane 8, ΔltaS; lane 9, Δspa/F; lane 10, ΔltaS; lane 11, ΔltaS; lane 12, ΔltaS. The ΔtagO, Δspa double mutant sample (lane 5) was enriched for a 25-kDa serum protein, and when complemented with a tagO-expressing plasmid, this enrichment was not observed (lane 6). (B) Comparison of the N-terminal amino acid sequence of “band a” with that of human SAP. (C) Binding of SAP to the parental S. aureus RN420 strain (parent) or the ΔtagO, Δspa double mutant (ΔtagO) in the presence or absence of calcium ion. (D) Binding of FITC-labeled SAP to the parental S. aureus RN420 strain (parent) or the ΔtagO, Δspa by flow cytometry, as described in Materials and Methods. The curves with gray areas underneath represent cell-only controls.

With addition of 0.2–0.8 mg s-PGN into 3 mg of is-PGN, s-PGN liberated approximately half of the bound SAP into the supernatant (lane 8), confirming the interaction of SAP with S. aureus PGN. To determine whether SAP can bind to additional bacterial PGNs, SAP was incubated with five other WTA-depleted is-PGN preparations from Bacillus subtilis, Enterococcus faecalis, Lactobacillus bulgaricus, Micrococcus luteus, and Escherichia coli (Supplemental Fig. 2A) and bound to all five (lanes 2–6). Among them, E. coli and E. faecalis PGNs interacted relatively weakly with SAP (lanes 3 and 6). Because the amino acid sequence of SAP shares 50% sequence homology with that of human CRP, we examined whether CRP might also bind S. aureus is-PGN; however, CRP was not observed to bind (Supplemental Fig. 2B, lanes 2 and 5). When we incubated a mixture of CRP and SAP (2 μg each) with is-PGN, SAP alone localized to the is-PGN fraction (lane 3), whereas CRP remained in the supernatant (lane 6). Taken together, these results suggest strongly that SAP binds specifically to WTA-depleted S. aureus PGN and that the presence of WTA inhibits SAP binding in vitro.

SAP forms a high-molecular mass complex in the presence of s-PGN

We previously demonstrated that clustering of insect PGRP-SA on the soluble polymeric form of S. aureus PGN is required for sensing bacterial PGN during activation of the melanin synthesis cascade, a major host innate immune response in insects (29).

FIGURE 2. Purified SAP binds specifically to bacterial PGNs. (A) The is-PGNs were purified from four different S. aureus strains including parental RN420 and WTA-, protein A–, and sortase-depleted mutant cells and treated with trypsin or TCA or both. Next, is-PGN preparations (1 mg) were incubated with 1 μg SAP for 1 h at 4°C, recovered by centrifugation, and washed. Proteins bound to the is-PGNs were eluted with buffer B (10 mM Tris-HCl (pH 7.4), 140 mM NaCl, and 10 mM EDTA) and analyzed by SDS-PAGE on a 15% gel under nonreducing conditions. (B) Measurement of the binding between FITC-labeled SAP and WTA-linked or WTA-depleted PGNs, using flow cytometry. The curves with gray areas underneath represent cells only as controls. (C) Competitive inhibition experiments. Various concentrations of purified s-PGN were added to reaction mixtures containing 3 mg is-PGN and 2 μg SAP and incubated for 1 h at 4°C. The is-PGNs were recovered by centrifugation and washed; SAP bound to is-PGNs was eluted in buffer B, and SAP released from is-PGN was recovered by TCA treatment. Samples of SAP bound and released were analyzed by SDS-PAGE on a 15% gel under nonreducing conditions.

Because of this observation, we hypothesized that SAP binding to soluble polymeric S. aureus PGN might perform a similar biological function. As expected, when a mixture of SAP and soluble polymeric S. aureus PGN was injected onto the size exclusion column, a peak with an apparent molecular mass of 560 kDa was observed; SAP alone elutes at a volume consistent with a molecular mass of 235 kDa (Fig. 3A). Western blot analysis using anti-SAP Ab confirmed that SAP elution coincided with the peaks (Fig. 3B), indicating that the SAP monomer (25 kDa) can assemble into a dimer (235 kDa) in the presence of calcium ion (13); moreover, SAP can form a larger-mass complex (560 kDa) with s-PGN.

SPR analysis reveals SAP binding to synthetic PGN fragments

To determine the number of repeating MurNac-GlcNac disaccharides in PGN required for SAP binding, the interaction of SAP with two different synthetic PGN fragments [monomeric PGN (MurNac-L-Ala-d-isoGln-GlcNac) and dimeric PGN (MurNac-L-Ala-d-isoGln-L-Lys-d-Ala-GlcNac)_2] was evaluated using SPR. SAP was immobilized on the surface of an SA chip, and varying concentrations of the two synthetic PGN fragments were passed over the chip (Fig. 3C). The estimated K_D for dimeric PGN binding to SAP was calculated to be 8.00 × 10^-3 ± 8.65 × 10^-3 M; the K_D for monomeric PGN binding to SAP was determined to be 5.97 × 10^-3 ± 7.31 × 10^-3 M. In a previous study, the K_D value between an identical dimeric PGN fragment and human PGRP-SA was...
either dimeric PGN \[(\text{MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala-GlcNAc})_2\], SPR sensorgrams were obtained by injecting various concentrations of each fraction was directly correlated with the magnitude of the trace. (Panel B) The presence of SAP in each fraction was examined by Western blot using an anti-SAP Ab. The intensity of the SAP band from each fraction was directly correlated with the magnitude of the trace. (Panel C) SPR sensorgrams were obtained by injecting various concentrations of either dimeric PGN \[(\text{MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala-GlcNAc})_2\], left panel) or monomeric PGN (MurNAc-L-Ala-D-isoGln-GlcNAc, right panel) over an SA chip containing immobilized SAP for 500 s.

