Identification of the *Staphylococcus aureus* MSCRAMM clumping factor B (ClfB) binding site in the αC-domain of human fibrinogen

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Clumping factor B (ClfB) of *Staphylococcus aureus* binds to cytokeratin 10 and to fibrinogen. In this study the binding site in human fibrinogen was localized to a short region within the C terminus of the Aα-chain. ClfB only bound to the Aα-chain of fibrinogen in a ligand-affinity blot and in solid-phase assays with purified recombinant fibrinogen chains. A variant of fibrinogen with wild-type Bβ- and γ-chains but with a deletion that lacked the C-terminal residues from 252–610 of the Aα-chain did not support adherence of *S. aureus* Newman expressing ClfB. A series of truncated mutants of the recombinant Aα-chain were tested for their ability to support adherence of *S. aureus* Newman ClfB+, which allowed the binding site to be localized to a short segment of the unfolded flexible repeated sequence within the C terminus of the Aα-chain. This was confirmed by two amino acid substitutions within repeat 5 of the recombinant Aα-chain which did not support adherence of Newman ClfB+. *Lactococcus lactis* expressing ClfB mutants with amino acid substitutions (N256 and Q235) located in the putative ligand-binding trench between domains N2 and N3 of the A-domain were defective in adherence to immobilized fibrinogen and cytokeratin 10, suggesting that both ligands bind to the same or overlapping regions.

INTRODUCTION

*Staphylococcus aureus* is an important opportunistic pathogen of humans that is responsible for a wide range of infections ranging from superficial skin infections to more serious invasive diseases such as endocarditis, osteomyelitis and septicemia. The primary habitat of *S. aureus* is the moist squamous epithelium of the anterior nares (Cole et al., 2001; Peacock et al., 2001). The success of *S. aureus* as a pathogen is due in part to its ability to adhere to a wide range of host tissues including host extracellular matrix proteins such as fibrinogen (Fg), fibronectin and collagen. Adhesion to host proteins is mediated by bacterial cell-wall-associated proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). *S. aureus* can express up to 20 different potential MSCRAMMs that are covalently anchored by sortase to peptidoglycan (Mazmanian et al., 1999; Navarre & Schneewind, 1994).

*S. aureus* expresses several different proteins that can bind specifically to Fg, including clumping factors A and B (ClfA and ClfB) and the bifunctional fibronectin- (and Fg-) binding proteins A and B, FnbpA and FnbpB (McDevitt et al., 1994; Ni Eidhin et al., 1998; Perkins et al., 2001; Wann et al., 2000). ClfA, FnbpA and FnbpB bind to the extreme C terminus of the γ-chain protruding from domain D of Fg. In contrast, the SdrG protein from *Staphylococcus epidermidis* binds to the fibrinopeptide-B protruding from domain E (Davis et al., 2001). *S. aureus* also secretes several proteins that bind Fg, notably coagulase, the extracellular Fg-binding protein (Efb) and MHC class II analogue protein (Map) (Jonsson et al., 1995; McGavin et al., 1993; Palma et al., 1998; Phonimdaeng et al., 1990).

ClfB is only expressed on the cell surface during the exponential phase of growth (McAleese et al., 2001). Ligand binding is specified by the A region, which is divided into three independently folded subdomains N1, N2 and N3 (Perkins et al., 2001). The *S. aureus* metalloprotease aureolysin cleaves ClfB between N1 and N2, resulting in the loss of Fg-binding activity at the end of the exponential phase of growth (McAleese et al., 2001; Perkins et al., 2001). ClfB is a bifunctional MSCRAMM. It binds to cytokeratin 10 (CK10) exposed on the surface of desquamated epithelial cells in addition to Fg. It is a major determinant of the ability of *S. aureus* to adhere to squamous cells and to colonize the anterior nares (O’Brien et al., 2002). The binding domain in CK10 was shown to be quasi-repeats of glycine and serine residues that occur as

Abbreviations: CK10, cytokeratin 10; Fg, fibrinogen; HRP, horseradish peroxidase; MSCRAMMs, microbial surface components recognizing adhesive matrix molecules.
unfolded loops located at the C terminus of the protein, which likely protrude from keratin filaments (Walsh et al., 2004).

