The Fibrinogen-binding MSCRAMM (Clumping Factor) of Staphylococcus aureus Has a Ca\textsuperscript{2+}-dependent Inhibitory Site

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The clumping factor (ClfA) is a cell surface-associated protein of Staphylococcus aureus that promotes binding of fibrinogen or fibrin to the bacterial cell. Previous studies have shown that ClfA and the platelet integrin α\textsubscript{IIb}β\textsubscript{3} recognize the same domain at the extreme C terminus of the fibrinogen γ-chain. α\textsubscript{IIb}β\textsubscript{3} interaction with this domain is known to occur in close proximity to a Ca\textsuperscript{2+}-binding EF-hand structure in the α-subunit. Analysis of the primary structure of ClfA indicated the presence of a potential Ca\textsuperscript{2+}-binding EF-hand-like motif at residues 310–321 within the fibrinogen-binding domain. Deletion mutagenesis and site-directed mutagenesis of this EF-hand in recombinant truncated ClfA proteins (Clf40, residues 40–559; and Clf41, residues 221–559) resulted in a significant reduction of affinity for native fibrinogen and a fibrinogen γ-chain peptide. Furthermore, Ca\textsuperscript{2+} (or Mn\textsuperscript{2+}) could inhibit the binding of the fibrinogen γ-chain peptide to Clf40 (40–559) and the adhesion of S. aureus cells to immobilized fibrinogen with an IC\textsubscript{50} of 2–3 mM. In contrast, Mg\textsuperscript{2+} (or Na\textsuperscript{+}) at similar concentrations had no effect on the ClfA-fibrinogen interaction. Far-UV CD analysis of Clf40 (40–559) and Clf41 (221–559) in the presence of metal ions indicated Ca\textsuperscript{2+}- and Mn\textsuperscript{2+}-induced differences in secondary structure. These data suggest that Ca\textsuperscript{2+} binds to an inhibitory site(s) within ClfA and induces a conformational change that is incompatible with binding to the C terminus of the γ-chain of fibrinogen. Mutagenesis studies indicate that the Ca\textsuperscript{2+}-dependent inhibitory site is located within the EF-hand motif at residues 310–321.

Staphylococcus aureus causes a wide range of opportunistic infections that range from superficial skin infections to life-threatening diseases including endocarditis, pneumonia, and septicemia. Adherence of bacteria to host matrix components that is mediated by bacterial surface adhesins is the initial critical event in the pathogenesis of most infections. The extracellular matrix (ECM) contains numerous glycoproteins and proteoglycans assembled into insoluble matrices that serve as substrata for the adhesion and migration of tissue cells. These processes involve integrins, a family of heterodimeric (αβ) cell-surface receptors that recognize specific ECM proteins. It has become increasingly evident that bacteria, including S. aureus, also utilize the ECM as substrata for their adhesion by way of a family of adhesins called MSCRAMM (microbial surface components recognizing adhesive matrix molecules) (1) that specifically recognize host matrix components.

One important component of the ECM, also occurring in soluble form in blood plasma, is fibrinogen, a 340-kDa hexamer composed of 2α-, 2β-, and 2γ-chains linked by disulfide bonds. This protein is recognized by several integrins including the platelet integrin α\textsubscript{IIb}β\textsubscript{3}. Activation of platelets and integrin α\textsubscript{IIb}β\textsubscript{3} results in fibrinogen-dependent aggregation in vitro and the formation of platelet-fibrin thrombi in vivo.

S. aureus contains several fibrinogen-binding proteins, one of which (clumping factor, ClfA) is primarily responsible for the clumping of bacteria in fibrinogen solutions and bacterial adhesion to fibrinogen substrata (2). The gene encoding the fibrinogen-binding protein of S. aureus has been cloned, sequenced, and characterized in our laboratory (2). The clfA gene encodes a 933-amino acid protein that contains structural features characteristic of many cell surface-associated proteins from Gram-positive bacteria including a typical cell wall attachment region comprising an LPDTG motif, a hydrophobic transmembrane sequence, and a positively charged C terminus (Fig. 1). In addition, the protein contains a repeat sequence (region R) of 308 alternating aspartate and serine residues located just outside the cell wall attachment region. Region R is required for the surface display of the 320-amino acid-long region A, which contains the fibrinogen-binding domain (3, 4). Recombinant region A bound fibrinogen and strongly inhibited bacteria-fibrinogen interactions, as did anti-region A antibodies. Analysis of PCR-generated truncated proteins localized the binding domain to between residues 221 and 559 (3).

