Staphylococcus aureus Fibronectin-Binding Protein (FnBP)–Mediated Adherence to Platelets, and Aggregation of Platelets Induced by FnBPA but Not by FnBPB

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Background. The ability of Staphylococcus aureus to adhere to platelets and to induce aggregation of platelets is considered to be a critical factor in S. aureus–associated infective endocarditis.

Methods. To identify and characterize further bacterial factors involved in the S. aureus–platelet interaction, we generated a phage-display library of S. aureus genomic DNA by use of the improved phagemid vector pG8SAET. The library was affinity-panned against gel-filtered, immobilized platelets.

Results. Repeatedly isolated clones contained overlapping DNA fragments encoding a portion of the S. aureus fibronectin (Fn)–binding proteins (FnBPs). In a flow cytometric adherence assay, Staphylococcus carnosus that heterologously expressed either fnbA or fnbB, which encode FnBPA and FnBPB, respectively, showed increased adherence to activated, gel-filtered platelets. Adherence was promoted by the addition of Fn or fibrinogen (Fg), which most likely act as bridging molecules. Interestingly, promotion of adherence mediated by Fn was in the same range with S. carnosus producing either FnBPA or FnBPB, whereas promotion of adherence mediated by Fg was significantly more pronounced with S. carnosus that produce FnBPA than with S. carnosus that produce FnBPB. Furthermore, FnBPA, but not FnBPB, mediated aggregation of platelets when present on S. carnosus cells.

Conclusion. Our results indicate a substantial functional difference between FnBPA and FnBPB in the S. aureus–platelet interaction.

In the past decade, Staphylococcus aureus has emerged as a dominant cause of acute infective endocarditis (IE), which often is fatal, with mortality of 20%–40% [1]. The pathogenesis of IE is characterized by a series of events: initial endocardial damage results in exposure of the subendothelium and subsequent deposition of platelets and fibrin. The fibrin-platelet matrix on the damaged valves may serve as foci for adhering bacteria that circulate in the blood [2]. Alternatively, bacteria may adhere to the undamaged endothelium either directly through adhesin-receptor interactions or through mediation by bridging ligands, such as the plasma and extracellular matrix proteins fibrinogen (Fg) and fibronectin (Fn). Attached bacteria may then cause further aggregation of platelets, leading to an enlargement of the infected vegetation on the valves [2].

The interaction of bacteria with platelets is critical for the development of IE. It has been shown in vitro that surface-immobilized activated platelets are able to promote adherence of S. aureus [3]. Extracellular matrix proteins, such as Fg, Fn, and thrombospondin, may also play a role in this process, by acting as bridging molecules and thus enhancing the S. aureus–platelet interaction [4, 5]. In addition, the direct binding of bacteria to platelet surface components was postulated to be a central mechanism in the pathogenesis of IE.
In recent years, significant progress has been made in the identification of *S. aureus* surface proteins that are able to mediate adherence to platelets and to promote endocarditis. In particular, *S. aureus* surface proteins of the MSCRAMM (microbial surface components recognizing adhesive matrix molecules) family [7] have been implicated in this process. Thus, protein A (encoded by *spa*), which binds IgG and von Willebrand factor [8], has been identified as a surface protein conferring *S. aureus* binding to platelets via the platelet membrane protein gC1qR/p33 [9]. Furthermore, the Fg-binding clumping factor A (CIFA) is involved in the development of experimental endocarditis, which seems, at least in part, to be mediated through its direct binding to a novel 118-kDa platelet membrane receptor [10–12].

The surface display of portions of bacterial adhesins on hybrid phages, followed by affinity selection, is a useful tool to identify and characterize protein-ligand interactions. We recently found that the C-terminal domain of the *S. aureus* coagulase and the N-terminal domain of the extracellular Fg-binding protein (Efb) are involved in platelet binding, probably via Fg as a bridging molecule, by use of the phage-display technique [13]. However, ~50% of the eluted phagemids did not contain an insert with an open-reading frame (ORF) and thus represented nonspecifically bound phagemids. Therefore, to identify and characterize further *S. aureus* adhesins involved in the *S. aureus*-platelet interaction, we generated a new phage-display library by use of the improved phagemid vector pG8SAET. This vector allows the identification of ORFs by detection of the artificial E-tag and has proven to be suitable when complex mixtures of ligands, such as eukaryotic cells, are used in the pannings [14, 15]. Analogous to our former study, platelet-binding hybrid phages were affinity-selected via panning against gel-purified, immobilized platelets. By use of this approach, we were able to identify the *S. aureus* Fn-binding proteins (FnBPs) as mediators of *S. aureus* adherence to platelets. Furthermore, we found that FnBPA, but not FnBPB, is able to induce aggregation of platelets.