\[ \text{the depletion process, we examined the concentrations of serum C1q and C1s proteins before and after depletion by Western blot analysis (Supplemental Fig. 1F, 1G), confirming that there is little reduction of serum C1q and C1s proteins by the depletion process. Next, we examined whether the depleted serum retained the necessary and functional complement factors (Fig. 4A). Whereas the depleted serum itself did not induce C3 deposition [Fig. 4A(c)], inclusion of anti-\(S. aureus\) Ig or MBL into the depleted serum did induce C3 depositions on the surface of parental \(S. aureus\) cells [Fig. 4A(e), 4A(f)]. This result suggests that all necessary serum complement factors are present and functional in the depleted serum. Nevertheless, addition of SAP (1 \(\mu g\)) to the depleted serum did not induce C3 deposition on either parental [Fig. 4A(d)] or \(\Delta tagO\) cells [Fig. 4A(j)]. Similarly, when we added SAP to complete human serum, no additional C3 deposition was observed [Fig. 4A(b), 4A(h)] when compared with a control [Fig. 4A(a), 4A(g)]. Whereas SAP can bind to \(S. aureus\) \(\Delta tagO\) mutant cells, SAP did not stimulate C4 deposition on these cells (Supplemental Fig. 2C). Taken together, these results reveal that SAP bound to

calculated to be \(3.69 \times 10^{-6} \pm 2.65 \times 10^{-6}\) M (34); therefore, SAP appears to possess a greater affinity than human PGRP-SA for the dimeric PGN fragment. Taken together, these results indicate that SAP has the ability to interact with bacterial PGN fragments ranging in size from monomers to oligomers.

\[ \text{SAP does not induce complement activation onto } S. aureus \text{ cells} \]

In previous studies, SAP was found to activate the classical complement pathway (11). To examine whether SAP is capable of activating the complement system upon binding to PGNs, we prepared human sera depleted of \(S. aureus\)-recognizing proteins, including SAP, MBL, or anti-\(S. aureus\) Ig. Recently, we prepared a human serum depleted of both anti-\(S. aureus\) Ig and MBL and demonstrated that addition of purified anti-\(S. aureus\) Ig or purified MBL to this depleted serum induces specific C3 deposition (26, 27). In this study, we needed also to deplete SAP. A serum depleted of all three components was prepared by adsorption of the intact serum with a mixture of WTA-intact parental \(S. aureus\) cells and WTA-depleted \(S. aureus\) \(\Delta tagO\) mutant cells. Depletion of MBL and SAP was first confirmed by Western blot analysis (Supplemental Fig. 1C, 1D). In addition, to confirm the depletion of SAP and MBL by absorption, we measured the amounts of SAP and MBL of the intact sera and depleted sera by ELISA (Supplemental Fig. 1E). As expected, before absorption, the amount of SAP and MBL in the intact serum was calculated to be 27.2 ng/\(\mu l\) and 3.7 ng/\(\mu l\), respectively. However, SAP and MBL were not detected in the depleted serum (Supplemental Fig. 1E). Furthermore, to exclude the possibility of reduced C1q concentration by

\[ \text{FIGURE 3.} \text{ SAP forms a high-molecular-mass adduct with } s-PGN \text{ and binds to the PGN monomer during SPR analysis. (A) A mixture of SAP (5 } \mu g \text{) and polymeric } s-PGN (200 } \mu g \text{) was injected onto a Toyopearl HW55-S column (2.6 } \times 155 \text{ cm) equilibrated with buffer A [10 mM Tris-HCl (pH 7.4), 140 mM NaCl, and 10 mM CaCl}_2. \text{ Two peaks (red trace) generated corresponded to molecular masses of 560 and 235 kDa. When SAP alone (black trace) was injected onto the same column, only the 235-kDa peak was observed. (B) The presence of SAP in each fraction was examined by Western blot using an anti-SAP Ab. The intensity of the SAP band from each fraction was directly correlated with the magnitude of the trace. (C) SPR sensorgrams were obtained by injecting various concentrations of either dimeric PGN [(\text{MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala-GlcNAc})_2, left panel] or monomeric PGN (\text{MurNAc-L-Ala-D-isoGln-GlcNAc, right panel}) over an SA chip containing immobilized SAP for 500 s.} \]

\[ \text{FIGURE 4.} \text{ SAP does not induce C3 deposition on } \Delta spa \text{ mutant or } \Delta tagO. \text{ } \Delta spa \text{ double mutant cells, but induces complement-independent phagocytosis of } S. aureus \text{ cells by PMNs. (A) Panels (a) and (g) show C3 deposition on parental and } \Delta tagO \text{ mutant cells after incubation with intact serum, respectively; (b) and (h)}, C3 \text{ deposition by incubation of SAP (1 } \mu g \text{) with intact serum; (c) and (i)}, C3 \text{ deposition in depleted serum; (d) and (j)}, C3 \text{ deposition after addition of 1 } \mu g \text{ SAP into depleted serum; (e) and (k)}, C3 \text{ deposition after addition of 1 } \mu g \text{ MBL into depleted serum; (f) and (l)}, 50 \text{ ng MBL added into depleted serum.} \]

\[ \text{The Journal of Immunology} \]