In earlier studies, Hawiger and co-workers (5, 6) showed that a synthetic peptide mimicking the extreme C terminus of the fibrinogen γ-chain inhibited fibrinogen-induced clumping of S. aureus cells. Recently, it was shown that purified recombinant ClfA protein specifically recognized these amino acid residues and that a synthetic peptide corresponding to this domain effectively inhibited binding of fibrinogen to recombinant ClfA (7). Interestingly, this same synthetic peptide also interacts with the α-subunit of the platelet integrin α\textsubscript{IIb}β\textsubscript{3} (8, 9). The γ-chain-binding site has been mapped to a region of the α\textsubscript{IIb} polypeptide that contains a sequence motif resembling the Ca\textsuperscript{2+}-binding EF-hand motif found in many eukaryotic Ca\textsuperscript{2+}-binding proteins (10). The EF-hand motif consists of 13 residues, with coordination typically supplied by oxygenated resi-
dues at positions 1, 3, 5, 7, and 12 and by a solvent molecule hydrogen-bonded to residue 9 (Fig. 2A) (11). These residues form a coordination sphere for the cation and are flanked by α-helices. Cooperative binding of multiple Ca\(^{2+}\) ions is not unusual, and more than one Ca\(^{2+}\)-binding motif often can be found within the same protein. Analysis of the α-subunit of α\(_{IIb}\)β\(_{3}\) revealed the presence of four functional motifs similar to EF-hands, although they lack an oxygenated residue at position 12 (Fig. 2B) (31, 39). Chemical cross-linking experiments provided direct evidence for the role of an α\(_{IIb}\) EF-hand-like sequence in fibrinogen binding (10) (Fig. 2B). In addition, a peptide corresponding to this EF-hand sequence bound to fibrinogen in a divalent cation-dependent manner (12). Taken together, these data provide evidence for the involvement of EF-hand-like sequences in the divalent cation-dependent binding of the fibrinogen C-terminal γ-chain peptide. Further biochemical characterization of integrin α\(_{IIb}\)β\(_{3}\) has demonstrated that Ca\(^{2+}\) binds to two distinct classes of sites: high affinity binding sites that promote ligand binding and low affinity binding sites that inhibit ligand binding (13).

In this report, we propose a mode of interaction between ClfA and the fibrinogen γ-chain peptide that exhibits some similarities to α\(_{IIb}\)β\(_{3}\)-ligand interactions. A potential divalent cation-binding EF-hand motif was identified in ClfA (Figs. 1 and 2C). It differs from the EF-hand consensus (Fig. 2, A and B) at only one residue, a non-cation-coordinating residue. We demonstrate that region A of ClfA can bind Ca\(^{2+}\) and that the interaction between region A and fibrinogen is inhibited by millimolar concentrations of Ca\(^{2+}\). In addition, we show using far-UV spectroscopy that Ca\(^{2+}\) ions affect the secondary structure of the fibrinogen-binding region of ClfA. Site-specific mutants of ClfA with a modified EF-hand were generated and shown to have a lower affinity for fibrinogen compared with the wild type. In addition, the effects of Ca\(^{2+}\) were reduced in these mutant proteins. Together, these studies indicate that Ca\(^{2+}\) plays a regulatory role in the interaction of fibrinogen and region A of ClfA.

**EXPERIMENTAL PROCEDURES**

**Bacteria and Growth Conditions**

*Escherichia coli* XL-1 Blue (14) was used as the bacterial host for plasmid cloning and protein expression. *E. coli* cells harboring plasmids were routinely grown in L-broth, Terrific broth, and L-agar (15). Ampicillin (100 µg/ml) was incorporated as appropriate. *S. aureus* strain Newman was grown in Trypticase soy broth or agar.

**Manipulation of DNA**

Restriction and DNA modification enzymes were purchased from New England Biolabs Inc. or Promega and were used according to the manufacturers’ instructions. DNA manipulations were performed using standard procedures (15).

**Amplification of clfA Gene Fragments**

PCR, with the oligonucleotides listed in Table I, was used to amplify specific clfA fragments from chromosomal DNA of *S. aureus* strain Newman. Genomic DNA was isolated as described previously (16). The oligonucleotides contained restriction enzyme cleavage sites at their

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**Fig. 1.** Schematic model showing the domain organization of *S. aureus* clumping factor (ClfA), as described previously (3). S, signal peptide; A, fibrinogen-binding region; R, repeat region; W, cell wall-spanning region; M, membrane-spanning domain; +, positively charged tail. The putative EF-hand-like motif (residues 310–321) is indicated in black. The minimum fibrinogen-binding domain is located between residues 221 and 559.

**A** Consensus EF-hand motif:

\[
\begin{align*}
1 & : D - X - (DNS) - (ILVFYW) - (DENSTG) - (DNQGHKR) - (GP) - \\
2 & : D - V - N - D - G - R - H - L - L - V \\
3 & : D - L - D - R - D - G - Y - N - D - I - A - V \\
4 & : D - I - D - D - N - G - Y - P - D - L - I - V
\end{align*}
\]

**B** Potential divalent cation-binding sites (EF-hands) from α\(_{IIb}\):

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|----|----|----|
| 1 | E | F | D | G | D | L | N | T | E | Y | V |
| 2 | D | V | N | D | G | R | H | L | L | V |
| 3 | D | L | D | R | D | G | Y | N | D | I | A | V |
| 4 | D | I | D | D | N | G | Y | P | D | L | I | V |

**C** Proposed divalent cation-binding motif in ClfA:

310-321: D S D G N V I Y T F T D

**Fig. 2.** Metal ion-binding motifs in integrins and *S. aureus* clumping factor. A, the EF-hand consensus showing preferred residues at each position (11, 38). Acceptable residues are in parentheses; unacceptable residues are in italics. X indicates any residue. The boldface letters indicate cation-coordinating residues. B, metal ion-binding sites in the α\(_{IIb}\) subunit of platelet integrin (adapted from Gulino et al. (39)). The asterisk indicates a site that interacts with the fibrinogen C-terminal γ-chain-binding site in α\(_{\text{IIIb}}\) (10). C, divalent cation-binding motif in region A of ClfA. The boldface letters indicate putative cation-coordinating residues.

