SdrG, a fibrinogen binding bacterial adhesin of the MSCRAMM subfamily from Staphylococcus epidermidis, targets the thrombin cleavage site in the Bβ chain

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Running title: Binding specificity of SdrG
SUMMARY

*Staphylococcus epidermidis* is an important opportunistic pathogen and is a major cause of foreign body infections. We have characterized the ligand-binding activity of SdrG, a fibrinogen-binding MSCRAMM from *S. epidermidis*. Western ligand blot analysis showed that a recombinant form of the N-terminal A-region of SdrG bound to the native Bβ chain of fibrinogen (Fg) and to a recombinant form of the Bβ chain expressed in *E. coli*. By analyzing recombinant truncates and synthetic peptide mimetics of the Fg Bβ chain, the binding site for SdrG was localized to residues 6-20 of this polypeptide. Recombinant SdrG bound to a synthetic 25 amino acid peptide (β1-25) representing the N-terminus of the Fg Bβ chain with a K_D of 1.4 x 10^{-7} M as determined by fluorescence polarization experiments. This was similar to the apparent K_D (0.9 x 10^{-7} M) calculated from an ELISA where SdrG bound immobilized Fg in a concentration dependent manner. SdrG could recognize fibrinopeptide B (residues 1-14), but with a substantially lower affinity than that observed for SdrG binding to synthetic peptides β1-25 and β6-20. However, SdrG does not bind to thrombin digested Fg. Thus, SdrG appears to target the thrombin cleavage site in the Fg Bβ chain. In fact, SdrG was found to inhibit thrombin-induced fibrinogen clotting by interfering with fibrinopeptide B release.
INTRODUCTION

Coagulase-negative staphylococci (CNS) are important opportunistic pathogens that are particularly associated with foreign body infections in humans. *Staphylococcus epidermidis* is the most common pathogenic species of CNS\(^1\) and accounts for 74-92% of the infections caused by this group of staphylococci (1).

The molecular pathogenesis of most infections is complex and involves multiple microbial factors and host components, but is generally initiated by the adherence of the microbe to host tissues. Bacterial adherence involves specific surface components called adhesins. Bacterial pathogens, such as staphylococci that live in the extracellular space of the host, target extracellular matrix (ECM) components, including fibrinogen (Fg) and fibronectin, for adherence and colonization. This process is mediated by a sub-family of adhesins that have been termed MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (2). *Staphylococcus aureus* expresses multiple MSCRAMMs of which several have been characterized in some detail (For a recent review see Ref. 3).

In addition to *S. epidermidis*, *S. aureus* also causes serious foreign body infections. *S. aureus* appears to adhere to the biomaterial through an indirect mechanism. Upon implantation, the foreign body rapidly becomes coated with host proteins derived primarily from plasma with Fg being a dominant component. *S. aureus* appears to adhere to the absorbed proteins rather than to the biomaterial itself using adhesins of the MSCRAMM family (4,5). At least four of the *S. aureus* MSCRAMMs recognize Fg. Two of these MSCRAMMs, clumping factor A and B (ClfA, ClfB), have Fg-binding A-regions followed by a long segment of Ser-Asp (SD) dipeptide repeats. The other two Fg-binding MSCRAMMs, contain a similar ligand binding A-region followed by a fibronectin binding motif that is repeated 5 times (6). Because the fibronectin binding activity was identified first, these two MSCRAMMs are known as fibronectin binding protein A and B (FnbpA...
and FnbpB) (7,8). Studies have demonstrated the importance of ClfA and ClfB in the adherence of *S. aureus* to plasma-coated biomaterials. *S. aureus* mutants deficient in one or both of these MSCRAMMs exhibited an impaired ability to adhere to plasma-coated catheters *in vivo* or *ex vivo* (9,10).

For *S. epidermidis*, adherence to foreign bodies could involve both specific and non-specific processes. The bacteria may initially associate directly with the foreign body through non-specific interactions, while the later stages of adherence may involve more specific interactions between bacterial adhesins and host ligands. *S. epidermidis* expresses polysaccharide adhesins including PS/A and PIA, which are encoded by the *ica* locus (11,12). In addition, we (13) and others (14) have recently shown that *S. epidermidis* contains surface proteins structurally related to *S. aureus* MSCRAMMs. Two of these *S. epidermidis* proteins, called SdrF and SdrG, have features typical of Gram-positive bacterial proteins that are anchored to the cell wall. Both proteins show significant amino acid sequence homology to ClfA and ClfB from *S. aureus* including an ~500 amino acid long A region, a SD dipeptide repeat region and features required for cell wall anchoring, including a LPXTG motif (Fig.1A). Recent studies by Pei, *et al.* suggest that one of these *S. epidermidis* proteins, SdrG (also called Fbe), can bind Fg and specifically recognizes the Bβ chain of this molecule (15). In the current study, we have localized the SdrG binding site in the Fg Bβ chain to the N-terminal segment of this polypeptide, proximal to the thrombin cleavage site. In fact, we have demonstrated that SdrG inhibits thrombin-induced fibrin clot formation by interfering with the release of fibrinopeptide B.
EXPERIMENTAL PROCEDURES