3323
S. aureus PGN does not induce activation of the complement system under these conditions.

SAP induces complement-independent phagocytosis of WTA-depleted ΔtagO mutant cells

Although SAP failed to activate the complement system, SAP might mediate phagocytosis via association with FcγRs. One earlier observation supporting this supposition is that SAP- or CRP-mediated opsonization of apoptotic cells enhances their phagocytosis by macrophages (15, 16). To examine the effect of SAP on phagocytosis of S. aureus cells by human PMNs, we directly counted the number of FITC-labeled ΔtagO cells engulfed by 100 PMNs (Fig. 4B). In the absence of depleted serum, SAP increased the number of phagocytosed ΔtagO mutant cells from 10 ± 4 (Fig. 4B, column 1) to 111 ± 13 (Fig. 4B, column 2), suggesting that SAP may activate FcγR-mediated phagocytosis. Addition of depleted serum did not increase the number of phagocytosed ΔtagO mutant cells (column 4), suggesting that serum complement factors do not play a role in SAP-mediated phagocytosis of ΔtagO mutant cells, a result consistent with the failure of SAP to activate the complement system against S. aureus ΔtagO cells (Fig. 4A). In the absence of depleted serum, SAP increased the number of parental S. aureus cells engulfed by 100 PMNs from 4 ± 2 (Fig. 4B, column 6) to 33 ± 9 (column 7), indicating that SAP only weakly induces phagocytosis of WTA-intact S. aureus cells. Again, addition of depleted serum had little effect on phagocytosis of parental cells (column 9). Notably, CRP did not induce phagocytosis of either parental or S. aureus ΔtagO cells under identical conditions (columns 5 and 10).

To expand upon these results, we investigated the possibility that SAP stimulates phagocytosis of WTA-depleted PGN (Supplemental Fig. 2D). Regardless of the presence or absence of depleted serum, SAP enhanced greater engulfment of FITC-labeled, WTA-depleted PGN than of WTA-intact PGN (Supplemental Fig. 2D, columns 2, 4 and columns 6, 8, respectively). Taken together, these results support the idea that SAP induces complement-independent phagocytosis of WTA-depleted S. aureus ΔtagO mutant cells as a result of its interaction with exposed PGN; in contrast, SAP does not affect phagocytosis of WTA-intact Δspa mutant cells.

SAP-mediated phagocytosis was FcγR dependent

Finally, to confirm whether the FcγRs of PMNs are involved in SAP-enhanced phagocytosis, commercially available mAbs targeting three different FcγRs—CD64 (FcγRII), CD32 (FcγRII), and CD16 (FcγRIII)—were tested. These three receptors are known to differ in their abilities to bind Ig and Ig-containing immune complexes (35). As a control, a combination of three anti-FcγR mAbs clearly inhibited anti-WTA Ig-mediated phagocytosis of parental S. aureus cells (Fig. 5, columns 11 and 12); however, the Ab mixture did not affect MBL-mediated opsonophagocytosis (Fig. 5, columns 14 and 15). With ΔtagO mutant cells, the mixture of three mAbs was able to inhibit SAP-mediated phagocytosis (Fig. 5, columns 2 and 3). With the use of different pairs of mAbs, the anti-CD64/CD32 combination exhibited more potent inhibition compared with the anti-CD32/CD16 and anti-CD64/16 combinations (Fig. 5, columns 4–6). When each anti-FcγR mAb was assayed independently, anti-CD64 exhibited a greater effect than either anti-CD32 or anti-CD16 (Fig. 5, columns 7–9). These results reveal that SAP-mediated phagocytosis is mediated by FcγRs present on the surface of PMNs.

To further confirm the requirement of FcγRs for SAP-mediated phagocytosis, we transfected the FcγRII gene into HEK293T cells. The expression of FcγRII on the HEK293T cells was confirmed by flow cytometry analyses, as described previously (30). As a positive