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**Construction of Expression Plasmids**

Amplified fragments of the clfA gene were cloned into the expression plasmid pQE30 (QIAGEN Inc.) to generate the constructs pCF40 (40–559) and pCF41 (221–559). Recombinant protein expressed from this vector contains an N-terminal extension of six histidine residues (His\(_{6}\)). The vector contains an N-terminal extension of six histidine residues (His\(_{6}\)). Construction of a Deletion Mutant Plasmid

Residues 310–321 of ClfA corresponding to a putative Ca\(^{2+}\)-binding EF-hand were deleted in Clf40 (40–559) and Clf41 (221–559). The deletion mutant plasmids were designated pCF51 and pCF64, respectively. Construction of pCF51 involved two separate PCRs. The first reaction amplified DNA encoding residues 40–309 using a reverse primer (DOCR1) with an XbaI site incorporated at the 5'-end and a forward primer (DOCF2) incorporating a BamHI site. The second reaction amplified DNA encoding residues 332–559 using a forward primer (DOCF1) incorporating a BamHI site and a reverse primer (DOCR1) incorporating a HindIII site. Following cleavage by XbaI and either BamHI or HindIII (depending on the reaction), the two products were ligated together and cloned into the expression vector pQE30 as de-
scribed above. Creation of the XhoI site introduced a serine and an arginine residue at the site of deletion. pCF64 was constructed in the same way using the appropriate forward primer (F5) in the first reaction.

Site-directed Mutagenesis

Plasmid DNA purified from E. coli XL1-Blue cells containing pCF40-(40–559) and pCF41-(221–559) served as the template in the mutagenesis studies. The residues were mutated to alanine as single, double, or quadruple mutations (Table I). A novel method of mutagenesis was developed that involved two separate PCRs. The first reaction employed a flanking reverse primer (incorporating a HindIII site) and a forward primer that introduced the nucleotide mismatch required for the desired mutation and, in addition, incorporated a novel restriction site at the 5′-end of the oligonucleotide that would not affect the amino acid sequence of the final gene product. In the second reaction, a reverse primer incorporating the same silent restriction site mutation was used in conjunction with a flanking forward primer (with a BamHI site). Both gene fragments were digested at the common restriction site, ligated, digested with BamHI and HindIII, cloned into the expression vector pQE30, and transformed into E. coli XL1-Blue cells. Transformants were screened for the proper plasmid construction. The DNA sequence was verified by the dideoxy termination method (15) using [α-32P]-dATP (Amersham Corp.) and Sequenase 4.0 (U. S. Biochemical Corp.). The oligonucleotides used to construct the site-directed mutants are listed in Table I.

Expression and Purification of Recombinant Proteins

Recombinant plasmids were transformed into E. coli XL1-Blue cells and expressed as described previously (3). Fusion proteins containing the His tag were purified by immobilized metal chelate affinity chromatography. A amidodiacetic acid-Sepharose 6B Fast Flow column (40–559) and pCF41-(221–559) served as the template in the mutagenesis and deletion mutagenesis reactions. Proteins were purified by immobilized metal affinity chromatography. A iminodiacetic acid-Sepharose 6B Fast Flow column (pH 7.4) was charged with 150 mM Ni2+ and 2 mM EDTA, and 20 mM Tris-HCl (pH 7.4) was added and applied to a Q-Sepharose column pre-equilibrated with the same buffer. Bound protein was eluted with a continuous linear gradient of NaCl (50–500 mM; total volume of 200 ml) in 20 mM Tris-HCl and 2 mM EDTA (pH 7.9). Eluted fractions were monitored by absorbance at 280 nm, and peak fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Western immunoblotting. The glutathione S-transferase-cfA41-(221–559) fusion protein was purified by glutathione-Sepharose (Pharmacia Biotech Inc.) affinity chromatography and cleaved with bovine thrombin as described previously (3). The recombinant MSCRAMM fragment was isolated after passing the digest through a glutathione-Sepharose column, followed by ion-exchange chromatography on a Q-Sepharose column as described above.

Analysis of Fibrinogen-binding Activity of Recombinant Proteins

Western Ligand Blot Assay—Fibrinogen-binding proteins fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane were detected using fibrinogen conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham Corp.) as described previously (3).

Bacterial Adherence Assay—The adherence of S. aureus Newman cells expressing CfA to microtiter wells coated with fibrinogen was assayed as described previously (6).