**Bacterial Strains and Growth Conditions**

*Escherichia coli* strain JM101 was used for plasmid cloning. *E. coli* strain Topp3 (Stratagene) was used for protein expression. Strains harboring plasmids were grown in Lennox L broth (Sigma) or on Lennox L agar (Sigma) supplemented with 100 µg/ml ampicillin.

**PCR Amplification of the sdrG Gene Fragment**

The gene fragment encoding the entire A-region was amplified by PCR using *S. epidermidis* K28 genomic DNA as a template. The oligonucleotide primers used were 5'-CCCGGATCCGAGGAGAATACAGTACAAGACG-3' and 5'-CCCGGTACCGATTTTTTCAGGAGGCAAGTCACC-3'. The restriction enzyme cleavage sites (underlined) *BamHI* and *KpnI* were incorporated into the forward and reverse primers, respectively. The reactions were carried out using a Perkin-Elmer DNA thermocycler. The reactions contained 50 ng of template DNA, 100 pmol of forward and reverse primers, 20 mM Tris-HCl (pH 8.8), 2 mM MgSO<sub>4</sub>, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 0.1% Triton X-100, 25 mM of each dNTP, and 5 units of *Pfu* DNA polymerase (Stratagene). Amplification was performed at 94°C for 1 min, 50°C for 1 min, 72°C for 4 min, for 25 cycles.

**Cloning of sdrG into the Expression Plasmid**

The amplified sdrG fragment was digested with *BamHI* and *KpnI* and ligated into the expression plasmid pQE30 (Qiagen Inc.) that had been digested with the same enzymes, yielding the construct pSdrG(50-597). The recombinant protein rSdrG(50-597) expressed from this plasmid contains an N-terminal extension of six histidine residues (His-tag).

**Expression and Purification of Recombinant MSCRAMM Protein**

*E. coli* transformed with pSdrG(50-597) was grown for ~2 h for the cultures to give an OD$_{600}$ of 0.6. rSdrG(50-597) expression was induced by the addition of isopropyl-β-thiogalactopyranoside (IPTG) (Gibco-BRL) (225 µM) and the cultures were incubated at 37°C for an additional 3 h.
Bacteria were pelleted and resuspended in phosphate buffered saline (PBS), pH 7.5 (140 mM NaCl, 270 µM KCl, 430 µM Na₂HPO₄, 147 µM KH₂PO₄) and frozen o/n at –20°C. Bacterial cells were thawed and mechanically lysed by using a French Pressure Cell (SLM Amnico). Cell debris was removed by centrifugation and filtration through a 0.45 µm filter membrane. The supernatant containing the recombinant protein was applied to a Ni²⁺ charged (87.5 mM) 5 ml Hi Trap chelating column (Amersham Pharmacia Biotech) connected to a FPLC system. The column was equilibrated with buffer A (0.1 M NaCl, 10 mM Tris-HCl, pH 8.0) before the application of the filtered lysate. The column was then washed with 10 bed volumes of buffer A containing 5 mM imidazole. Bound protein was eluted with a continuous linear gradient of imidazole (5 -120 mM; total volume 160 mls) in buffer A. Fractions were monitored for protein by determining the absorbance at 280 nm and fractions containing rSdrG(50-597) were identified by SDS-PAGE (16). These fractions were pooled and dialyzed against PBS, pH 7.5. The dialyzed protein was then applied to a Q-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 25 mM Tris-HCl, pH 8.0. Bound protein was eluted with a continuous linear gradient of NaCl (0-0.5 mM; total volume 160 mls) in 25 mM Tris-HCl, pH 8.0. Fractions containing the purified rSdrG(50-597) were identified by determining the absorbance at 280 nm and by SDS-PAGE. The truncated A region of ClfA was purified as previously reported (17).