Fg is a 340 kDa plasma protein that plays a crucial role in haemostasis. It is composed of two identical disulfide-linked subunits, each of which is composed of three non-identical polypeptide chains, Aα, Bβ and γ (Doolittle, 1984; Henschens & MCDonagh, 1986; Herrick et al., 1999). The removal of C termini of the Aα-chains (residues 220–610) by proteolysis results in generation of αC fragments, representing the whole or parts of the αC-domain (Weisel & Medved, 2001). The αC-domains are involved in fibrin assembly and clot formation (Cierkiewski & Budzynski, 1992; Gorkun et al., 1994; Medved et al., 1985) and control activation of factor XIII (Credo et al., 1981). The αC-domains and the N-terminal portions of the Bβ-chains are the parts of the Fg molecule for which the 3D structure has not been established (Yang et al., 2001). However, recent studies have shown that the αC-domain consists of two structurally distinct regions, a flexible connector region from residues 221–391 and an independently folded, compact portion from residues 392–610 (Fig. 1) (Burton et al., 2006; Tsurupa et al., 2002). In human Fg the flexible NH$_2$-connector region is unordered and is composed of a 43-residue segment followed by ten 13-residue tandem repeats.

In this study we investigated the binding of ClfB to human Fg and have localized the binding site to one of the tandem repeats within the flexible connector region of the αC-domain.

**METHODS**

**Bacterial strains and growth conditions.** *Escherichia coli* strain XL-1 Blue was used as the host for plasmid cloning and was routinely grown in L-broth or agar with ampicillin (100 μg ml$^{-1}$) and tetracycline (10 μg ml$^{-1}$) as appropriate. Strain TOPP3 (Stratagene) was used as the host for recombinant ClfB A-domain (rClfB 45–542) or rClfA A-domain (rClfA 221–559) protein expression. *E. coli* strain JM101 was used for expression of recombinant Fg proteins.

**Fig. 1.** Structure of fibrinogen. Fg consists of two identical disulfide-linked subunits, each of which is composed of three non-identical polypeptide chains, Aα, Bβ and γ. Fg can be divided into four major regions, the central E region, two identical terminal D regions and the αC-domains. The αC-domains (residues 221–610) contain two distinct regions, a compact C-terminal half and an unordered NH$_2$-terminal half or connector region.

The *S. aureus* strains are mutants of strain Newman (Duthie & Lorenz, 1952) defective in ClfA (DU5876 clfA::Tn917) (McDevitt et al., 1994) and a double mutant defective in ClfA and ClfB (DU5944 clfA::Tn917 clfB::Tn917) (Ni Eidhin et al., 1998). Bacteria were routinely cultured in trypticoye broth or on agar. For optimum expression of ClfB, *S. aureus* was grown to exponential phase (OD$_{660}$ 0.6) in 50 ml brain heart infusion broth in a 250 ml conical flask shaken at 200 r.p.m. at 37 °C (McAleese et al., 2001; Ni Eidhin et al., 1998).

*Lactococcus lactis* strain NZ9800 carrying the nisin-inducible expression plasmid pNZ8037 expressing clfB or clfB Q235A was described previously (Miajlovic et al., 2007). L. lactis pNZ8037clfB N526A was constructed by site-directed mutagenesis as described for *L. lactis* pNZ8037clfB Q235A (Miajlovic et al., 2007) using the primer shown in Table 1. *L. lactis* strains were grown statically at 28 °C in M17 (Difco) broth incorporating 0.5% (w/v) glucose, chloramphenicol (Sigma, 10 μg ml$^{-1}$) with nisin at 3.2 ng ml$^{-1}$ to stimulate maximum induction (Miajlovic et al., 2007).

**Manipulation of DNA.** Restriction and DNA modification enzymes were purchased from New England Biolabs or Roche Molecular Biochemicals and were used according to the manufacturers’ instructions. DNA procedures were carried out according to standard protocols (Sambrook et al., 1989).

**Cloning and PCR amplification of Fg constructs.** *E. coli* strains expressing human Fg Aα-, Bβ- and γ-chains were provided by Professor Susan Lord, Department of Pathology, University of North Carolina at Chapel Hill. A 1878 bp fragment from plasmid p166.9 (Lord, 1985) and a 1236 bp fragment from p253 (Bolyard & Lord, 1988) were subcloned into the plasmid pQE30 (Qiagen) to produce recombinant mature Aα- and γ-chains with N-terminal His tags. Subcloning of the mature recombinant Bβ-chain was described previously (Davis et al., 2001). The recombinant plasmids were transformed into *E. coli* strain JM101 for protein expression.

