Lessons from the Crystal Structure of the S. aureus Surface Protein Clumping Factor A in Complex With Tefibazumab, an Inhibiting Monoclonal Antibody

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The Staphylococcus aureus fibrinogen binding MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules), ClfA (clumping factor A) is an important virulence factor in staphylococcal infections and a component of several vaccines currently under clinical evaluation. The mouse monoclonal antibody aurexis (also called 12-9), and the humanized version tefibazumab are therapeutic monoclonal antibodies targeting ClfA that in combination with conventional antibiotics were effective in animal models but showed less impressive efficacy in a limited Phase II clinical trial. We here report the crystal structure and a biochemical characterization of the ClfA/tefibazumab (Fab) complex. The epitope for tefibazumab is located to the “top” of the N3 subdomain of ClfA and partially overlaps with a previously unidentified second binding site for fibrinogen. A high-affinity binding of ClfA to fibrinogen involves both an interaction at the N3 site and the previously identified docking of the C-terminal segment of the fibrinogen γ-chain in the N2N3 trench. Although tefibazumab binds ClfA with high affinity we observe a modest IC50 value for the inhibition of fibrinogen binding to the MSCRAMM. This observation, paired with a common natural occurring variant of ClfA that is not effectively recognized by the mAb, may partly explain the modest effect tefibazumab showed in the initial clinic trial. This information will provide guidance for the design of the next generation of therapeutic anti-staphylococcal mAbs targeting ClfA.

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1. Introduction
Staphylococcus aureus (S. aureus) causes a number of opportunistic infections that range from relatively benign skin infections to life-threatening diseases including endocarditis, pneumonia and sepsis (Kristinsson, 1989; Lowy, 1998). Clumping factor A (ClfA) is a fibrinogen (Fg) binding microbial surface component recognizing adhesive matrix molecules (MSCRAMM), and an important virulence factor of S. aureus. ClfA plays a role in the molecular pathogenesis of several types of experimental infections such as septic arthritis, infective endocarditis, kidney abscesses and sepsis/septicemia (Flick et al., 2013; Joseffson et al., 2001; Mcadow et al., 2011; Sullam et al., 1996). Furthermore ClfA is important for S. aureus colonization of biomaterials, which presumably becomes coated with plasma proteins such as Fg once implanted (Vaudaux et al., 1995). ClfA binds to the carboxy terminal of the γ-chain of Fg (McDevitt et al., 1995; McDevitt et al., 1997), a region that is important for platelet aggregation and coagulation (Heemskerk et al., 2002; Jackson, 2007; Kamath et al., 2001) and recombinant ClfA has been reported to inhibit the interaction of Fg with the platelet integrin αIIbβ3 (Liu et al., 2007; Liu et al., 2005). However, the virulence potential of ClfA in a mouse model of septicemia does not appear to correlate with altered platelet aggregation or Fg coagulation but rather seems to be a function of impaired bacterial clearance (Flick et al., 2013). In fact ClfA can protect S. aureus against phagocytosis by macrophages (Palmqvist et al., 2004) and it appears that Fg binding to the MSCRAMM is required for the ClfA mediated inhibition of phagocytosis (Higgins et al., 2006). In addition, ClfA has been reported to bind complement factor I. This interaction may also play a role in ClfA dependent resistance to bacterial clearance (Hair et al., 2010; Hair et al., 2008).

Due to the importance of ClfA as a virulence factor, the protein has been explored as a potential vaccine candidate. Recombinant ClfA induced an antibody response in mice (Joseffson et al., 2008) and mice immunized with ClfA presented with less severe arthritis compared to mice immunized with a control antigen (Joseffson et al., 2001).

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Moreover, passive immunization with polyclonal ClfA antibodies generated in rats or rabbits protected mice against *S. aureus* induced sepsis and arthritis (Josefsson et al., 2001). Recently, a multi-mechanistic mAb targeting ClfA and the Alpha toxin was shown to be protective against *S. aureus* infection in a mouse model (Tkaczyk et al., 2016). A combination therapy of vancomycin with high titers of human polyclonal Abs or a mouse monoclonal antibody (mAb) called aurexios or 12-9 against ClfA was effective in a catheter induced infective endocarditis model in rabbits where treating with vancomycin alone was less effective (Patti, 2004; Vernachio et al., 2003; Weems et al., 2006). However, when tefibazumab, a humanized version of aurexios, was used together with antibiotics in a limited phase II clinical trial the results were less impressive (Patti, 2004; Weems et al., 2006).