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Anti-FcγR mAbs inhibit SAP-mediated phagocytosis. Ethanol-killed ΔtagO mutant (columns 1–9) and parental S. aureus cells (columns 10–15) were labeled with FITC (0.02 mM). As a positive control of FcγRs-mediated phagocytosis, anti-WTA Ig (1 µg) was incubated with FITC-labeled parental cells (3.8 × 10^6 cells) and human PMNs (1.5 × 10^5 PMNs) in the absence (column 11) or presence (column 12) of three different anti-FcγRs mAbs, anti-CD64 (anti-FcγRII mAb), anti-CD32 (anti-FcγRI mAb), and anti-CD16 (anti-FcγRIII mAb). MBL-mediated opsonophagocytosis was used as a negative control. MBL (50 ng) was incubated with FITC-labeled parental cells (3.8 × 10^6 cells) and depleted serum (2 µl) in the absence (column 14) or presence (column 15) of three different anti-FcγRs mAbs. FITC-labeled ΔtagO mutant S. aureus cells (3.8 × 10^6 cells) were incubated with SAP (1 µg) and PMNs (1.5 × 10^5 cells) in the presence of different combinations of anti-FcγRs mAbs (each 0.35 µg) in 40 µl RPMI 1640 medium at 37°C for 1 h (columns 3–9). Phagocytosed S. aureus cells in 100 PMNs were counted under fluorescence–phase contrast microscopy. Data are the means ± SD of results of three independent experiments.
control, anti-WTA Ig-induced phagocytosis of parental S. aureus cells by the FcyRII-expressing HEK293T cells (Fig. 6, column 12) and anti-CD32 (FcyRII) mAb clearly inhibited the anti-WTA Ig-mediated phagocytosis of parental S. aureus cells (Fig. 6, column 13). In this condition, SAP increased the number of ΔtagO mutant cells engulfed by 100 FcyRII-expressing HEK293T cells from 8 ± 5 (Fig. 6, column 1) to 86 ± 7 (Fig. 6, column 2), indicating that

SAP only weakly induces phagocytosis of WTA-deficient ΔtagO S. aureus cells. Addition of anti-CD32 mAb into column 2 solution inhibited SAP-mediated phagocytosis (Fig. 6, column 3), but anti-CD64 mAb did not (Fig. 6, column 4). As in PMNs, FcyRII-expressing HEK293T cells did not induce SAP-mediated phagocytosis toward parent cells (Fig. 6, column 9). Taken together, these results demonstrated the involvement of FcyRII in SAP-mediated phagocytosis against ΔtagO mutant cells.

Phagocytosed S. aureus cells lose WTA and were recognized by SAP

Next, we tried to get an insight into when WTA-deficient S. aureus cells can be generated in vivo. Because it was known that S. aureus WTA is easily released from PGN at the acidic pH (36), we supposed that S. aureus WTA would be released when S. aureus cells were transferred into acidic phagolysosomes after engulfment by PMNs. Before checking this possibility, we first incubated parental S. aureus cells in vitro with different pH conditions at 37˚C for 2 h. Bacterial cells were recovered by centrifugation, washed, and incubated with SAP to determine whether it can bind to bacterial cells preincubated with acidic conditions (Fig. 7A). As expected, SAP bound to S. aureus cells preincubated at pH 2, 3, and 4 (Fig. 7A, lanes 2–4), but SAP weakly bound bacteria preincubated at pH 5, 6, and 7 conditions (Fig. 7A, lanes 5–7), indicating that S. aureus WTA was removed at acidic conditions, leading to SAP binding to the exposed PGN of S. aureus. Then, we tested SAP binding to engulfed S. aureus cells. Parental S. aureus cells were first opsonized by incubation of Δspa-tagO-treated serum (10%) and anti-WTA Ig, and were incubated with PMNs for the indicated times (Fig. 7B). Then, the cell wall fraction of phagocytosed S. aureus cells was recovered by lysis of PMNs and incubation with 0.5% SDS solution to remove the proteins originating from PMNs. When we examined SAP-binding abilities toward cell walls derived from phagocytosed parental S. aureus cells, SAP bound to recovered cell walls with longer incubation conditions. The SAP band was visualized by Western blot analysis, using anti-SAP polyclonal Ab. (B) Ethanol-killed parental S. aureus Δspa cells (2 × 10^9 CFU) were opsonized with 10% Δspa-tagO-treated serum and 50 ng anti-WTA Igs in 20 µl buffer A at 37˚C for 1 h, and washed with the same buffer three times. Bound SAP was eluted with SDS-PAGE loading buffer, and separated with 15% SDS-PAGE with nonreducing conditions. The SAP band was visualized by Western blot analysis, using anti-SAP polyclonal Ab. (B) Ethanol-killed parental S. aureus Δspa cells (2 × 10^9 CFU) were opsonized with 10% Δspa-tagO-treated serum and 50 ng anti-WTA Igs in 20 µl buffer, and its 5-µl portion was then incubated with PMNs (4 × 10^6 cells) at 37˚C with the indicated time to induce phagocytosis in 40 µl RPMI media. Then, PMNs were lysed with water twice and washed with 0.5% SDS twice to solubilize any remaining PMN debris. Recovered S. aureus cell walls were washed with buffer A three times and incubated with 100 ng SAP at 37˚C for 1 h. Bound SAP was eluted with SDS-PAGE loading buffer, and bound and unbound SAP were separated with 15% SDS-PAGE with nonreducing conditions. The SAP band was visualized by Western blot analysis, using anti-SAP polyclonal Ab. (C) Ethanol-killed parental S. aureus Δspa cells (2 × 10^9 CFU) were opsonized with 10% Δspa-tagO-treated serum and 50 ng anti-WTA Igs at 37˚C for 30 min. A portion of S. aureus cells were then incubated with PMNs (4 × 10^6 cells) at 37˚C for the indicated time (0–3 h). PMNs were lysed by incubation with 0.5% SDS at 60˚C for 30 min. Precipitants containing bacterial cell walls were washed twice with 0.5% SDS and then with buffer A three times. Then, the bacterial cell walls were incubated with 50 ng anti-WTA Igs in buffer G [10 mM Tris-HCl (pH 7.4), 140 mM NaCl, and 1% BSA] on ice for 2 h to detect WTA on the cell walls. Bound anti-WTA Igs on the bacterial cell walls were detected via flow cytometry using anti-human IgG mAb (1: 5000; Sigma-Aldrich) and goat F(ab′)2 anti-mouse IgG mAb conjugated with FITC (diluted 1:200; Beckman Coulter). The amount of WTA on the recovered bacterial cell walls decreased in an incubation time-dependent manner.