Segments of the Aα-chain were amplified by PCR from the plasmid containing the full-length Aα-chain (residues 1–625 in the common Fg Aα-chain, NCBI accession number AAA17055). Although Fg Aα-chain DNA encodes 625 residues, in plasma the Aα-chain is only 610 residues long due to a post-translational modification. The oligonucleotide primers used for PCR are listed in Table 1. Restriction sites were incorporated at the 5’ ends of the primers to facilitate directional cloning. PCR amplification was carried out in a DNA thermal cycler (Perkin-Elmer Cetus) with Phusion DNA-polymerase (Bioline). Reactions were carried out with a 30 s denaturation step at 98 °C, a 10 s annealing step (the temperature of which depended on the individual primers) and elongation at 72 °C depending on the length of the PCR product. This standard cycle was repeated 25 times followed by incubation at 72 °C for 10 min. PCR products were purified using the Favourgen Gel/PCR Purification Kit (Favourgen Biotech), cleaved with the appropriate restriction enzyme and cloned into plasmid pQE30.

**Mutagenesis of Aα-chain of Fg.** Site-directed mutagenesis was carried out on pQE30 carrying the gene encoding Aα–1–625 using Quickchange (Stratagene). The primers used in the mutagenesis protocol are listed in Table 1. Two mutants were created: Aα–S317P and Aα–T322P.

Tandem repeat 5 of Aα–S317P was made to resemble repeat 3. Repeat 5 of Aα–T322P resembles repeats 1 and 2, which have proline residues in the middle of their repeat sequences.

**Construction of recombinant αα-chain mutants.** Sections of the Aα-chain were deleted by inverse PCR using oligonucleotide primers.

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Table 1. Synthetic oligonucleotides used to amplify Az-chain fragments and for site-directed and deletion mutants of the Az-chain

<table>
<thead>
<tr>
<th>Expression constructs</th>
<th>Primer name</th>
<th>Primer sequence</th>
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<tr>
<td>Truncated proteins</td>
<td>Az-1F</td>
<td>CGG GTA GCC GTA AGT GGA GGT G</td>
</tr>
<tr>
<td></td>
<td>Az-232F</td>
<td>GAC GCA TCA AAG ATG CCG CAG ATG AG</td>
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<td></td>
<td>Az-367F</td>
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<td></td>
<td>Az-283R</td>
<td>CGG GGA AGC TTA ATG CAG GAA CAG CAG AGC AGC CT</td>
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<tr>
<td></td>
<td>Az-574R</td>
<td>CGG GGA GTT TTA GTG TCT TCT GAA ACT CAG G</td>
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<tr>
<td></td>
<td>Az-625R</td>
<td>CGG GGA GTT TTA GGA GGA CAG GAA AGC CT</td>
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<td>Deletion mutagenesis</td>
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<td>GCC CTT GAT GAC TGA GCA GAG ACC AAA ACC CTG GGA G</td>
</tr>
<tr>
<td></td>
<td>Az-341F</td>
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<td></td>
<td>Az-344F</td>
<td>GCC CTT GAT GAC TGA GCA GAG ACC AAA ACC CTG GGA G</td>
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<td>Az-373F</td>
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<td>Az-286R</td>
<td>ATC TCG GGT TCC AGT ACT TCC AG</td>
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<tr>
<td></td>
<td>N526R</td>
<td>CCA CCA TAA CGT ACA ACA GCC TCA TTA TTC CAA C</td>
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Restriction endonuclease sites are underlined. The nucleotides in boldface indicate the location of the desired mutation. F corresponds to forward primer; R corresponds to reverse primer.

in Table 1. The forward primers incorporated a complete EcoRV site and the reverse primers contained half an EcoRV site at their 5’ ends, respectively. The PCR products were digested with EcoRV, religated and transformed into E. coli XL-1 Blue cells. Amino acid residues D and I were inserted in place of the region of DNA that was deleted from the az-domain.

**Sequencing of recombinant plasmids.** All recombinant plasmids were sequenced by GATC Biotech.

**Expression and purification of recombinant proteins.** Recombinant rClfA 221–559, rClfB 45–542 and rClfB 197–542 were purified by Ni²⁺ affinity chromatography as described previously (O’Connell et al., 1998; Perkins et al., 2001). Purification of the recombinant Fg Az-, Bf⁻ and γ-chains, and each of the recombinant Az-chain deletion and truncated proteins, was carried out by Ni²⁺ affinity chromatography (Perkins et al., 2001) with the addition of 6 M urea (Sigma) to the bacterial cell suspension prior to lysis.