Synthetic Peptides-The synthetic Fg peptides β1-25, β1-25S, β1-20, β6-25, were custom ordered from Research Genetics and the fibrinopeptides A and B (FpA and FpB) were from Bachem. Peptides β6-20 and β11-20 were synthesized in our laboratory using a multiple peptide synthesizer by Advanced Chemtech. For the following peptides the residue numbers are given and the sequence follows (Residue 1 corresponds to the first residue of the mature Bβ chain.): peptide β1-25, is composed of the first 25 amino acid residues of the N-terminus of the Bβ chain of Fg (QGVNDNEEGFSARGHRPLDKKREE), peptide β1-20 (QGVNDNEEGFSARGHRPLD), peptide β6-25
(NEEGFFSARGHRPLDKKREE), peptide β1-25S is a scrambled version of peptide β1-25 (FSERKDLHQEGNPFREVENDAKGR), peptide β6-20 (NEEGFFSRGHRPLD), peptide β11-20 (FSARGHRPLD), FpA (ADSESEGDFLAEGGGVR), and FpB (QGVNDNEEGFFSAR). Peptides were purified by HPLC and analyzed by MALDI mass spectrometry.

**ELISA**-Microtiter plates (Immulon 4, Dynatech Laboratories Inc.) were coated with 1 µg of Fg (Enzyme Research Labs) in PBS, pH 7.5 for 18 h at 4°C. Plates were washed three times with PBS, 0.05% Tween 20 (PBST) and blocked with 1 % (w/v) bovine serum albumin (BSA) for 1 h at room temperature. Plates were washed three times with PBST and rSdrG(50-597), diluted into PBS, was added to the wells and the plate was incubated for 1 h at room temperature. Plates were washed three times with PBST and bound rSdrG(50-597) was detected by adding a 1:2000 dilution of an anti-His-tag mAb (Clontech) in PBST, 0.1% BSA. Plates were incubated for 1 h at room temperature and then washed three times with PBST. A 1:2000 dilution of goat anti-mouse alkaline phosphatase (AP)-conjugated polyclonal antibodies (Bio-Rad) in PBST, 0.1% BSA were added to the wells and the plate was incubated for 1 h at room temperature. Plates were washed three times with PBST and developed with p-nitrophenyl phosphate (Sigma) in 1 M diethanolamine, 0.5 mM MgCl₂, pH 9.0 at room temperature for ~30 min. Plates were read at 405 nm using an ELISA plate reader (Thermomax microplate reader, Molecular Devices).

In the inhibition experiments, 50 nM rSdrG(50-597) in PBS was pre-incubated with the indicated amounts of selected peptides for 1 h at room temperature. The sample mixtures were added to the Fg-coated wells and bound rSdrG(50-597) was detected as described above.

For the ELISA with thrombin-digested Fg, the plate was coated with Fg and blocked as described above. The plate was washed three times with PBST and 50 µl of 1.0 NIH
unit/ml of thrombin was added to the Fg coated wells. The plate was incubated at 37°C for 30 min. The plate was washed three times with PBST and 50 µl of 1.0 NIH unit/ml of hirudin (Sigma) was added to the wells and incubated at 37°C for 30 min. The plate was washed three times with PBST and blocked with 1% BSA for 1 h at room temperature. After washing three times with PBST, 100 µl of biotin labeled rSdrG(50-597) (25-1000 nM) or a rSdrG(50-597)/hirudin (1.0 NIH unit/ml) mixture was added to the wells and incubated for 1 h at room temperature. The plate was washed three times with PBST and a 1:5000 dilution of streptavidin-AP conjugated (Boehringer Mannheim) in PBST/0.1% BSA was added to the wells for 1 h at room temperature. The plate was washed three times with PBST and developed as described above.

Construction of Fg Bβ Chain Truncates-An E. coli strain harboring plasmid p668 which contains the cDNA for the Fg Bβ chain was kindly provided by Dr. Susan T. Lord (University of North Carolina, Chapel Hill, NC). The 1525 bp fragment from p668 was subcloned into the plasmid pQE30 to produce recombinant mature Bβ chain with a N-terminal His-tag. Additional Bβ chain constructs (Fig. 3A) were made by subcloning into either pQE30 or pGEX-KG (Pharmacia) to produce recombinant proteins with a N-terminal His-tag or Glutathione S-transferase (GST) fusion.