Enzyme-linked Immunosorbent Assay—The ability of recombinant protein to bind to fibrinogen was analyzed using the enzyme-linked immunosorbent assay. Microtiter wells (Sarstedt, Inc.) were coated with 5 µg/ml fibrinogen (Kabi Pharmacia/Chromogenix) in coating solution (0.02% sodium carbonate buffer (pH 9.6)) for 18 h at room temperature. The plates were washed three times with PBS, 0.05% Tween 20, and 0.1% bovine serum albumin (PBS-TB). A solution of 2.5% bovine serum albumin and 0.05% Tween 20 in PBS was added to the wells to block any remaining protein-binding sites. After 1 h at 37 °C, the wells were washed again three times with PBS-TB, and purified recombinant protein in PBS was added and incubated for 2 h at 37 °C. The wells were again washed with PBS-TB and incubated with polyclonal antiserum raised against CfA41-(221–559), diluted 1:800, for 1 h. After further washing, 100 µl of horseradish peroxidase-labeled protein A (1:1000; Sigma) was added. Following incubation for 1 h at 37 °C and washing with PBS-TB, 100 µl of chromogenic substrate (580 µl tetramethylbenzidine and 0.0001% H2O2 in 0.1 M sodium acetate buffer (pH 5.0)) was added per well and developed for 10 min, and the reaction was stopped by the addition of 50 µl of 2 M H2SO4. Plates were read at 450 nm in an enzyme-linked immunosorbent assay plate reader (Lab-
systems Multiskan Plus).

Fluorescence Polarization—A fluorescence polarization assay was developed to determine the equilibrium constants for the interaction of the recombinant proteins with a synthetic peptide conjugated with a fluorescent probe. This peptide consisted of a 17 C-terminal residue of the gamma-chain in fibrinogen (i.e. GEGQQHHHLGGAKQGAVD) and was synthesized by a solid-phase method on a β- benzoylbenzyl alcohol resin using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry and a Model 396 multiple peptide synthesizer (Advanced ChemTech Inc.). The peptide was labeled with fluorescein as follows. The peptide (1 mg dissolved in 100 μl of dimethyl sulfoxide) at 37 °C for 60 min in the presence of coupling buffer (100 mM KH₂PO₄ (pH 7.0)). The reaction was quenched by the addition of 100 μl of 1 M Tris-HCl (pH 8.0), vortexed, and left at room temperature for 30 min. The fluoresceinated peptide was fractionated by reverse-phase chromatography on a Delta-Pak C₁₈ Radial-Pak cartridge HPLC column connected to a Waters 486 multi-wavelength detector. The labeling procedure can yield three forms of fluoresceinated peptide with the probe attached to 1) the N-terminal amino group, 2) the amino group of the internal lysine residue, and 3) the amino groups in both positions. Fractionation by HPLC yielded two major and one minor fluorescein-containing peak. Substitutions at the internal lysine residue should yield a peptide resistant to trypsin digestion. The separated peptides were incubated with trypsin, followed by HPLC analysis. Only one of the original major peaks contained peptide susceptible to trypsin (data not shown). This peptide, which contains the fluorescein probe linked to the N-terminal amino group, was used in the fluorescence polarization studies. A scrambled peptide consisting of the 17 C-terminal residues of the γ-chain in a random sequence (i.e. GHEHGLQGQGAVKDGAQ) was used as a control.

Recombinant protein in 10 mM Tris-HCl (pH 7.4) was incubated with fluorescein succinimidyl ester (1 mg dissolved in 100 μl of dimethyl sulfoxide) at 37 °C for 60 min, vortexed, and left at room temperature for 30 min. The fluoresceinated peptide was quenched by the addition of 100 μl of 1 M Tris-HCl (pH 8.0), vortexed, and left at room temperature for 30 min. The fluoresceinated peptide was fractionated by reverse-phase chromatography on a Delta-Pak C₁₈ Radial-Pak cartridge HPLC column connected to a Waters 486 multi-wavelength detector. The labeling procedure can yield three forms of fluoresceinated peptide with the probe attached to 1) the N-terminal amino group, 2) the amino group of the internal lysine residue, and 3) the amino groups in both positions. Fractionation by HPLC yielded two major and one minor fluorescein-containing peak. Substitutions at the internal lysine residue should yield a peptide resistant to trypsin digestion. The separated peptides were incubated with trypsin, followed by HPLC analysis. Only one of the original major peaks contained peptide susceptible to trypsin (data not shown). This peptide, which contains the fluorescein probe linked to the N-terminal amino group, was used in the fluorescence polarization studies. A scrambled peptide consisting of the 17 C-terminal residues of the γ-chain in a random sequence (i.e. GHEHGLQGQGAVKDGAQ) was used as a control.

The interaction of Clf40-(40–559) with a set amount of fluorescent peptide was measured in the presence of metal ions (5 mM) (Fig. 3). Incubation of Clf40-(40–559) with 5 mM Mn²⁺ caused complete loss of γ-chain peptide-binding activity. On the other hand, Mg²⁺ or Na⁺ at similar concentrations had no effect on protein-peptide interaction (Fig. 4A). In addition, Mg²⁺ (5 mM) did not counteract the inhibitory effect of Ca²⁺ (data not shown). Incubation of Clf40-(40–559) with EDTA (5 mM) increased the affinity of MSCRAMM for the γ-chain peptide by 2-fold (KD = 11.5 ± 0.6 μM) compared with non-EDTA-treated protein (KD = 20.8 ± 2.5 μM). It is likely that contaminating Ca²⁺ and Mn²⁺ in the assay solutions and protein preparations contribute to inhibition of the protein-peptide interaction. Incubation of EDTA removes these ions. Furthermore, these data show that divalent cations are not required to promote γ-chain peptide binding. Results similar to those described for Clf40-(40–559) were obtained when these experiments were repeated with the smaller construct, Clf41-(221–559). Thus, in the absence of added metal ions, Clf41-(221–559) bound the γ-chain peptide with a dissociation constant of 15.0 ± 1.1 μM (Fig. 3), and the addition of 5 mM EDTA slightly reduced the KD to 11.1 ± 0.4 μM. When these experiments were repeated with Clf41-(221–559) without an N-terminal extension of histidine residues, identical results were obtained, indicating that the effects of cations on region A function are independent of the purification tag (data not shown).