**SDS-PAGE and Western immunoblotting.** Recombinant proteins were analysed by SDS-PAGE by standard procedures (Laemmli, 1970) on gels containing 10–15% acrylamide. Gels were stained with Coomassie blue. Human Fg (Calbiochem) was separated by SDS-PAGE and transferred electrophoretically to PVDF Western blotting membranes (Roche Applied Science) by the wet system (Bio-Rad) in Tris/HCl (0.02 M), glycine (0.15 M) and methanol (20 %, v/v). Membranes were blocked for 15 h at 4 °C in 10 % (v/v) non-dry fat milk and then incubated with recombinant ClfA or rClfB (10 μg ml⁻¹) for 1 h with shaking. The membranes were washed three times with PBS containing Tween 20 (0.01 %, w/v) and then incubated with polyclonal antiserum to ClfA (1:2000) (McDevitt et al., 1995) or ClfB (1:2000) (McAleese et al., 2001) as appropriate. Horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (Dako, 1:2000) was used to detect bound antibody. Membranes were developed using LumiGLO chemiluminescent substrate (New England BioLabs) according to the manufacturer’s instructions and exposed to X-ray film.

**Fibrinogen.** Native human Fg was from Calbiochem. Recombinant Fg Az251 contains Az-chains truncated at residue 251 but is otherwise identical to normal human Fg. It was purified from CHO cells media (Gorkun et al., 1998). Plasmin digestion of native Fg was performed to isolate fragment D-domains (Rudchenko et al., 1996). Briefly, Fg (70 mg) in Tris-buffered saline (TBS) pH 7.4 was treated with plasmin [0.0015 unit (mg Fg⁻¹)] for 6 h at room temperature. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (Sigma) to 0.5 mM final concentration and dialysed overnight at 4 °C into TBS pH 7.4. The sample was then applied to a MonoQ HP anion-exchange column and eluted with a gradient of 0–1 M NaCl. SDS-PAGE and Western blotting with anti-Fg domain D and anti-Fg domain E polyclonal antibodies (Cambio) showed the D- and E-domains eluting at 250 mM and 350 mM NaCl, respectively.

**Adherence of bacteria to immobilized proteins.** Adherence of S. aureus or L. lactis to immobilized proteins was performed as described previously (Walsh et al., 2004). Nunc-Immuuno MaxiSorb microtitre plates were coated with the protein in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and incubated overnight at 4 °C. Wells were washed with PBS, BSA (5 mg ml⁻¹) was added and the plates were incubated for 2 h at 37 °C. The plates were washed three times with PBS. A bacterial cell suspension (OD600 1.0) was added (100 µl per well) and the plates were incubated for 1 h at room temperature. Plates were washed three times with PBS and bound cells were fixed with formaldehyde (25 %, v/v) for 30 min and stained with crystal violet (0.5 %, v/v 100 µl per well) for 1 min. Following three washes with PBS, acetic acid (5 %, v/v) was added (100 µl per well) for 10 min at room temperature. The absorbance...
Clumping factor B binding to fibrinogen Aα-chain

w was measured at 570 nm in an ELISA plate reader (Labsystems Multiskan Plus). Inhibition of bacterial adherence by rClfB 197–542 was performed as described by Perkins et al. (2001).

**Binding of rClfB 45–542 to immobilized proteins.** ELISA plates were coated with the appropriate protein in carbonate buffer overnight at 4 °C. Wells were washed twice with PBS and incubated at 37 °C with BSA in PBS for 2 h at 37 °C. They were then washed with PBS and varying concentrations of rClfB 45–542 in PBS with Tween 20 (0.01 %, w/v) were added. The plates were then incubated for 1 h at room temperature. Any unbound protein was removed by washing with PBS, and plates were incubated with rabbit anti-ClfB 45–452 antibodies for 1 h at room temperature. Wells were washed and HRP-labelled goat anti-rabbit IgG (1 : 2000) was added for 1 h at room temperature. Any unbound protein was removed by washing two times with PBS, and plates were incubated with rabbit anti-ClfB antibodies. The rClfB protein bound specifically to the Aα-domain (rClfB 45–542). Binding of rClfB was measured at 570 nm in an ELISA plate reader (Labsystems Multiskan Plus). Inhibition of bacterial adherence by rClfB 197–542 was performed as described by Perkins et al. (2001).