Western Ligand Blot Analysis-Whole E. coli lysates harboring each respective Fg Bβ chain construct were fractionated by SDS-PAGE and the separated proteins were transferred to nitrocellulose membrane with a semi-dry transfer cell (Bio-Rad). The membrane was incubated overnight with 5% (w/v) non-fat dry milk in PBS, pH 7.5 at 4°C to saturate non-specific binding sites. After blocking, the membrane was washed three times with PBST and then incubated with biotin labeled rSdrG(50-597) (0.5 µM) for 1 h at room temperature. rSdrG(50-597) was biotin labeled using EZ Link-sulfo-NHS-LC biotin (Sigma) according to the manufacturers' instructions. After three more washes with PBST,
the blot was incubated with a 1:5000 dilution of streptavidin-AP conjugated for 1 h at room temperature and developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Biorad) in carbonate:bicarbonate buffer (14 mM Na₂CO₃, 36 mM NaHCO₃, 5 mM MgCl₂·6H₂O, pH 9.8) for ~15 min at room temperature.

**Fluorescence Polarization**-Fluorescence polarization was used to determine the equilibrium dissociation constant (Kₐ) for the interaction of rSdrG(50-597) with peptide β1-25. The peptide was labeled with fluorescein as previously described (18). Increasing concentrations of rSdrG(50-597) in PBS, pH 7.5, were incubated with 10 nM labeled peptide for 3 h in the dark at room temperature. Reactions were allowed to reach equilibrium. Polarization measurements were taken with a Luminescence Spectrometer LS50B (Perkin Elmer) using FL WinLab software (Perkin Elmer). Binding data was analyzed by nonlinear regression used to fit a binding function as defined by the following equation:

\[ \Delta P = \frac{\Delta P_{\text{max}} \cdot [\text{protein}]}{K_0 + [\text{protein}]} \]

where \( \Delta P \) corresponds to the change in fluorescence polarization, \( \Delta P_{\text{max}} \) is the maximum change in fluorescence, and \( K_0 \) is the equilibrium dissociation constant of the interaction. A single binding site was assumed in this analysis.

**Fg Clotting Assay**-150 µl of a 3.0 µM Fg solution was incubated with 10 µl of rSdrG(50-597) or BSA (1.0-6.0 µM) and 50 µl of thrombin (Sigma) (1.0 NIH unit/ml) in microtiter wells at room temperature. Clot formation was monitored by measuring the increase in optical density (OD) at 405 nm over time and expressed as \( V_{\text{max}} \) (mOD/min). A plate reader (Thermomax microplate reader, SOFTmax software, Molecular Devices) was used to monitor OD. Using the kinetic mode with one wavelength (L1=405 nm), samples were
read every 10 sec for 5 min. In this assay, 1.0 NIH unit/ml of thrombin incubated with 3.0 µM Fg produced a fibrin clot in 5 min at room temperature.

Release of Fibrinopeptides by Thrombin-The thrombin catalyzed release of fibrinopeptides A and B was analyzed as follows. Fg solutions were diluted to 0.3 µM in 20 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM ε-aminocaproic acid, and 1.0 mM CaCl₂. ε-aminocaproic acid was included to inhibit any possible plasmin contaminant activity. Thrombin was added to a final concentration of 0.05 NIH units/ml. rSdrG(50-597) was added to a final concentration of 0.3 µM, 0.6 µM, or 1.5 µM. The tubes were mixed by inversion and 500 µl aliquots were removed for the 15 and 60 min time points. The aliquots were incubated at room temperature and then immersed in boiling water for 15 min to halt the reaction. The aliquots were stored on ice for the remainder of the time course. At the end of the reaction the samples were centrifuged for 15 min at 4°C, and the supernatants were removed and stored at -20°C overnight prior to analysis by high performance liquid chromatography.

The fibrinopeptides released were monitored by reverse phase HPLC essentially as described (19). The samples were loaded onto a Waters Delta-Pak C₁₈ column equilibrated with buffer A (25 mM NaH₂PO₄/Na₂HPO₄, pH 6.0). Fibrinopeptides were eluted with a linear gradient from 100% buffer A to 40% buffer B (Buffer A with 50% acetonitrile) and monitored by absorbance at 205 nm. Fibrinopeptide peak area was determined using the software Waters Millennium³².

RESULTS

Expression and Purification of Recombinant SdrG A-region- In order to characterize the ligand binding activity of SdrG, a recombinant form of the putative ligand binding A-region (residues 50-597) (Fig. 1C) was expressed in E. coli with a N-terminal His-tag. This protein construct, rSdrG(50-597), was purified by metal chelate affinity chromatography followed
by ion-exchange chromatography. The purity of the recombinant protein was confirmed by SDS-PAGE analysis, where it migrated with an apparent molecular mass of ~97 kDa (Fig. 1B). This is larger than the theoretical molecular weight of 63.7 kDa predicted from the primary amino acid sequence of this protein. Analysis of rSdrG(50-597) by MALDI mass spectrometry indicated a molecular mass of 63.3 kDa. Aberrant migration in SDS-PAGE has also been observed with recombinant MSCRAMMs derived from S. aureus, and may be explained by the hydrophilic nature of these proteins (9,13).