FIGURE 7. Phagocytosed S. aureus cells lose WTA and were recognized by SAP. (A) Ethanol-killed parental S. aureus Δspa cells (2 × 10^9 CFU) were incubated at 37˚C for 2 h in a 20 mM sodium citrate buffer having different pH, as indicated, and then cells were recovered by centrifugation and washed with buffer A three times. Washed cells were incubated with 100 ng SAP in 50 µl buffer A at 37˚C for 1 h, and washed with the same buffer three times. Bound SAP was eluted with SDS-PAGE loading buffer, and separated with 15% SDS-PAGE with nonreducing conditions. The SAP band was visualized by Western blot analysis, using anti-SAP polyclonal Ab. (B) Ethanol-killed parental S. aureus Δspa cells (2 × 10^9 CFU) were opsonized with 10% Δspa-tagO-treated serum and 50 ng anti-WTA Igs in 20 µl buffer, and its 5-µl portion was then incubated with PMNs (4 × 10^6 cells) at 37˚C with the indicated time to induce phagocytosis in 40 µl RPMI media. Then, PMNs were lysed with water twice and washed with 0.5% SDS twice to solubilize any remaining PMN debris. Recovered S. aureus cell walls were washed with buffer A three times and incubated with 100 ng SAP at 37˚C for 1 h. Bound SAP was eluted with SDS-PAGE loading buffer, and bound and unbound SAP were separated with 15% SDS-PAGE with nonreducing conditions. The SAP band was visualized by Western blot analysis, using anti-SAP polyclonal Ab. (C) Ethanol-killed parental S. aureus Δspa cells (2 × 10^9 CFU) were opsonized with 10% Δspa-tagO-treated serum and 50 ng anti-WTA Igs at 37˚C for 30 min. A portion of S. aureus cells were then incubated with PMNs (4 × 10^6 cells) at 37˚C for the indicated time (0–3 h). PMNs were lysed by incubation with 0.5% SDS at 60˚C for 30 min. Precipitants containing bacterial cell walls were washed twice with 0.5% SDS and then with buffer A three times. Then, the bacterial cell walls were incubated with 50 ng anti-WTA Igs in buffer G [10 mM Tris-HCl (pH 7.4), 140 mM NaCl, and 1% BSA] on ice for 2 h to detect WTA on the cell walls. Bound anti-WTA Igs on the bacterial cell walls were detected via flow cytometry using anti-human IgG mAb (1: 5000; Sigma-Aldrich) and goat F(ab′)2 anti-mouse IgG mAb conjugated with FITC (diluted 1:200; Beckman Coulter). The amount of WTA on the recovered bacterial cell walls decreased in an incubation time-dependent manner.

FIGURE 8. Two putative host immune responses against S. aureus infection. Left, SAP recognizes the exposed PGN of S. aureus ΔtagO mutant cells and then induces FcγRs-dependent phagocytosis by PMNs. Right, Serum MBL/MASP complex in infants who have not yet fully developed their adaptive immunity recognizes S. aureus WTA and then induces the C1q-mediated classical complement pathway, leading to the clearance of S. aureus by opsonophagocytosis. However, anti-WTA Igs in adults who have developed their adaptive immunity recognize S. aureus WTA and then induce the C1q-mediated classical complement pathway, leading to the clearance of S. aureus by opsonophagocytosis. Green pentameric moieties indicate SAP.
time (Fig. 7B, lanes 3–5), but not to nonphagocytosed S. aureus cells (Fig. 7B, lane 2). Conversely, unbound SAP was gradually decreased by an increase of incubation time with PMNs (Fig. 7B, lanes 12–15). To confirm this observation, we performed the same experiments in the presence of NH4Cl, an inhibitor of endosome acidification (37). As expected, SAP could not bind to bacterial cell walls recovered from phagocytosed S. aureus cells in the presence of NH4Cl (Fig. 7B, lanes 8–10), and the amounts of unbound SAP were not changed (Fig. 7B, lanes 17–20). These results demonstrated that inhibition of acidification by NH4Cl prevented both WTA release and SAP binding to bacterial cell walls derived from phagocytosed S. aureus cells. In addition, removal of WTA in phagocytosed S. aureus cells was confirmed by FACS analyses (Fig. 7C). As expected, anti-WTA Ig bound to cell walls recovered from parental S. aureus cells [Fig. 7C(a)], but its binding ability was almost lost after 3 h of phagocytosis [Fig. 7C(d)]. Therefore, these results also suggest that phagocytosed S. aureus cells lost WTA and were able to be recognized by SAP (Fig. 8).