The interaction of Clf40-(40–559) with a set amount of fluoresceinated γ-chain peptide across a concentration range of Ca²⁺ and Mg²⁺ was also measured. With Mg²⁺, the binding of the γ-chain peptide to Clf40-(40–559) was unaffected across manner, whereas a scrambled version of the peptide had no effect at similar concentrations (Fig. 3, inset). Incubation of Ca²⁺ (5 mM) significantly reduced the binding affinity for the peptide (KD > 200 μM) (Fig. 3). Incubation of Clf40-(40–559) with 5 mM Mn²⁺ caused complete loss of γ-chain peptide-binding activity. On the other hand, Mg²⁺ or Na⁺ at similar concentrations had no effect on protein-peptide interaction (Fig. 4A). In addition, Mg²⁺ (5 mM) did not counteract the inhibitory effect of Ca²⁺ (data not shown). Incubation of Clf40-(40–559) with EDTA (5 mM) increased the affinity of MSCRAMM for the γ-chain peptide by 2-fold (KD = 11.5 ± 0.6 μM) compared with non-EDTA-treated protein (KD = 20.8 ± 2.5 μM). It is likely that contaminating Ca²⁺ and Mn²⁺ in the assay solutions and protein preparations contribute to inhibition of the protein-peptide interaction. Incubation of EDTA removes these ions. Furthermore, these data show that divalent cations are not required to promote γ-chain peptide binding. Results similar to those described for Clf40-(40–559) were obtained when these experiments were repeated with the smaller construct, Clf41-(221–559). Thus, in the absence of added metal ions, Clf41-(221–559) bound the γ-chain peptide with a dissociation constant of 15.0 ± 1.1 μM (Fig. 3), and the addition of 5 mM EDTA slightly reduced the KD to 11.1 ± 0.4 μM. When these experiments were repeated with Clf41-(221–559) without an N-terminal extension of histidine residues, identical results were obtained, indicating that the effects of cations on region A function are independent of the purification tag (data not shown).

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the entire concentration range tested. In contrast, concentrations of Ca\(^{2+}\) above 2 mM inhibited the interaction (Fig. 4A). At concentrations greater than 5 mM, very little binding of the γ-chain peptide to region A was observed. Very similar results were obtained when the effects of different cations on S. aureus cell adhesion to immobilized fibrinogen were measured (Fig. 4B). Concentrations of Ca\(^{2+}\) above 2 mM inhibited bacterial adhesion to fibrinogen, whereas Mg\(^{2+}\) did not affect cell adhesion throughout the concentration range analyzed. These results indicate the existence of an inhibitory Ca\(^{2+}\)-binding site in region A of ClfA with an apparent KD similar to the physiological concentration of Ca\(^{2+}\) present in normal human sera. Thus, under some physiological conditions, this site may be occupied. Inclusion of Mg\(^{2+}\) had no effect on Ca\(^{2+}\)-induced inhibition (data not shown), suggesting that Mg\(^{2+}\) is unable to compete with Ca\(^{2+}\) for binding to the inhibitory binding site and that there is a certain degree of specificity for Ca\(^{2+}\).

**Effect of Ca\(^{2+}\) Ions on the Structure of Region A:** Circular dichroism spectroscopy was used to investigate if the binding of Ca\(^{2+}\) ions affects the secondary structures of the Clf40-(40–559) and Clf41-(221–559) proteins. Cation binding by integrins has been shown to be associated with conformational changes. For example, expression of the epitope recognized by monoclonal antibody 24 on α\(_2\)β\(_1\) was dependent on Mg\(^{2+}\), and monoclonal antibody 24 expression correlates with the ability to bind ligand (24, 25).

The CD spectra of Clf41-(221–559) were dominated by a large minimum at 215 nm. Inclusion of Ca\(^{2+}\) and Mn\(^{2+}\) had a reproducible effect on the protein far-UV CD spectra at 200 nm (Fig. 5). This effect on secondary structure was dependent on the concentration of divalent cation and on the particular cation used. Ca\(^{2+}\) altered the CD spectra of Clf41-(221–559) by reducing the signal at 200 nm in a concentration-dependent manner (Fig. 5). The presence of Mn\(^{2+}\) resulted in a qualitatively similar change, although the effect was more pronounced (data not shown). Identical results were obtained with Clf41-(221–559) without an N-terminal extension of six histidine residues, indicating that the effects of Ca\(^{2+}\) and Mn\(^{2+}\) on the secondary structure of region A are independent of the purification tag (data not shown).

The CD spectra of Clf40-(40–559) were dominated by a large minimum at 200 nm, suggesting differences in secondary structure compared with Clf41-(221–559). The spectroscopic differences observed reflect the effect of the additional 180 amino acid residues present in Clf40-(40–559). However, deconvolution of the spectra using SELCON (26) indicated only minor differences in the percentage α-helix and β-sheet of each construct (estimated α-helix/β-sheet is 15/35% for Clf40-(40–559) and 15/40% for Clf41-(221–559)). As with Clf41-(221–559), the addition of Ca\(^{2+}\) (and Mn\(^{2+}\)) to Clf40-(40–559) altered the far-UV spectra at 200 nm in a concentration-dependent manner (Fig. 6, upper panel). Cation-induced changes in the far-UV spectra of Clf40-(40–559) and Clf41-(221–559) were completely reversible by the addition of EDTA (10 mM).