**SdrG Binds the Fg Bβ Chain** - SdrG is closely related to the recently described fibrinogen binding MSCRAMM Fbe (15). Therefore, we initially examined the ligand binding specificity of SdrG for Fg in an ELISA. In this assay, rSdrG(50-597) bound immobilized Fg, but failed to bind to other immobilized ECM proteins such as fibronectin, collagen types I and IV, vitronectin, laminin and thrombospondin (data not shown). Binding of increasing concentrations of rSdrG(50-597) to absorbed Fg exhibited saturation kinetics (Fig. 2). Together these observations demonstrate the specificity of the SdrG-Fg interaction. Furthermore, biotin labeled rSdrG(50-597) recognized the Bβ chain, but not the Aα or γ chains of Fg when analyzed by Western ligand blotting (data not shown).

**Localization of the SdrG Binding Site in the Fg Bβ Chain** - The observation that rSdrG binds the Fg Bβ chain fractionated under reducing and denaturing conditions in Western ligand blot analysis suggests that the MSCRAMM recognizes a specific linear amino acid sequence in the Bβ chain. To explore this possibility and locate the SdrG binding site, a recombinant mature Fg Bβ chain and a series of truncated forms of the Bβ chain expressed in E. coli were analyzed by Western ligand blot. The recombinant Bβ chain constructs were expressed as either His-tag or glutathione S-transferase (GST) fusion proteins (Fig. 3A). The fractionated proteins were transferred to a supporting membrane and probed with biotin labeled rSdrG(50-597) (Fig. 3B). rSdrG(50-597) recognized the mature
recombinant Bβ chain (residues 1-462) as well as the recombinant truncates encompassing residues 1-341, 1-220, 1-195 and 1-95. However, rSdrG(50-597) failed to bind to the two recombinant truncates that lacked the N-terminal 25 amino acid residues of the Bβ chain, rβ(25-95) and rβ(25-195) (Fig. 3B). These observations demonstrate that rSdrG(50-597) recognizes a linear sequence in Fg and suggests that this site lies within the N-terminal region of the Bβ chain.

Inhibition of rSdrG(50-597) Binding to Fg by Synthetic Peptides- To further define the rSdrG(50-597) binding site in the Fg Bβ chain, we used a peptide mimetic approach. A series of peptides representing segments of the N-terminal region of the Fg Bβ chain were synthesized and tested for their ability to inhibit the binding of rSdrG(50-597) to Fg in an ELISA (Fig. 4). In Fig. 4A, peptides β1-25 and β6-25 were shown to inhibit the binding of rSdrG(50-597) to Fg in a concentration dependent manner, whereas the scrambled version of β1-25, peptide β1-25S, did not interfere with the binding of rSdrG(50-597) to Fg. Effective inhibition of rSdrG(50-597) binding to Fg was also observed with peptide β6-20 and, to a somewhat lesser degree, with β1-20. Peptide β11-20 was essentially inactive in this assay (Fig. 4B).

The thrombin cleavage sites in Fg lie between residues 14 (Arg) and 15 (Gly) in the Bβ chain and between 16 (Arg) and 17 (Gly) in the Aα chain. Upon cleavage of Fg by thrombin the fibrinopeptides, FpA and FpB, are sequentially released. The fibrinopeptides were examined as inhibitors of rSdrG(50-597) binding to Fg in an ELISA. FpB inhibited the binding of rSdrG(50-597) in a concentration dependent manner, but this peptide was at least 10 fold less active than the synthetic peptide β1-25 (Fig. 4C). FpA was essentially inactive and behaved similar to the scrambled peptide β1-25S. Taken together, this suggest that rSdrG(50-597) recognizes a linear amino acid sequence in the
Bβ chain located within residues 6-20. This recognition site appears to overlap the thrombin cleavage site in this polypeptide.

**rSdrG Binding to Thrombin-Digested Fg**-The rSdrG binding site seems to lie within close proximity to the thrombin cleavage site, therefore, we investigated if rSdrG(50-597) could bind to Fg in which the thrombin cleavage site was abolished. Fg coated microtiter wells were pretreated with thrombin or thrombin plus hirudin (which inhibits thrombin activity) in order to remove FpB and destroy the cleavage site. The ability of rSdrG(50-597) to bind to this thrombin digested Fg was significantly impaired (Fig. 5), suggesting that the thrombin cleavage site residues Bβ 14 (Arg), 15 (Gly) and residues within FpB (1-14) are essential for rSdrG(50-597) to bind Fg.