Discussion

Screening of S. aureus mutant strains for microbial molecular patterns enabled the identification of a novel ligand of SAP. SAP binding to bacterial PGN was abolished by the presence of S. aureus WTA. We were unable to reproduce a SAP-mediated complement activation reported in previous studies. This inconsistency may be attributable to the different methods used to prepare human sera and/or the bacterial species tested. However, in this article, we clearly demonstrate that SAP opsonization enhances phagocytosis, specifically of ΔtagO mutant cells by PMNs, in an FcγRs-dependent manner. The requirement of FcγRs for SAP-mediated phagocytosis was confirmed independently, using FcγRII-expressing HEK293T cells and anti-FcγRII (CD32) mAb. In addition, we provided evidence that WTA-deficient S. aureus can be generated in phagolysosomes of PMNs. Recently, Sun et al. (30) reported that serum anti-PGN Abs induce delivery of S. aureus PGN into the phagocytes via FcγRs to activate intracellular nucleotide-binding and oligomerization domain (NOD) proteins that are known as NOD-like receptors (NLRs). In this study, because SAP also functions as an S. aureus PGN recognition protein and an inducer of FcγRs-dependent phagocytosis in serum, like anti-PGN Abs, it will be quite plausible for serum SAP to play a role similar to that of anti-PGN Abs.

From these results, together with those from our recent studies (26, 27), we developed a model describing the recognition mechanisms of the host innate immune responses against S. aureus invasion (Fig. 8). In human infants, who have not developed adaptive immunity, the serum MBL/MASP complex recognizes S. aureus WTA and induces activation of the complement via the lectin pathway, leading to clearance of S. aureus by opsonophagocytosis. Human adults have developed anti-WTA IgGs that directly recognize S. aureus WTA and activate the classical complement pathway, which triggers opsonophagocytosis of S. aureus (Fig. 8, right). Clearance of S. aureus can also be achieved following SAP recognition of PGN in the absence of WTA; SAP-mediated clearance occurs via FcγRs-dependent and complement-independent phagocytosis (Fig. 8, left). One possible explanation for the function of SAP in immunity is that recognition of cell wall component PGNs that are common to both Gram-negative and Gram-positive bacteria—namely, PGN recognition by a serum PRR—represents an ancient means of defense. In response, Gram-positive bacteria may have evolved WTA to conceal PGN to escape the SAP-induced host innate immune responses. An alternative model is that a host may possess an ability to remove WTA to allow SAP-mediated opsonophagocytosis.

At present, two PGN-recognizing protein families have been characterized for the mammalian immune system. Members of the first family, the so-called PGN recognition proteins (PGRPs), bind to and, in some cases, hydrolyze PGNs of the bacterial cell wall (38). A combined genomic and experimental approach has led to the identification of four PGRP family members, which are conserved in insects, mice, and humans (39). These proteins share a conserved 160-aa-long PGRP domain with significant sequence similarity to a family of bacteriophage and bacterial type 2 amidases that catalyze hydrolysis of the amide bonds in PGNs (40). The PGN binding domain of PGRPs binds the muramyl pentapeptide or tetrapeptide fragment of PGN with high affinity. Mammalian PGRPs were initially identified as PRRs regulating host innate immunity. Notably, recent studies have demonstrated that all four known PGRPs modulate the acquisition and maintenance of normal gut microbiota, which protects the host from inflammation, tissue damage, and colitis (41). Members of the second PGN-recognizing protein family contain NOD and NLRs (42). The NLRs, NOD1 and NOD2, are intracellular receptors for bacterial PGN fragments (42). The ligands of NOD1 and NOD2 were determined to be α-glutamyl-meso-diaminopimelic acid (43) and muramyl dipeptide, respectively; muramyl dipeptide is a PGN motif conserved widely among both Gram-positive and Gram-negative bacteria (44). Recent studies have demonstrated that NOD1 and NOD2 recognize a subset of pathogenic microorganisms able to invade a host cell and multiply intracellularly. Once activated, NOD1 and NOD2 trigger intracellular signaling pathways that elicit expression of inflammatory genes (45). In this article, we add SAP to the list of PGRP-like proteins that induce phagocytosis of bacteria by PMNs. Our SPR analysis revealed that SAP recognizes the monomeric unit of the PGN fragment. Despite this, the functional significance of SAP remains ambiguous. Our most significant observation is that WTA blocks the recognition of PGN by SAP; therefore, PGN is a cryptic ligand of SAP. Biochemical evidence that host PMNs remove WTA to allow detection of S. aureus PGN by SAP is provided by showing intracellular generation of WTA-deficient S. aureus cells inside PMNs after phagocytosis.

Acknowledgments

We thank Dr. Myung-Hee Nam (Korea Basic Science Institute, Seoul, Korea) for helping with the measurement of Biacore analyses.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Fig. S1

(A) Incubation
\[ \Delta tagO, \Delta spa + \text{intact serum} \]
\[ \Delta tagO, \Delta spa \text{ EDTA eluate} \]
10mM Tris 140mM NaCl 10mM EDTA pH 8.0

Buffer change
10mM Tris 140mM NaCl pH 7.4

Protein G sepharose column
10mM Tris 140mM NaCl pH 7.4

Eluate (Ig) Flow-through

Blue Sepharose column
10mM Tris 140mM NaCl pH 7.4

Flow-through Eluate (Ig)

Superdex 200 column
10mM Tris 140mM NaCl pH 7.4

Concentration

(B) Control \[ \Delta tagO, \Delta spa \text{ eluate} \]
After Protein G / Blue Sepharose Purified SAP

(C) SH(-), WB, anti-hSAP pAb

(D) SH(-), WB, anti-hMBL mAb

(E) Sera | SAP (ng/µl) | MBL (ng/µl) |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact serum</td>
<td>27.2</td>
<td>3.7</td>
</tr>
<tr>
<td>Depleted serum</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(F) C1q | Intact serum | Depleted serum |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SH(-), WB, anti-hC1q pAb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(G) C1s | Intact serum | Depleted serum |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>SH(-), WB, anti-hC1s pAb</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Supplemental Fig. S1. The purification procedures of SAP from human serum, and preparation of a depleted serum and its biochemical characterization.