**MATERIALS AND METHODS**

**Bacterial strains, phagemid vector, helper phage, media, and reagents.** The clinical isolate *S. aureus* 4074 [13] was used as the DNA donor strain, and the phagemid pG8SAET [15] served as the vector in the construction of the phage-display library. *Escherichia coli* TG1 was used to construct the library and to produce the enriched phage stocks [16]. Phage R408 (Promega) was used as a helper phage. *Staphylococcus carnosus* (pFNBA4) and *S. carnosus* (pFNBB4), which express *fnbA* and *fnbB* from *S. aureus* 8325-4 [17], respectively, were used to analyze the platelet-binding potential of FnBPA and FnBPB. Plasmids pFNBA4 and pFNBB4 have been described elsewhere [18]. *S. aureus* Cowan 1 (ATCC 12598) and *S. carnosus* TM300 [19] served as positive and negative controls, respectively.

Staphylococcal strains were grown on blood agar plates or on brain-heart infusion (BHI) plates, in BHI broth (Difco) or B-medium [19], containing 10 μg/mL chloramphenicol when appropriate. *E. coli* strains were cultivated in Luria-Bertani (LB) medium (Difco) or on LA plates (LB medium with 1.5% agar; Difco), which contained 50 μg/mL ampicillin when appropriate.

Bovine serum albumin (BSA), α-thrombin (bovine), and Fn from human plasma were purchased from Sigma. The peptide GPRP (Gly-Pro-Arg-Pro) was purchased from Bachem AG. Human Fg was purchased from Enzyme Research Labs. Monoclonal antibody against human CD42a (GP IX) (conjugated with phycoerythrin [PE]) was purchased from Exalpha via NatuTec. Syto 13 for labeling of *S. aureus* was purchased from Molecular Probes via Mobitec.

**DNA manipulations, transformation of *E. coli*, preparation of phage stocks, and DNA sequencing.** DNA manipulations, transformation of *E. coli*, and preparation of phage stocks were performed according to standard procedures [20]. Preparation of chromosomal and plasmid DNA, polymerase chain reaction (PCR), and DNA sequence analysis were performed as described elsewhere [13]. The following primers were used to amplify the inserts of isolated hybrid phagemids by PCR and for DNA sequencing: CH60 (5′-TGGTGGCGTAACACCTGC-3′) and CH61 (5′-GATCCCTAGGCACGGGTTCGCC-3′).

**Construction of the phagemid library.** The phagemid library was constructed essentially as described elsewhere [13], except that the vector was linearized with SnaBI.

**Preparation of platelets.** Blood was obtained from healthy adult volunteers who had not taken any medication affecting platelet function for at least 2 weeks before the study. All experiments were performed with the informed consent of the blood donors. Platelets for panning were purified as described elsewhere [13]. For flow-cytometric analysis, platelet-rich plasma (PRP) was prepared from anticoagulated blood by centrifugation, as described elsewhere [21], and the platelets were gel-filtered on a Sephadex CL-2B column [21]. In some experiments, Fn or Fg (25, 50, or 100 μg/mL) was added. To inhibit fibrin polymerization, experiments were performed in the presence of the peptide GPRP (1.25 mmol/L) [21]. The platelets were labeled by incubation for 30 min with a monoclonal antibody against CD42a (GP IX) conjugated with PE, at saturated concentrations.

**Panning procedure.** Panning against platelets was performed as described elsewhere [13], except that 3 instead of 2 rounds of panning were performed.

**Screening for expression of the E-tag.** Expression of the E-tag was detected as described elsewhere [15], except that nitrocellulose membranes were blocked with 1% BSA in Tris-
Preparation of bacteria for flow-cytometric platelet-adherence assay and measurement of the formation of S. aureus–platelet associates. Bacteria grown to exponential phase (OD_{600nm} of 0.8) or to stationary phase (16 h) in BHI broth were washed in TBS (pH 7.4), briefly sonicated (10 cycles, each of 1 s, 50 W; Branson), and diluted with TBS (containing 2 mmol/L Ca^{2+}) to a concentration of 250,000 bacteria/µL. Microorganisms were labeled with the fluorescent dye Syto 13 (emission similar to fluorescein isothiocyanate [FITC]), at a concentration of 2 µmol/L, for 10 min, washed in TBS, and again briefly sonicated as described above.