**Determination of Equilibrium Dissociation Constants (K_D)**- An equilibrium dissociation constant (K_D) for the interaction of rSdrG(50-597) with the Fg Bβ chain peptide β1-25 was determined. By analyzing the binding of increasing concentrations of rSdrG(50-597) to the fluorescein-labeled β1-25 peptide in a fluorescence polarization assay, rSdrG(50-597) binding to the labeled peptide exhibited saturation kinetics with a K_D of 1.4 ± 0.01 x 10^{-7} M (Fig. 6A). To demonstrate the specificity of this interaction, the binding of rSdrG(50-597) to the labeled β1-25 peptide was measured in the presence of increasing amounts of unlabeled peptide (β1-25) or scrambled peptide (β1-25S). The unlabeled β1-25 peptide, but not peptide β1-25S inhibited binding of rSdrG(50-597) to the fluorescein-labeled β1-25 peptide, in a concentration dependent manner (Fig. 6B). The apparent K_D determined for the binding of rSdrG(50-597) to the fluorescein labeled peptide β1-25 is similar to the apparent K_D (0.9 x 10^{-7} M) for the interaction of rSdrG(50-597) with immobilized, intact Fg as determined by ELISA (Fig. 2).
rSdrG(50-597) Inhibits Thrombin-Induced Fibrin Clot Formation- In the final stages of the blood coagulation cascade, thrombin cleaves Fg releasing the fibrinopeptides and producing fibrin monomers. These fibrin monomers then polymerize to form a fibrin clot (20). The localization of the SdrG binding site described above raises the possibility that rSdrG(50-597) may be able to inhibit thrombin-induced fibrin clot formation, perhaps by directly competing with thrombin for binding to the N-terminus of the Bβ chain of Fg or by binding to a proximal site and sterically blocking thrombin's proteolytic attack on the Bβ chain. To test this hypothesis, we designed a fibrin clot inhibition assay in which 3.0 µM Fg, 0-6.0 µM rSdrG(50-597) and 1.0 NIH unit/ml of thrombin were incubated and the formation of a fibrin clot was monitored by measuring the increase in optical density at 405 nm. Fig. 7 shows that rSdrG(50-597) inhibited fibrin clot formation in a concentration dependent manner, whereas BSA had no effect. This suggests that rSdrG(50-597) can interfere with thrombin activity by binding to a site in the Fg Bβ chain that is proximal to or overlaps the binding site for thrombin.

Analysis of Fibrinopeptide B Release by HPLC- The release of FpA and FpB from the N-terminus of the Aα and Bβ chains of Fg by thrombin can be monitored and quantitated by high performance liquid chromatography (19,21,22). We examined the effect of rSdrG(50-597) on fibrinopeptide release by measuring the peak areas of FpA and FpB, as detected by HPLC. The HPLC chromatograms shown in Fig. 8 show the expected fibrinopeptide release following digestion of Fg with thrombin superimposed with the fibrinopeptide release when Fg and thrombin are incubated with rSdrG(50-597). A significant decrease in the amount of FpB release was shown with a 1:1 ratio of rSdrG(50-597) to Fg (Table I) whereas, a 5:1 ratio was effectively able to inhibit the release of FpB (Fig. 8). This effect was seen at an incubation time of 15 min and 60 min. There was no apparent interference of FpA release by rSdrG(50-597).
DISCUSSION

In this study, we have shown that SdrG binds the N-terminus of the Bβ chain of Fg with a high degree of specificity. The binding of SdrG to an N-terminal Fg peptide exhibits a K_d of 1.4 x 10^{-7} M, which is significantly lower than the K_d determined for the binding of ClfA to a γ chain peptide (2.0 x 10^{-5} M) (18). Thus, SdrG appears to have a higher affinity for its respective synthetic Fg peptide target compared to the S. aureus MSCRAMM. The K_d determined for the binding of SdrG to the synthetic peptide β1-25 is similar to the apparent K_d estimated for the binding of SdrG to intact Fg absorbed onto microtiter wells. This observation suggests that the SdrG binding site in the synthetic peptide is presented in a nearly optimal form and that additional segments of Fg do not significantly contribute to the formation of the SdrG binding site.