The domain organization of ClfA is prototypic for the MSCRAMM subfamily of cell wall anchored staphylococcal proteins (Foster et al., 2014). The N-terminus contains a signal sequence followed by the ligand-binding A region that is composed of three subdomains N1, N2 and N3. C-terminal of the A region is the serine-aspartate repeat (Sdr) domain which can become glycosylated (Thomer et al., 2014; Hazenbos et al., 2013) followed by the LPXTG motif and other features required for cell wall anchoring. A segment composed of subdomains N2 and N3 binds a peptide mimicking the C-terminus of Fg γ-chain (γ-peptide) (McDevitt et al., 1997) and a segment containing amino acids 229–545 of ClfA (ClfACC) was shown to represent the minimal protein necessary for appreciable Fg binding (Ganesh et al., 2008).

Many of the staphylococcal MSCRAMMs appear to bind their ligands by variations of the Dock, Lock and Latch (DLL) binding mechanism (for a recent review see Foster et al., 2014). This dynamic binding mechanism was first proposed after analyzing crystal structures of both the apo (open) and the ligand-bound (closed) forms of the N2N3 ligand-binding segment of the Staphylococcus epidermidis Fg-binding MSCRAMM SdrG (Ponnuraj et al., 2003). Subsequent biochemical studies confirmed the major steps of the DLL mechanism for SdrG (Bowden et al., 2008). ClfACC (ClfA229–545) is a variant of ClfA that has a double amino acid substitution to lock ClfA in the closed conformation through formation of a disulfide bridge (Ganesh et al., 2008). While the corresponding SdrGCC is unable to bind ligand due to the closure of the docking trench, ClfACC surprisingly exhibits a higher affinity for the Fg γ-peptide than the wild-type ClfA229–545 (Ganesh et al., 2008). Subsequent structural and biochemical characterization revealed that ClfA binds to Fg by a variant of the DLL mechanism where locking and latching can precede ligand docking (Ganesh et al., 2008).

In order to understand the detailed mechanism of action of tefibazumab, we used a combination of structural, biochemical and biophysical approaches to gain insight into the molecular details of the mAb’s interaction with ClfA and the effects on the MSCRAMM’s Fg binding. We determined the crystal structure of ClfACC in complex with a Fab fragment of tefibazumab. In the co-crystal structure, tefibazumab is bound to the “top” of the N3 domain of ClfA. This mAb binding site is distinct from the trench between N2 and N3 subdomains where the Fg γ-peptide docks (Ganesh et al., 2008) and suggests that residues outside the docking trench on ClfA are also important for Fg binding. Further biochemical studies demonstrate the presence of a second Fg binding site on top of N3 that is critical for an overall high affinity Fg/ClfA interaction. These results reveal that tefibazumab inhibits ClfA binding to Fg by targeting a previously unknown Fg binding site on the MSCRAMM and provide additional target sites for future design of effective inhibitors of the ClfA/Fg interaction.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids and Primers

Amino acid substitutions in the tefibazumab epitope of ClfA were generated in the plasmid pQE30 vector expressing ClfA229–545 from *S. aureus* strain Newman (Ganesh et al., 2008) by site-directed mutagenesis using the primers listed in Supplementary Table ST6. Plasmid pCF41 (O’Connell et al., 1998) carrying DNA encoding ClfA221–559 served as template for introducing Y515A, P457A and W518A substitutions. Overlapping complementary primers containing the desired nucleotide changes (Supplementary Table ST6) were used to amplify the plasmid.

The PCR reaction was incubated with 1 U of the restriction enzyme DpnI (New England Biolabs) for 1 h at 37 °C to digest methylated DNA used as template and transformed into *E. coli* TG1 (Zymo Research). *E. coli* was grown at 37 °C in Luria-Bertani (LB) broth supplemented with ampicillin (100 μg/ml, Sigma-Aldrich). Plasmids were extracted with Wizard Plus SV Minipreps DNA purification system (Promega) and the mutation was confirmed by DNA sequencing (Genewiz).

2.2. Recombinant Proteins

The recombinant proteins were expressed in *E. coli* Top3 (Bayou Biolabs) and purified by nickel chelate chromatography and anion exchange chromatography as previously described (Wann et al., 2000). The GST tagged γ-peptide was expressed and purified as described earlier (O’Connell et al., 1998). Proteins and peptide used in this study and their specific names are listed in Supplementary Table ST1.