To check that native and mutant Fg A251 were coating the ELISA plates efficiently, HRP-labelled anti-human Fg antibody (Dako http://mic.sgmjournals.org 553) was added to the plates following the initial blocking step. The plates were incubated at room temperature for 1 h with shaking. After washing with PBS, bound antibody was detected by adding tetramethylbenzidine and H2SO4 and reading the absorbance at 450 nm.

**RESULTS**

**ClfB binds to the Aα-chain of human Fg**

Native whole human Fg was separated into individual Aα-, Bβ- and γ-chains by denaturing SDS-PAGE, transferred to PVDF membranes and probed with recombinant (r) ClfB region A-domain (rClfB 45–542). Binding of rClfB was detected with anti-ClfB region A antibodies. The rClfB protein bound specifically to the Aα-chain (Fig. 2a). In contrast, rClfA region A-domain rClfA 40–559 reacted with the γ-chain of human Fg (Fig. 2a), confirming previous reports (McDevitt et al., 1997).

Purified recombinant Fg Aα-, Bβ- and γ-chains were immobilized and tested for their ability to support the adherence of ClfB-expressing *S. aureus* cells. The ability of *S. aureus* Newman cells defective in ClfA to adhere to Fg is exclusively due to expression of ClfB. Newman does not express the bifunctional fibronectin- and Fg-binding proteins FnBPA and FnBPA (Wann et al., 2000). Newman clfA adhered to recombinant Aα-chain (rAα 1–625) in a dose-dependent and saturable manner but did not adhere to either the recombinant Bβ-chain or the γ-chain (Fig. 2b). The double clfA clfB mutant did not adhere to Fg or the Aα-chain (Fig. 2c). These data clearly show that ClfB binds only to the Aα-chain of Fg and not to the Bβ-chain as was reported previously (Ni Eidhin et al., 1998). A truncated but functional form of rClfB 197–542 (Perkins et al., 2001) inhibited adherence of Newman clfB to rAα 1–625 in a dose-dependent manner (Fig. 3), indicating that recombinant ClfB and ClfB expressed by *S. aureus* compete for the same binding site(s).

![Fig. 2. ClfB binds to Fg Aα-chain. (a) Ligand affinity blotting. Human Fg was fractionated by SDS-PAGE and stained with Coomassie blue (lane 1), or transferred to a PVDF-membrane and probed with rClfB 45–542 (lane 2) or rClfA 220–559 (lane 3). Binding of rClf proteins was detected with anti-Clf polyclonal antisera. (b) Adherence of *S. aureus* Newman ClfA– to immobilized recombinant Aα– (●), Bβ– (▲) or γ– (▼) chains of Fg. (c) Adherence of *S. aureus* Newman ClfA– (○) and Newman ClfA– ClfB– (●) to immobilized recombinant Aα-chain of Fg. Cells were grown to exponential phase and suspensions (1×10⁸ c.f.u.) were added to wells. Adherent bacteria were detected by crystal violet staining. The experiment was performed twice with similar results and values represent means ± SD of triplicate wells.](http://mic.sgmjournals.org)

**ClfB binds to a site within the AαC-domain of human Fg**

Mutant recombinant human Fg with a deletion of residues 252–625 comprising the C terminus of the Aα-chain (Fg Aα251) was purified from CHO cells (Gorkun et al., 1998). Native human Fg and Fg Aα251 were coated onto the wells of a microtitre dish and tested for their ability to support adherence of *S. aureus* cells and binding by rClfB protein. *S. aureus* Newman clfA adhered strongly to native Fg but
did not bind to Fg A251 (Fig. 4a). Also rClfB 45–542 bound very weakly to Fg A251 in an ELISA-based assay (Fig. 4b). Plasmin treatment of Fg cleaves the protein into an E-fragment, two D-fragments and two zC-domains. Purified D- and E-domains of Fg did not inhibit the adherence of S. aureus cells to Fg (data not shown). These data suggest that the binding site for ClfB in Fg lies in the C-terminal region of the Aα-chain of Fg between residues T251 and V610.