Several studies have examined the role of Fg binding MSCRAMMs from S. aureus as virulence factors in animal models. Strains in which the genes encoding ClfA or ClfB have been inactivated are less virulent compared to the wild type strain in a rat model of catheter-induced endocarditis (23,24). These results suggest that ClfA- and ClfB-mediated adherence is required for the maximum virulence potential of S. aureus to be expressed. ClfB has been shown to promote S. aureus adherence to ex vivo hemodialysis tubing, further confirming that ClfB contributes to bacterial attachment to biomaterials coated with host proteins (9). In a recent study, Stutzmann Meier, et al. showed that heterologous expression of ClfA on Streptococcus gordonii, which is generally considered a non-virulent bacterium, rendered this organism pathogenic in a rat endocarditis model (25). With the discovery that SdrG is a Fg binding MSCRAMM expressed by S. epidermidis, the possibility arises that SdrG can act as a virulence factor in S. epidermidis-induced infections and plays a role similar to that of the Fg binding MSCRAMMs in S. aureus-induced infections.
We have mapped the binding site of rSdrG(50-597) in the Fg Bβ chain to a linear sequence in the N-terminal region of this polypeptide. Peptide β6-20 is a potent inhibitor of the binding of rSdrG(50-597) to Fg, whereas FpB (1-14) has poor inhibitory activity. Because peptide β6-20, but not β11-20 is recognized by this MSCRAMM, the N-terminal border of the binding site must lie between residues 6 and 11 of the Bβ chain. The observation that rSdrG(50-597) is unable to bind to thrombin digested Fg, i.e. the fibrinopeptides are absent, suggests that the C-terminus of this binding site is located between residues 14 and 20 of the Bβ chain.

It is striking that many of the identified staphylococcal MSCRAMMs appear to specifically recognize Fg, although the sites targeted in Fg by these proteins vary. ClfA, FnbpA and FnbpB of S. aureus all recognize the C-terminus of the Fg γ chain (6,27). ClfB from S. aureus targets an as yet unidentified site in the Aα chain (9) and SdrG is here shown to bind to the N-terminus of the Bβ chain. Thus, these MSCRAMMs use a conserved A region to bind different sites in Fg. Furthermore, the MSCRAMMs appear to target sites in Fg that are important in the molecular physiology of this key component of hemostasis. The C-terminus of the γ chain is recognized by the platelet integrin α_{Ibb}β_3, and ClfA is a potent inhibitor of Fg-induced platelet aggregation (26,27). Here, we show that the binding site in the Bβ chain for rSdrG(50-597) appears to overlap the thrombin cleavage site and that rSdrG(50-597) can interfere with fibrin clot formation by inhibiting the thrombin-induced release of FpB. Fg may play an important role in the host’s defense against microbial infections and interfering with this function gives the bacteria an advantage and the ability to survive in a hostile environment. One such potential advantage may be related to the observed chemotactic activity of FpB for human peripheral blood leukocytes (28-30). We have shown that rSdrG(50-597) can prevent the release of FpB, thus one can speculate that the reason S. epidermidis possesses a protein that can bind to this region of the Fg Bβ
chain is to prevent the release of chemotactic elements. This may reduce the influx of phagocytic neutrophils and help to ensure the survival of the bacteria in the host.

REFERENCES


**FOOTNOTES**

1 The abbreviations used are: CNS, coagulase-negative staphylococci, ECM, extracellular matrix, Fg, fibrinogen, MSCRAMM, microbial surface component recognizing adhesive matrix molecules, ClfA and ClfB, clumping factors A and B, FnbpA and FnbpB, fibronectin-binding proteins A and B, SdrF and SdrG, serine-aspartate repeat proteins F and G, FpA and FpB, fibrinopeptides A and B, ELISA, enzyme-linked immunosorbent assay, HPLC, high performance liquid chromatography, PCR, polymerase chain reaction, PAGE, polyacrylamide gel electrophoresis, $K_D$, equilibrium dissociation constant.

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**FIGURE LEGENDS**

Fig. 1 **Structural organization of SdrG.** A. Cartoon presentation of SdrG. The number of amino acid residues contained in each region is indicated below each segment. S, signal sequence, A, N-terminal Fg binding region, B1 & B2, repeats of unknown function, R,
serine-aspartate repeat region, W, wall-spanning region, M, membrane-spanning region. The positively-charged tail and LPXTG motif involved in cell-wall anchoring are also indicated. B. A model of the recombinant His-tag construct rSdrG(50-597), representing the A region. C. Coomasie stained SDS-PAGE of purified rSdrG(50-597).

Fig. 2  **rSdrG(50-597) binding to immobilized Fg.** Increasing concentrations of rSdrG(50-597) (●) were incubated with immobilized Fg in an ELISA. After incubation in the wells for 1 h at room temperature, bound protein was detected as described in the materials and method section. The apparent $K_D$ was 0.9 x 10^{-7} M. Values represent the mean ± standard deviation of triplicate wells.