The effect of Ca\(^{2+}\) and Mn\(^{2+}\) on secondary structure correlates with the effect that these metal ions have on the ability of the protein to bind the C-terminal γ-chain peptide. These data indicate that when Ca\(^{2+}\) and Mn\(^{2+}\) bind to region A of ClfA, alterations to the secondary structure of the protein occur, with Mn\(^{2+}\) having the largest effect. These structural alterations may be responsible for the inhibition of ligand binding.

**Interaction of ClfA Region A Mutants with the Fibrinogen**
**γ-Chain Peptide**—The role of the putative Ca\(^{2+}\)-binding EF-hand at residues 310–321 in ligand binding was investigated using deletion mutagenesis and site-directed mutagenesis. Deletion of amino acids 310–321 from Clf40-(40–559) and Clf41-(221–559) resulted in complete loss of fibrinogen-binding activity. These mutant proteins did not bind fibrinogen in a Western ligand blot assay (data not shown). They also failed to bind to the fibrinogen C-terminal γ-chain peptide in a fluorescence polarization assay (Fig. 7A).

The EF-hand motif was further investigated by mutating putative cation-coordinating residues in Clf40-(40–559) and Clf41-(221–559) and characterizing the interactions of these mutant proteins with the fluorescently labeled C-terminal γ-chain peptide. The amino acid residues investigated were replaced with alanine, a residue that was not expected to interfere with existing secondary structure, but would be unable to coordinate cations. The corresponding base changes were made using a novel PCR mutagenesis technique as described under “Experimental Procedures.” The recombinant proteins containing the mutations were purified to homogeneity by metal ion chromatography and anion exchange chromatography. Structural analysis of the isolated mutant proteins by CD spectroscopy indicated differences in secondary structure resulting from the introduction of mutations (Fig. 6). Interestingly, the observed effect of Ca\(^{2+}\) on structure was significantly less in the mutants as compared with the wild-type protein (Fig. 6).

The effects of the mutations on the interaction of Clf40-(40–559) with the fibrinogen γ-chain peptide are shown in Fig. 7A. Similar effects were observed when the mutations were introduced into the smaller construct (Clf41-(221-559) (data not shown). Thus, it is apparent that substitutions within the putative EF-hand affect peptide-binding affinity. Substitution of four residues (D310A/D312A/T318A/D321A) exhibited the most dramatic decrease. Because of the very low affinity of this quadruple mutant for the peptide, the dissociation constant...
could not be measured accurately. The D310A/D312A double mutant bound the peptide over three times more weakly than the wild-type protein ($K_d = 66.5 \pm 7.4 \mu M$). Mutation of the first aspartate in the EF-hand (D310A) had no effect on $\gamma$-chain peptide-binding activity.

The effects of $Ca^{2+}$ on the interaction of the D310A single mutant and the D310A/D312A double mutant with the $\gamma$-chain peptide were also measured. As shown in Fig. 7B, the degree of inhibition induced by the inclusion of $Ca^{2+}$ is significantly less with the mutants compared with the wild-type protein. These results show that the introduced mutations have (a) reduced the ability of region A of ClfA to bind to the fibrinogen $\gamma$-chain peptide and (b) reduced the ability of $Ca^{2+}$ to inhibit this interaction. One interpretation of these data is that the inhibitory $Ca^{2+}$-binding site and the fibrinogen $\gamma$-chain peptide-binding site share contact points within amino acid sequence 310-321.

Interaction of Region A Mutants with Fibrinogen—An enzyme-linked immunosorbent assay was developed (as described under “Experimental Procedures”) to assess the effects of the mutations on the interaction of ClfA(221–559) with native fibrinogen (Fig. 8). Deletion of the EF-hand motif or substitution of four residues within it had the severest effect on fibrinogen-binding ability. The D310A/D312A double mutant also displayed significant reduction in the ability to bind fibrinogen. The D310A single mutant reduced binding to 60% of the wild type. These results further implicate amino acids 310–321 of region A as playing an important part in the ligand-binding function of ClfA.

**DISCUSSION**

The adhesion of microorganisms to host tissues is the critical first step in the series of events that lead to clinically manifested infections. It has become evident that eukaryotic adhesive ECM components that support adhesion of host cells also serve as ligands for pathogenic microorganisms (1). Fibrinogen, the blood plasma coagulation protein, is also found in the ECM and plays important roles in wound healing. During coagulation, fibrinogen is proteolytically converted to fibrin, which forms the structure of the blood clot. In addition, fibrinogen is the major blood protein deposited on implanted biomaterial (27). Immobilized fibrin/fibrinogen in a blood clot, in the ECM, or present on the surface of biomaterial can serve as a substrate for the adherence of *S. aureus* cells (28, 29).