To investigate further the binding site for ClfB within the C-terminus of the Aα-chain, a series of truncated forms of the recombinant α-chain were expressed (Fig. 5a). The proteins were purified, immobilized in ELISA wells and tested for their ability to support the adherence of S. aureus Newman clfA. Bacteria adhered to the protein corresponding to the C-terminal half of the Aα-chain (rAα 232–625) but did not adhere to the N-terminal region (rAα 1–283, Fig. 5a), confirming that the ClfB-binding site in the Fg α-chain is in the C-terminal domain. The C-terminal α-chain construct (rAα 232–625) was further truncated in order to locate the binding site for ClfB (Fig. 5b). The truncated proteins were tested for their ability to support the adherence of ClfB+ S. aureus cells. The smallest truncate that supported binding of ClfB was rAα 315–574, whereas rAα 367–625 did not.
suggesting that ClfB binds to a site between residues W315 and W367 in the \(\alpha\)-C-domain.

**ClfB binds to repeat region 5 within the flexible connector region of the \(\alpha\)-chain**

The structural organization of the \(\alpha\)-C-terminal domain (residues 221–610) of human Fg has not yet been fully established. It has been shown that each \(\alpha\)-C-domain consists of two structurally distinct regions, a compact C-terminal half connected to the rest of the molecule via an unordered NH\(_2\)-terminal connector region (Fig. 1) (Burton *et al.*, 2006; Tsurupa *et al.*, 2002). In human Fg the flexible connector region starts with a 43-residue segment followed by ten 13-residue tandem repeats (Fig. 6) (Tsurupa *et al.*, 2002). Using r\(\alpha\)-chain truncates we located the binding site for ClfB to between residues W315 and W367 (Fig. 5), corresponding to tandem repeats 5–8. Deletion mutants that had lost one or more of the tandem repeats were created in the plasmid expressing \(\alpha\) 1–625. The ability of *S. aureus* ClfB\(^+\) cells to adhere to the recombinant mutant proteins was investigated (Fig. 7). *S. aureus* cells adhered in a dose-dependent and saturable manner both to the wild-type recombinant \(\alpha\)-chain and to an \(\alpha\)-chain construct that contained a deletion of most of repeat 6 (\(\alpha\) 1–625 \(\Delta\)332–343). Cells also adhered to a construct with deletion of repeats 7 and 8 (\(\alpha\) 1–625 \(\Delta\)342–372), although the binding appeared to be weaker (Fig. 7). However, ELISA with anti-Fg antibodies showed that r\(\alpha\) 1–625 \(\Delta\)342–372 coated the plates poorly compared to all of the other proteins (data not shown), which might explain the lower adherence. Crucially, a deletion lacking repeat 5 (r\(\alpha\) 1–625 \(\Delta\)316–328) failed to support adherence, suggesting that the ClfB-binding site is located in this region of the \(\alpha\)-chain of Fg.

**Altering residues in repeat 5 of the \(\alpha\)-chain disrupts binding to ClfB**

The possibility that an additional ClfB-binding site occurs in repeats 1–3 (residues 264–302) was investigated by

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**Fig. 6.** Schematic diagram showing the organization of the \(\alpha\)-C-domain of human Fg. (a) The unordered NH\(_2\)-terminal half (residues 221–391) is composed of a 43 amino acid segment followed by ten 13-residue tandem repeats. The recombinant \(\alpha\)-chain proteins used in this study are shown by lines and correspond to truncated or deleted variants. (b) Amino acid sequence of the N-terminal part of the \(\alpha\)-C-domain. The ten tandem repeats begin at T264 and are shown in alternating normal and bold type.
isolating two proline substitutions within repeat 5 of rFg Aa1–625. Purified recombinant Aa1–625 chain and two mutants (S317P and T322P) were tested for their ability to support adherence of Newman clfA. ELISA with anti-6xHis antibody (Roche) showed that each protein coated the microtitre plates equally well (data not shown). Newman clfA bound to the wild-type Aa-chain in a dose-dependent and saturable manner (Fig. 8). Newman clfA was not able to adhere to the Aa-T322P mutant, while adherence to the Aa-S317P mutant was reduced. The inability of Aa-T322P to support binding to ClfB suggests that there is a single binding site for ClfB in the α-chain of Fg located in repeat 5.

Expression of ClfB mutants by L. lactis

In order to investigate if the trench located between domains N2 and N3 of ClfB is important in binding to the Fg Aa-chain, several residues with side chains that were predicted to be located close to or within the trench were converted to alanine and expressed on the surface of L. lactis NZ9800 from the nisin-inducible vector pNZ8037. L. lactis-expressing mutants Q235A and N256A were defective in adherence to immobilized Fg (Fig. 9) and CK10 (data not shown) compared to the wild-type control. Western immunoblotting indicated that the proteins were expressed at the same level as the wild-type and were intact. This suggests that CK10 and Fg likely bind to the same region of ClfB.