(A) shows the general purification procedures of SAP. To purify SAP from human serum, S. aureus ΔtagO, Δspa double mutant cells (3.3 x 10^{11} cells) was fixed with 1% formaldehyde in the PBS at 37°C for 1 h. The fixed S. aureus cells were recovered by centrifugation (6,000 xg), washed twice with PBS, and incubated at 37°C for 30 min with ethanolamine (final concentration of 100 mM) to inactivate remaining formaldehyde. After washing with PBS three times, the fixed S. aureus cells were incubated with 50 ml of human serum at 4°C for 2 h, and washed twice with buffer A [10 mM Tris-HCl (pH 7.4) containing 140 mM NaCl, and 10 mM CaCl₂]. Bound proteins were eluted with buffer B [10 mM Tris-HCl (pH 7.4) containing 140 mM NaCl, and 10 mM EDTA]. After buffer change by ultrafiltration kit (10,000 MW cut off, Satorius), in order to deplete serum Igs, the proteins were loaded onto Protein-G Sepharose column (0.5 x 7 cm, GE healthcare) equilibrated with buffer C [10 mM Tris-HCl (pH8.0) and 140 mM NaCl], and then the flow-through fractions were loaded to Blue-Sepharose column (0.5 x 7 cm, GE healthcare) equilibrated with buffer C. The flow-through fractions of the Blue-Sepharose column (see lane 3 in B) were then loaded onto Superdex 200 column equilibrated with buffer C. The eluted fractions was analyzed by SDS-PAGE, and then fractions containing 25 kDa SAP were pooled and concentrated by ultrafiltration kit (10,000 MW cut off, Satorius). The purity of SAP was confirmed by SDS-PAGE (see lane 5 in B) and by determination of N-terminal amino acid sequence. From 50 ml human serum, 500 μg of pure SAP was obtained.

(B) shows 15% SDS-PAGE with CBB staining of obtained proteins from column chromatographies. Lanes 1-5 indicate the mixture of each 1 μg of Ig, HSA and SAP, EDTA-eluate from S. aureus ΔtagO, Δspa double mutant cells (10 μg), flow-through fraction of Blue-Sepharose columns (5 μg), a side fraction of Superdex 200 column (5 μg) and finally purified SAP (1 μg), respectively.

(C) Western blot analysis (WB) of SAP in a depleted serum. The depleted serum was obtained from a human intact serum (1 ml) by three-times successful incubations with a mixture of formaldehyde-fixed S. aureus ΔtagO, Δspa double mutant cells (1.3x 10^{10} cfu) and Δspa mutant cells (1.3x 10^{10} cfu) on ice for 30 min as described in materials and methods. Control SAP (50 ng, lane 1), intact serum (1μl, lane 2) and the depleted serum (1μl, lane 3) were separated by SDS-PAGE under non-reducing conditions [SH(-)], and SAP was detected using anti-SAP polyclonal Ab (pAb).

(D) MBL during preparation of the depleted serum. Control MBL (2 ng, lane 1), each elution fraction from the S. aureus pellet incubated with the serum (lanes 2-4), the depleted serum (2 μl, lane 5), and an intact serum (2 μl, lane 6) were separated, and MBL was detected using anti-MBL mAb (Dobeel, Seoul, Korea)

(E) Level of SAP and MBL in the intact and depleted serum were determined by ELISA. Soluble PGN (5 μg) or mannan (5 μg)-coated microplates were blocked with 200 μl of a buffer D [20 mM Tris-HCl (pH 7.4) 150 mM NaCl, and 1% BSA] for 2 h at room temperature and then incubated with 1-2 μl human intact or depleted sera in 50 μl of buffer E [10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 10mM CaCl₂, 1% BSA] for 2 h at 4°C. Wells were washed with washing buffer. After washing, the wells were incubated with rabbit anti-human SAP pAb (1:500) for SAP detection and anti-human MBL mAb (1:1000) for MBL detection in 50 μl of buffer E for 1 h at room temperature. After washing with buffer F [10 mM Tris-HCl, 140 mM NaCl (pH 7.4)], plates were incubated with secondary antibodies [goat anti-rabbit IgG (H-L) conjugated with horseradish peroxidase (HRP) for SAP (Enzo, 1:5000) and goat anti-mouse IgG (H-L) conjugated with HRP for MBL (Beckman Coulter, 1:10,000 dilution for MBL)] for 1 h at room temperature. After washing with buffer A, the resulting plates were developed with the substrate, 3,3’,5,5-tetramethylbenzidine (Zymed Laboratories Inc.) in the dark and stopped with 2 N H₂SO₄. The concentration was estimated from standard curve that was obtained from purified SAP and MBL. Absorbance at 450 nm was recorded using a microplate reader (Thermo Scientific).