Fig. 3  **Localization of the rSdrG(50-597) binding site in Fg using recombinant Bβ chain constructs.** A. Cartoon models of the recombinant truncates of the Fg Bβ chain constructed using the pQE30 His-tag vector or the GST fusion vector pGEX-KG. B. Whole *E. coli* cell lysates containing the recombinant proteins were loaded onto a 10% SDS-polyacrylamide gel. The gel was transferred to a nitrocellulose membrane and the blot was probed with biotin labeled rSdrG(50-597) and developed as described in the materials and methods section. *Lane 1*, native Fg, *lane 2*, rβ(1-462), *lane 3*, rβ(1-341), *lane 4*, rβ(1-220), *lane 5*, rβ(1-195), *lane 6*, rβ(25-195), *lane 7*, rβ(1-95), *lane 8*, rβ(25-95).

Fig. 4  **Inhibition of rSdrG(50-597) binding to immobilized Fg by synthetic peptides.** rSdrG(50-597) (50 nM) was pre-incubated with increasing concentrations of peptides for 1 h at room temperature and transferred to microtiter wells coated with 1 µg human Fg. After incubation in the wells for 1 h at room temperature, bound SdrG was detected as described in the materials and methods section. A. β1-25 (●), β6-25 (∆), β1-25S (◆). B. β6-20 (■),
β1-20 (▼) and β11-20 (○). C. FpA (□), FpB (▲), β1-25 (●), β1-25S (◆). Values represent the mean ± standard deviation of triplicate wells.

Fig. 5 rSdrG(50-597) binding to thrombin digested Fg.
Fg coated microtiter wells were pretreated for 30 min at 37°C with thrombin (▲), thrombin and hirudin (●), hirudin alone (◆) or untreated (■). Plates were blocked, washed and incubated with biotin labeled rSdrG(50-597) (25-1000 nM) for 1 h at room temperature. Bound SdrG was detected as described in materials and methods. Values represent the mean ± standard deviation of triplicate wells.

Fig. 6 Quantitative analysis of rSdrG(50-597) binding to intact immobilized Fg or Fg peptide β1-25. A. Increasing concentrations of rSdrG(50-597) were incubated with the fluorescein-labeled N-terminal Bβ chain peptide β1-25 (10 nM) for 3 h in the dark at room temperature. Equation 1 was used to fit the binding data. From three experiments the $K_d$ for the interaction of rSdrG(50-597) with peptide β1-25 was calculated to be $1.4 ± 0.01 \times 10^{-7}$ M. B. Binding of the fluorescein-labeled β1-25 to rSdrG(50-597) in the presence of increasing concentrations of unlabeled β1-25 (●) or the scrambled Bβ chain peptide β1-25S (▲). Values are the mean of duplicate reactions.

Fig. 7 Inhibition of fibrin clot formation by rSdrG(50-597). Thrombin (1.0 NIH unit/ml) was added to a mixture of Fg (3.0 µM) and rSdrG(50-597) (●) (0-6.0 µM) or BSA (▲) (0-6.0 µM) in microtiter wells. Fibrin clot formation was monitored by measuring an increase in optical density at 405 nm. Values represent the mean ± standard deviation of quadruple wells.
Fig. 8 Inhibition of FpB release by rSdrG(50-597). Superimposed chromatograms show the amount of fibrinopeptide released when the Fg-thrombin sample has no SdrG present (upper curve) and when the Fg-thrombin sample is incubated with 1.5 μM rSdrG(50-597) (lower curve) at the 60 min time point. The decrease in the amount of FpB released with SdrG present is shown in the lower curve.

Table I

Percentage of FpB released in the presence of SdrG

Fg (0.3 μM) was incubated with SdrG and 0.5 NIH units/ml of thrombin at room temperature and the samples were analyzed by HPLC. The amount of fibrinopeptide released was determined by measuring the area under the peaks on the HPLC chromatograms. The data was normalized in order to compare the data from separate chromatograms assuming that the release of FpA is not affected by the presence of SdrG. The peak area representing FpB in the absence of SdrG was set to 100%.

<table>
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<th>60 min</th>
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<td>&lt;0.001</td>
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</table>
Polarization Change (mP)

[rSdrG(50-597)] (μM)
Absorbance (405 nm)

[Protein] (µM)
SdrG, a fibrinogen binding bacterial adhesin of the MSCRAMM subfamily from *Staphylococcus epidermidis*, targets the thrombin cleavage site in the Bβ chain

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