Platelets were activated with α-thrombin at the given concentrations for 4 min, and labeled bacteria were added subsequently. Bacteria and platelets (10:1) were coincubated for 15 min at room temperature, and formation of conjugates was measured immediately thereafter (FACSCalibur flow cytometer; Becton Dickinson). Associates were identified by use of double labeling with Syto 13 (FL-1; “FITC-like” signal) and anti-CD42a-PE (FL-2; PE signal) and are given as the rate of bacteria-positive platelets. Shown are the mean values of 3 independent experiments.

Measurement of aggregation of platelets. Aggregation of platelets was assayed essentially as described elsewhere [22], except that staphylococcal cells were added to PRP adjusted with platelet-poor plasma to a concentration of platelets/µL or to gel-filtered platelets (GFPs) adjusted to a concentration of 2×10^6 platelets/µL. Platelet function was confirmed according to the criteria of Clarke and Carbon [23], the library was representative of the S. aureus genome. Therefore, portions of all proteins encoded by the genome should be expressed on the phage surfaces in fusion to the major coat protein VIII (pVIII) of the M13 phage.

Panning of the Library against Platelets
To identify further platelet-binding domains of bacterial receptors, the library was affinity-panned against immobilized GFPs. After the third panning, an ∼20-fold enrichment of platelet affinity-selected phages was observed. Previous results have shown that this enrichment is specific when bacteria possess a receptor for the ligand used in the panning and that there is no enrichment observed when bacteria do not express a specific receptor, as shown in pannings against human serum albumin [13, 24]. To identify potentially correct clones (i.e., phagemid clones carrying an insert containing an ORF that corrects the reading frame so that the artificial E-tag and, thus, gene VIII [encoding pVIII] is expressed), we performed colony blot analysis and further analyzed E-tag–positive clones.

Analysis of Affinity-Selected Clones by Panning against Platelets
Size determination of inserted DNA fragments. Plasmid DNA of 57 E-tag–positive colonies obtained after the third panning against platelets was prepared and used to amplify the inserted DNA fragment by use of PCR. Analysis of the fragments by electrophoresis in 2% agarose gels revealed that all clones were isolated repeatedly and contained 1 of the following DNA fragments: 700, 580, 390, 330, 300, or 290 bp.

Nucleotide sequence analysis of inserted DNA fragments. The nucleotide sequence of the amplified DNA fragments was determined. Sequence analysis of the 330-bp (represented by clone Pf) and 300-bp (clone Pg) fragments revealed that they share identical nucleotide sequences encoding the same protein domain in fusion with pVIII. Comparison of the deduced amino acid sequences of clones Pf (112 aa) and Pg (100 aa) with sequences of known proteins in the Swiss-Prot database (available at: http://www.ebi.ac.uk/swissprot) revealed identity or high similarity with a portion of the central domain of the S. aureus FnBPs (figure 1A). Alignment of the clones Pf and Pg with the respective domains of the FnBPs revealed 100% identical amino acids with the D domain of FnBP from strain MW2 [26], 98% identical amino acids with the same domain of FnBP from strain N315 and Mu50 [27], 98% identical amino acids (Pf) or 99% identical amino acids (Pg) with the homologous D domain of FnBPA from strain 8325-4 [28], and 97% identical amino acids with the homologous D domain of FnBPB from strain 8325-4 [29] (figure 1B). All other DNA fragments encoded either the N-terminal domain of Efb or the C-terminal portion of the coagulase, confirming our previous results [13].

Flow-Cytometric Analysis of the Platelet-Binding Capacity of S. aureus FnBPA and FnBPB
To elucidate the role that the FnBPs play in the S. aureus–platelet interaction, we performed a flow-cytometric platelet

Adherence of S. aureus to Platelets • JID 2004:190 (15 July) • 323
Figure 1. A. Schematic map of fibronectin (Fn)-binding protein A (FnBPA) of *Staphylococcus aureus* strain 8325-4 aligned with the polypeptide sequences expressed by the phagemid clones Pf and Pg, which were affinity-selected by panning against immobilized platelets. SP, signal sequence; A, Fg-binding domain; B, region containing 2 repeats with unknown function; C, region containing the Du repeat, which has Fn-binding activity; D, region containing 4 repeats with Fn-binding activity; W, cell wall–spanning region; LPXTG, motif that is the target of the sortase, which anchors the protein to the cell-wall peptidoglycan [25]; M, membrane-spanning region.