*S. aureus* has long been known to form clumps in the presence of blood plasma. Hawiger et al. (5) identified fibrinogen as the plasma protein responsible for this phenomenon. Further study revealed that the domain of fibrinogen that interacts with the fibrinogen receptor of *S. aureus* is located at the carboxyl-terminal of the $\gamma$-chain (6, 7). Recently, this was confirmed by showing that purified ClfA protein binds to the extreme C terminus of the $\gamma$-chain of fibrinogen and that a synthetic C-terminal $\gamma$-chain peptide fully inhibits this interaction (7). The 12 residues at the C terminus of the fibrinogen $\gamma$-chain also bind to the integrin receptor ($\alpha_{IIb}\beta_3$) on the surface of platelets, resulting in aggregation *in vitro* and the formation of platelet-fibrin thrombi (9, 18–20, 30). These residues mediate initial contact with nonstimulated platelets and on activation are sufficient to promote stable adhesion to fibrinogen (21). The mode of interaction between fibrinogen and $\alpha_{IIb}\beta_3$ has been studied in some detail. The $\gamma$-chain peptide of fibrinogen binds in a divergent cation-dependent manner to a region of $\alpha_{IIb}$ corresponding to an EF-hand-like sequence (10, 12). A requirement of divalent cations for ligand binding to integrins is often observed. However, inhibition of integrin-ligand binding by $Ca^{2+}$ has also been reported (13, 25, 32–34), although there is no evidence to indicate that $Ca^{2+}$ inhibits the interaction between $\alpha_{IIb}\beta_3$ and the fibrinogen $\gamma$-chain peptide. Recently, Hu et al. (13) identified two classes of cation-binding sites in $\beta_3$-containing integrins: sites that, when occupied by $Ca^{2+}$, promote ligand binding and sites that inhibit ligand binding. The location of the inhibitory cation-binding site(s) has not been identified.

Analysis of the primary structure of region A of ClfA identified a potential divergent cation-binding EF-hand motif at residues 310–321, which differs from the EF-hand consensus at only one residue, a non-cation-coordinating site (Fig. 2b). The 12 residues at the C terminus of the fibrinogen $\gamma$-chain also bind to the integrin receptor ($\alpha_{IIb}\beta_3$) on the surface of platelets, resulting in aggregation *in vitro* and the formation of platelet-fibrin thrombi (9, 18–20, 30). These residues mediate initial contact with nonstimulated platelets and on activation are sufficient to promote stable adhesion to fibrinogen (21). The mode of interaction between fibrinogen and $\alpha_{IIb}\beta_3$ has been studied in some detail. The $\gamma$-chain peptide of fibrinogen binds in a divergent cation-dependent manner to a region of $\alpha_{IIb}$ corresponding to an EF-hand-like sequence (10, 12). A requirement of divalent cations for ligand binding to integrins is often observed. However, inhibition of integrin-ligand binding by $Ca^{2+}$ has also been reported (13, 25, 32–34), although there is no evidence to indicate that $Ca^{2+}$ inhibits the interaction between $\alpha_{IIb}\beta_3$ and the fibrinogen $\gamma$-chain peptide. Recently, Hu et al. (13) identified two classes of cation-binding sites in $\beta_3$-containing integrins: sites that, when occupied by $Ca^{2+}$, promote ligand binding and sites that inhibit ligand binding. The location of the inhibitory cation-binding site(s) has not been identified.

Analysis of the primary structure of region A of ClfA identified a potential divergent cation-binding EF-hand motif at residues 310–321, which differs from the EF-hand consensus at only one residue, a non-cation-coordinating site (Fig. 2b). Secondary structure analysis of the primary sequence of ClfA predicts this putative EF-hand to be flanked by $\alpha$-helices, which are required for correct EF-hand conformation (22). The proposed cation-binding EF-hand motif lies within the minimum 329-residue segment of region A that retains fibrinogen-binding activity (residues 221–550) (3). Also present within the fibrinogen-binding domain is a putative MIDAS motif, a cation-binding sequence contained within the I-domain of integrins (35). Analysis of this motif by site-directed mutagenesis indicated that it plays a role in ClfA binding to fibrinogen. The I-domain of an integrin-like protein from *Candida albicans*, which has homology to the I-domain of the leukocyte integrin $\alpha_{IIb}\beta_2$, also has homology to the fibrinogen-binding domain of ClfA (36).

We have shown that $Ca^{2+}$ plays a role in the interaction between ClfA and fibrinogen. Preliminary experiments showed...
that Ca\(^{2+}\) at high concentrations prevented clumping of *S. aureus* cells in the presence of fibrinogen. In addition, clumping of bacteria (due to the interaction of ClfA and fibrinogen) could be reversed by the addition of Ca\(^{2+}\) (data not shown). These effects were prevented by the addition of EGTA or EDTA. In the studies reported in this work, we have used purified components and a quantitative binding assay. Ca\(^{2+}\) dramatically inhibits the interaction between the γ-chain peptide and region A of ClfA. Mn\(^{2+}\) is a more potent inhibitor than Ca\(^{2+}\), suggesting that Mn\(^{2+}\) has a higher affinity for the inhibitory cation-binding site. Alternatively, Mn\(^{2+}\) may bind to a different cation-binding site. The protein-peptide interaction is not affected by Mg\(^{2+}\) or monovalent cations (Figs. 3 and 4A). Ca\(^{2+}\) also inhibited the adherence of *S. aureus* cells to immobilized fibrinogen (Fig. 4B) at concentrations similar to those that affect the γ-chain peptide interaction. These observations are consistent with the presence of an inhibitory site(s) in ClfA that allows Ca\(^{2+}\) and Mn\(^{2+}\) (but not Mg\(^{2+}\)) to bind. When Clf40-(40–559) and Clf41-(221–559) were incubated with the concentrations of Ca\(^{2+}\) that inhibited ligand binding, distinct differences in secondary structure were evident (Figs. 5 and 6). Thus, Ca\(^{2+}\) binding to an inhibitory binding site(s) in region A of ClfA induces a conformational change in the ligand-binding site that apparently results in inhibition of the ClfA-ligand interaction. It is noted that inclusion of EDTA/EGTA slightly increased the affinity of the fibrinogen γ-chain peptide for ClfA, indicating that divalent cations are not required to promote peptide binding. This feature represents a significant difference from the observed metal ion dependence of the α\text{III}β\text{II}-fibrinogen interaction (12).