Fig. 7. Localization of the ClfB-binding site in the tandem repeat region of the αC-domain of Fg using recombinant Aa-chain deletions. Increasing concentrations of recombinant α-chain constructs were immobilized on ELISA plates. Exponential-phase Newman clfA– cells (1×10⁸ c.f.u.) were added to the wells and adherent bacteria were detected by staining with crystal violet. Error bars represent standard deviations for three replicates. The experiment was performed twice with similar results.

Fig. 8. Adherence of S. aureus Newman clfA– cells to immobilized recombinant Fg Aa– (○), AaS317P– (□) and Aa–T322P– (▲) chains. Increasing concentrations of recombinant α-chain constructs were immobilized on ELISA plates. Exponential-phase Newman clfA– cells (1×10⁸ c.f.u.) were added to the wells and adherent bacteria were detected by staining with crystal violet. Bars represent standard deviations for three replicates. The experiment was performed twice with similar results.

Fig. 9. Fg binding by L. lactis ClfB + and L. lactis ClfB mutants. L. lactis ClfB + (▲), L. lactis ClfB Q235A (□), L. lactis ClfB N526A (●) and L. lactis pNZ8037 (▽) were induced with nisin and grown to stationary phase. Adherence of washed cultures to ELISA plates coated with Fg (10 μg ml⁻¹) was assessed by crystal violet staining; the values represent the means ± SD of triplicate wells.
DISCUSSION

Many aspects of haemostasis and wound healing involve the αC-domains of Fg playing a central role. The two self-interacting αC-domains are formed by the C-terminal two-thirds of the two Aα-chains (residues 220–610) (Burton et al., 2006; Medved et al., 1983). Compared to the rest of the Fg molecule, the αC-domains are very sensitive to proteases and are readily cleaved into smaller fragments (Doolittle, 1984; Henschen & MCDonagh, 1986; Weisel & Medved, 2001), and they are the first portions of fibrin to be removed upon fibrinolysis (Tsurupa & Medved, 2001).

This paper presents data that define the binding site in Fg for ClfB as being located in repeat 5 of the flexible region of the Aα-chain. Only the Aα-chain and not the Bβ- or γ-chains could support binding of ClfB. Fg with a deletion of the entire αC region and a recombinant Aα-chain mutant lacking the C-terminal αC region did not support binding. A series of recombinant truncated proteins narrowed down a binding domain to repeat 5 located between residues 316 and 328.

In human Fg each individual tandem repeat is composed of 13 amino acids (Fig. 6). Up to eight residues in the repeats are glycine or serine. Repeat 5 (NGSSGTGSTGNQ) may form a loop similar to the Tyr-(Gly/Ser)₅ Ω loops present in the tail region of CK10, to which ClfB also binds (Walsh et al., 2004). Since the repeats that apparently do not support ClfB binding contain proline and/or arginine residues (Fig. 6), it is possible that these residues interfere with the potential of ClfB to bind to other parts of the repeat region. To examine this, two mutants in repeat 5 mimicking the presence of proline residues located in other putative non-ClfB-binding repeats were isolated in a recombinant Fg α-chain construct that contained each of repeats 1–8. The S317P mutant had reduced affinity for ClfB whereas the T322P mutant was unable to bind ClfB. This suggests that repeat 5 is the only site in the Fg α-chain that ClfB binds to, and that T322 is crucially important for this. The presence of a P in the putative Ω loops in other repeats, in particular repeat 2, might explain their apparent inability to support binding in the T322 mutant. The S317P substitution creates a sequence in repeat 5 that resembles repeat 3. It is unclear why wild-type repeat 3 did not support reduced ClfB binding in the S317P mutant similar to the T322P substitution in repeat 5. Perhaps the sequences at the C terminus of repeat 3 that differ from repeat 5 are responsible.

This study suggests that Fg and CK10 have the same or differing sequences at the C terminus of repeat 3 that resemble repeat 3. It is unclear why wild-type repeats 1, 2, and 3 did not support reduced ClfB binding in the S317P mutant similar to the T322P substitution in repeat 5. Perhaps the sequences at the C terminus of repeat 3 that differ from repeat 5 are responsible.

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REFERENCES


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