B. Alignment of the amino acid sequences expressed by the phagemid clones Pf and Pg with the respective sequences from FnBPB from *S. aureus* strain MW2, FnBPA and FnBPB from *S. aureus* strain 8325-4, and FnBPA from *S. aureus* strain N315. The positions of the respective D domains of FnBPA from strain 8325-4 are indicated. Asterisks indicate identical amino acids; colons indicate highly similar amino acids; and periods indicate somewhat similar amino acids. Gaps (—) were filled in to maximize homologies.

adherence assay. Because of the redundancy of mechanisms that are involved in the binding of *S. aureus* to platelets, it is difficult to assess the function of an isolated factor in the *S. aureus* background. Indeed, we could not find a difference in platelet adherence between the *S. aureus* wild-type (wt) strains Newman and 8325-4 and their isogenic fnbA/fnbB double-knockout mutant strains (data not shown). Therefore, we heterologously expressed the genes encoding FnBPA and FnBPB (*fnbA*, encoded by the plasmid pFNBA4, and *fnbB*, encoded by the plasmid pFNBB4) in the nonpathogenic and non–platelet-binding host strain *S. carnosus* [17]. The platelet-binding activity of *S. carnosus* (pFNBA4) and *S. carnosus* (pFNBB4), compared with that of the *S. carnosus* wt strain as a negative control and that of *S. aureus* Cowan 1 as a positive control, was analyzed. Adherence of stationary-phase *S. aureus* Cowan 1, *S. carnosus* (pFNBA4), *S. carnosus* (pFNBB4), and *S. carnosus* TM300 to nonactivated platelets in PRP was low and within the same range (~2.5%–3.5% bacteria-positive platelets) (figure 2A). After platelet activation with different concentrations of α-thrombin, adherence of strains *S. aureus* Cowan 1 (16% bacteria-positive platelets, 1 U/mL thrombin; ) and *S. carnosus* (pFNBB4) (9.5% bacteria-positive platelets, 1 U/mL thrombin; ) was significantly increased. In contrast, adherence of *S. carnosus* TM300 was not altered. Adherence of strains *S. aureus* Cowan 1, *S. carnosus* (pFNBA4), and *S. carnosus* (pFNBB4) was not increased or was only slightly increased by the addition of 50 μg/mL Fn (figure 2B).

To clarify the role that plasma proteins play in that interaction, we performed the flow-cytometric adherence assay us-
Mediation of staphylococcal adherence to activated platelets by fibronectin (Fn)-binding protein A (FnBPA) and FnBPB. Adherence of staphylococcal strains to nonactivated (0 U/mL, thrombin) or activated (0.1, 0.2, 0.5, and 1 U/mL thrombin) platelets in platelet-rich plasma without (A) or with (B) the addition of 50 μg/mL Fn was determined by use of flow cytometry. Bacteria-platelet associates were identified by staining with Syto 13 and glycoprotein CD42a phycoerythrin-labeled antibody. Staphylococcus carnosus TM300; ◆, S. carnosus (pFNBA4); □, S. carnosus (pFNBB4); ×, Staphylococcus aureus Cowan 1. Data are expressed as mean ± SD (n = 3).

DISCUSSION

In the present study, we have described the successful construction of a S. aureus–derived phage-display library by use of the improved vector pG8SAET. After panning against immobilized GFPs, we repeatedly isolated clones that contained overlapping DNA fragments (Pf and Pg) encoding the Fn-binding domain of S. aureus FnBPs. In our previous investigation, we could not identify the FnBPs as platelet-binding adhesins, thus confirming the suitability of the pG8SAET vector for use in a phage-display library when complex ligands, such as eukaryotic cells, are used in the panning experiments [14, 15]. S. aureus produces 2 closely related FnBPs, FnBPB and FnBPB. FnBPs have been implicated in the pathogenesis of S. aureus infection by facilitating attachment of the bacteria to host cells and to Fn-coated polymer material and by acting as invasins to cultured host cells [31–33].

Adherence of S. aureus to Platelets • JID 2004:190 (15 July) • 325
However, to our knowledge, a role for the FnBPs in the \textit{S. aureus}–platelet interaction has not been suggested before.

To dissect the role that the FnBPs play in the \textit{S. aureus}–platelet interaction, we used a flow-cytometric adherence assay and \textit{S. carnosus} strains that heterologously express the \textit{S. aureus} genes \textit{fnbA} and \textit{fnbB} from strain 8325-4 encoding FnBPA and FnBPB, respectively. We found that \textit{S. carnosus} (pFNBA4) and \textit{S. carnosus} (pFNBB4) showed increased adherence to activated platelets, compared with the \textit{S. carnosus wt} strain. Previous results suggested that FnBPs do not play a role in the induction of endocarditis by \textit{S. aureus} [34]. However, the results of the present study strongly suggest that FnBPs play a role in the induction and/or propagation of IE caused by \textit{S. aureus} and thus support the recent findings of Que et al. [12], who reported that \textit{Lactococcus lactis} cells expressing the \textit{fnbA} gene have a 100-fold higher infectivity than do \textit{L. lactis} cells harboring an empty vector, in an experimental rat endocarditis model, and thus correspond to the infectivity of \textit{S. aureus} [12].