Deletion mutagenesis and site-directed mutagenesis were used to identify the Ca\(^{2+}\)-dependent inhibitory site. Deletion of the EF-hand (residues 310–321) or substitution of four of the putative cation-coordinating residues resulted in almost complete loss of both fibrinogen-binding activity and γ-chain peptide-binding activity (Figs. 7A and 8). A single substitution (D310A) and a double substitution (D310A/D312A) caused a significant reduction in ligand-binding affinity (Fig. 8). In addition, the inclusion of Ca\(^{2+}\) with these mutant proteins only marginally affected their binding to the γ-chain peptide (Fig. 7B). The proteins may have retained some ability to bind Ca\(^{2+}\), explaining the small reduction in activity in the presence of the ion. Incomplete EF-hand motifs in other proteins can chelate Ca\(^{2+}\), for example, the platelet integrin α\text{II}β\text{III} (31, 37). The reduced inhibitory effect of Ca\(^{2+}\) with the D310A and D310A/D312A mutant proteins compared with the wild type indicates that the ion interacts with the inhibitory site less effectively. This conclusion is supported by the observation that Ca\(^{2+}\)-induced changes in secondary structure were significantly less in the mutants compared with the wild-type protein (Fig. 6). Taken together, these results implicate the EF-hand motif at residues 310–321 as an inhibitory Ca\(^{2+}\)-binding site. Substitution of four cation-coordinating residues or deletion of the EF-hand motif should result in complete loss of cation-binding ability, but as these proteins have no fibrinogen-binding activity, inhibition of function by Ca\(^{2+}\) and Mn\(^{2+}\) could not be assessed.

In summary, these findings indicate that changes in amino acid sequence 310–321 of region A compromise the protein’s ability to bind fibrinogen and to bind Ca\(^{2+}\) at an inhibitory cation-binding site. There are several possible explanations for this observation. One interpretation is that the inhibitory binding site and the fibrinogen γ-chain peptide-binding site share contact points within sequence 310–321. A model can be proposed in which Ca\(^{2+}\), at millimolar concentrations, binds to an inhibitory binding site within region A of ClfA that overlaps with the ligand-binding site (Fig. 9A). Occupation of this Ca\(^{2+}\)-binding site directly interferes with ligand binding. In an alternative model, the inhibitory site and the fibrinogen-binding site are physically distinct (Fig. 9B). Interaction of Ca\(^{2+}\) with the inhibitory site induces a conformational change in the ligand-binding site that affects the affinity for fibrinogen. Further work is needed to differentiate between these models.

Does Ca\(^{2+}\) have a role in the regulation of ClfA activity *in vivo*? The IC\(_{50}\) determined for the interaction of *S. aureus* cells with fibrinogen is similar to the total concentration of Ca\(^{2+}\) present in normal human sera, although the concentration of ionized Ca\(^{2+}\) is lower (maintained between 1.0 and 1.3 mM) (40). Even at these lower concentrations, Ca\(^{2+}\) may regulate ligand-binding function in a subset of ClfA molecules bound to the cell wall of *S. aureus*. Indeed, the concentration of Ca\(^{2+}\) in the local environment of ClfA may vary significantly due to the protein’s acidic aspartate-serine repeat sequence and the extent of the acidic cell wall and capsule, both of which associate with divalent cations. Thus, the ability of Ca\(^{2+}\) to affect ClfA function may be a direct consequence of the protein’s microenvironment on the cell surface, an environment that changes during *in vivo* growth. One can speculate that Ca\(^{2+}\)-dependent regulation of ClfA activity prevents all of the receptors on intravascular *S. aureus* cells from being occupied by soluble fibrinogen, thus allowing the bacteria (under the right conditions) to adhere to solid-phase fibrinogen or fibrin clots. In addition, this process may allow cells to detach from the initial vegetation, allowing microbial proliferation.

We have shown that ClfA contains a class of Ca\(^{2+}\)-binding sites that, when occupied, regulate fibrinogen-binding activity. Regulation of integrin-ligand binding by divalent cations is well documented, and this phenomenon represents an intriguing similarity between eukaryotic and prokaryotic adherence proteins.
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The Fibrinogen-binding MSCRAMM (Clumping Factor) of Staphylococcus aureus Has a Ca^{2+}-dependent Inhibitory Site

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