(F) and (G) show Western blot (WB) of C1q and C1s, respectively. C1q (50 ng, a high molecular weight complex) or C1s (50 ng, 76 kDa) in lane 1, intact serum (1 μl, lane 2) or depleted serum (1 μl, lane 3) was separated and detected using anti-C1q polyclonal Ab (pAb) or anti-C1s pAb, respectively.
SUPPLEMENTAL FIG. S2. Purified SAP binds specifically to bacterial PGNs and induces complement-independent phagocytosis of WTA-depleted S. aureus PGN by PMNs.

(A) SAP binding abilities to PGNs purified from S. aureus and five other bacteria were tested. Insoluble PGNs (is-PGNs) were purified as described previously (1) with some modification. Briefly, cultured bacterial cells were disrupted using glass beads and centrifuged at 500 x g for 10 min. Supernatants were centrifuged again at 20,000 x g for 10 min, and resulting precipitates were re-suspended in 20 mM sodium citrate (pH 4.7) containing 0.5% (w/v) SDS, heated at 60°C for 30 min and centrifuged at 20,000 x g for 10 min. After removal supernatant, new precipitates were washed twice with buffer and distilled injection water. The resulting precipitates were re-suspended in 5% (w/v) trichloroacetic acid, incubated at room temperature for 18 h to remove WTA and centrifuged at 6,500 x g for 30 min. The resulting precipitates, which contained the is-PGN, were washed four times with distilled injection water and three times with acetone. Finally, precipitates were dried and suspended with distilled injection water, then lyophilized as purified is-PGNs. Soluble-PGN (s-PGN) was obtained as described previously (2) with some modification. Briefly, the prepared in-PGN (20 mg) was digested with lysostaphin (200 μg) for 14 h at 37°C and fractionated on a size exclusion column (Toyopearl HW-55S column, 2.6 x 155 cm). Fractions exhibiting insect phenoloxidase activity were collected and concentrated using a rotary evaporator at 4°C. The concentrated solution was fractionated a second time using the same column, previously equilibrated with distilled water at a flow rate of 0.5 ml/min. Fractions containing s-PGN were pooled and stored at 4°C until use. SAP binding to each PGN was examined as described in Fig. 2A.

(B) Comparison of SAP and CRP for binding to is-PGN. Following incubation of 2 μg SAP or CRP with 1 mg is-PGN for 1 h at 4°C as indicated, SAP or CRP in the supernatant or co-precipitated with is-PGN was analyzed by SDS-PAGE on a 15% gel under reducing conditions.

(C) SAP does not induce C4 deposition on S. aureus cells. Panels (a) and (g) show the relative C4 deposition on parental S. aureus Δspa and its ΔtagO mutant cells by intact serum, respectively; (b) and (h), C4 deposition after addition of SAP (1 μg) into (a) and (g), respectively; (c) and (i), C4 deposition after incubation of parental and ΔtagO mutant cells with a depleted serum, respectively. The depleted serum was as characterized in Supplemental Fig. S2. Panels (d) and (j), (e) and (k), (f) and (l) show C4 deposition after addition of SAP (1 μg), S. aureus-recognition Ig (1 μg) and MBL (50 ng) into panels (c) and (i), respectively. The serum concentration was 10%. C4 bound to S. aureus cells was detected by flow cytometry as described in the Materials and Methods. The gray area represents data from bacterial cells only.

(D) SAP induces complement-independent phagocytosis of WTA-depleted S. aureus PGN by PMNs. All the conditions are the same with Fig. 4B except for addition of WTA-depleted purified PGN (10 μg, columns 1-4) and WTA-attached purified PGN (10 μg, columns 5-8) instead of FITC-labeled S. aureus cells. Each PGN was labeled with 0.02 mM FITC (Sigma) in 0.1 M Na₂CO₃ (pH 8.5) for 30 min at room temperature, centrifuged, and suspended in in Hanks' balanced salt solution (HBSS). Resulting FITC-labeled PGN was incubated with SAP (1 μg) and depleted serum (10%) in a 20 μl buffer, respectively. A 5 μl portion of opsonized FITC-labeled PGN were incubated with human PMNs (1.5 x 10⁵ PMNs) in 40 μl RPMI 1640 medium at 37°C for 1 h. Phagocytosed FITC-labeled PGN in at least 100 PMNs were counted using a dye exclusion test. Data are the means ± SD of results of three independent experiments. Used PMNs are prepared as described previously (3); Briefly, four ml whole blood containing 40 μl heparin (1,000 units/ml) was layered on top of 4 ml Polymorphprep solution (Nycomed Pharma AS, Torshov, Norway). Following centrifugation at 450 x g for 30 min at 20°C, both the plasma and mononuclear phases were carefully discarded. The PMN phase (1.5 ml) was transferred into another tube and mixed with 1.5 ml of RPMI medium 1640 (50%). Then, PMNs were pelleted by centrifugation at 250 x g for 5 min at 20°C, washed three times with 4 ml RPMI medium 1640, and resuspended in 1 ml RPMI medium 1640 containing 0.4% HSA and 10 mM HEPES. PMNs were counted using a Burker-Turk cell counting chamber and diluted to 4.3 x 10⁶ cells/ml with RPMI medium 1640 containing HSA and HEPES. PMN viability was determined to be greater than 95% using a dye exclusion test.

References