Because we isolated hybrid phages expressing the Fn-binding domain of FnBPs, we analyzed the capacity of Fn to act as a bridging molecule and to enhance the FnBP-mediated platelet binding. We found that the addition of Fn to activated platelets greatly enhanced binding of \textit{S. carnosus} (pFNBA4) and \textit{S. carnosus} (pFNBB4) to platelets, strongly suggesting that Fn indeed acts as a bridging molecule. \textit{S. aureus} FnBPs are also known to contain an Fg-binding site in their A domain [35]. Therefore, we analyzed the ability of Fg to promote FnBP-mediated adherence to platelets. Our results demonstrate that Fg also is able to promote the adherence of \textit{S. carnosus} producing either FnBPA or FnBPB to platelets, probably by acting as a bridging molecule. Of interest, promotion of adherence to activated GPs mediated by Fn is very similar with \textit{S. carnosus} (pFNBA4) and \textit{S. carnosus} (pFNBB4), whereas promotion of adherence to activated GPs mediated by Fg is more pronounced with \textit{S. carnosus} (pFNBA4) than with \textit{S. carnosus} (pFNBB4). This difference seems to be statistically significant, at least when 50 \( \mu g \)/mL Fg was added (\( P = .01 \)) (figure 3D). The Fg-binding activity in FnBPA was located in the 500-aa A domain [35]. In that
Adherence of S. aureus to Platelets

Figure 4. Mediation of platelet aggregation by fibronectin-binding-protein A (FnBPA), but not by FnBPB. Staphylococcal cells were added to platelet-rich plasma, and platelet aggregation was assayed by use of light transmission using a P.I.C.A. aggregometer (Chrono-log). The results of 1 representative experiment are shown.

Figure 5. Model of the proposed events involved in the fibronectin (Fn)–binding protein A (FnBPA)–mediated (A) or FnBPB-mediated (B) interaction of Staphylococcus aureus (SA) with activated platelets. I: The D domain of FnBPA (A) or FnBPB (B) from SA binds to the integrin $\alpha_\beta_1$, via Fn as a bridging molecule. II: The D domain of FnBPA (A) or FnBPB (B) from SA binds to the GPIIb/IIIa platelet receptor via Fn as a bridging molecule. III: A portion of the A domain of FnBPA (A) or FnBPB (B) from SA binds to the GPIIb/IIIa platelet receptor via fibrinogen (Fg) as a bridging molecule. IV: A portion of the A domain of FnBPA (A), but none of FnBPB (B), binds directly to an unidentified platelet receptor ($\times$). Afterward, FnBPA (A), but not FnBPB (B), induces platelet aggregation. The events I to III (at least in part) also seem to be required for the induction of platelet aggregation, because the presence of plasma proteins is essential (see above).
as suggested by the results of a Western ligand assay [17]. Furthermore, S. carnosus (pFNBA4) and S. carnosus (pFNBB4) both exhibit invasion of 293 cells to a similar extent [17].

The addition of either Fn or Fg in the flow-cytometric adherence assay greatly enhanced the adherence of S. carnosus (pFNBA4) and S. carnosus (pFNBB4) to platelets and that S. carnosus (pFNBA4) induce aggregation of platelets only in PRP, but not in GFPs, suggests that the plasma proteins function as bridging molecules between S. aureus FnBPs on one side and a platelet receptor on the other side (figure 5). Platelet receptors that are potentially involved in that interaction are the Fg receptor GPIIb/IIIa, which also binds Fn and represents the most abundant receptor on platelets (60,000–100,000 copies/platelet), and the Fn receptor integrin α5β1. During activation of platelets, Fg and Fn are secreted from α-granules and then are bound by the GPIIb/IIIa platelet receptor. Previously, it was suggested that S. aureus–mediated aggregation of rabbit platelets is dependent on Fg but independent of GPIIb/IIIa, because it was not inhibited by the RGDS (Arg-Gly-Asp-Ser) peptide, which is an antagonist of GPIIb/IIIa [36]. However, recently, ClfA, ClfB, and the serine-aspartate repeat protein SdrE were found to be able to aggregate human platelets when the respective genes were expressed in the nonaggregating host L. lactis, which is necessary to develop new strategies to combat invasive S. aureus disease, such as IE.

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References