2.3. Fibrinogen

Human fibrinogen (Catalog # FIB3, Enzyme Research Laboratories, South Bend, IN) was used in all experiments and dialyzed against 150 mM NaCl, 10 mM KCl, 25 mM Tris pH 7.4 (TBS buffer) unless prepared as described. Fibrinogen D-fragment was purchased from Millipore (Calbiochem, catalog #341600).

2.4. Generation of Fab Fragments

Purified tefibazumab (a generous gift of Inhibitex, Inc.) was dialyzed against 20 mM sodium phosphate pH 7.0 and adjusted to a final concentration of ~10 mg/ml. Beads containing immobilized papain (Thermo Scientific, Rockford, IL) were washed 3 times with phosphate buffer and a 50% slurry was made with the digestion buffer, 20 mM phosphate, 20 mM cysteine, 10 mM EDTA, pH 7.0. Five hundred μl of slurry was added to 2 ml sample supplemented with 20 mM of cysteine and the mixture was incubated for 8 h at 37 °C. The papain beads were then removed by centrifugation and the digest was dialyzed against phosphate buffer. Subsequently undigested IgG and generated Fc fragments were removed by passing the mixture through a protein A column (Thermo Scientific, Rockford, IL).

2.5. Crystallization, Structure Solution and Refinement

The isolated Fab fragments were mixed with purified ClfACC at an equal molar ratio and left for 1 h at 4 °C. The complex was then concentrated to ~ 10 mg/ml for crystallization experiments. Two microliters of the sample was mixed with 2 μl of reservoir solution containing PEG 4000, 2% isopropanol, 0.1 M Hepes pH 7.0 and allowed to equilibrate in a limbro plate at 4 °C. Several crystals were collected, washed 3 times with stabilizing solution, then dissolved and run on an SDS-page gel to confirm the presence of both proteins. The X-ray diffraction data was collected at Advanced Photon Source at Argonne National laboratory for 210° with an oscillation width of 1°. Data was processed using HKL2000 (Otwinowski and Minor, 1997). The structure was solved by the molecular replacement (MR) method using ClfACC (pdbid; 1VR3) as the search model. To determine the MR solution for the Fab fragment, several poly-alanine models of Fv fragments from the PDB database were attempted of which pdb id; 1FT8 (Fokin et al., 2000) yielded a reasonable MR solution. The model was rebuilt using Coot (Emsley and Cowtan, 2004) and refined using PHENIX (Adams et al., 2002) and Refmac 5.0 (Murshudov et al., 1997) to a final R-factor of 0.207 and an
R free of 0.259. Several regions in the constant domain of the mAb showed poor density and therefore backbone atoms were modeled wherever possible. The data collection and refinement statistics are summarized in Table 1.

2.6. Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) experiments were carried out using a VP-ITC instrument (MicroCal). In experiments where the interaction of soluble Fg with ClfACC was characterized the two proteins were co-dialyzed against TBS. The titration was performed at 30 °C using a preliminary injection of 5 μl followed by 29 injections of 10 μl with an injection speed of 0.5 μl/s and a stirring speed of 260 rpm. The cell contained 7 mM Fg (dimer concentration) and the syringe contained 150 μM ClfACC. Since Fg is a dimeric molecule, a single site binding model with 14 μM concentration of Fg was used for data fitting and analyzed using Origin version 5 software (MicroCal).

For ClfA/P16 peptide interaction, the protein was dialyzed and the peptide dissolved in the binding buffer and 10 μl aliquots of 0.5 mM P16 peptide were injected into the cell containing 30 μM ClfA/P16.

2.7. Surface Plasmon Resonance

Surface plasmon resonance-based binding experiments were performed at 25 °C on a Biacore 3000 (GE Healthcare/Biacore, Uppsala, Sweden). Phosphate buffered saline (PBS-T: 8.06 mM Na2HPO4 and 1.94 mM KH2PO4 (pH 7.4), 2.7 mM KCl, 137 mM NaCl, and 0.005% Tween-20) was used as running buffer for immobilization and a higher rate of 30 or 50 μl/min was used during immobilization and a higher rate of 30 or 50 μl/min for binding experiments. The sensor surfaces of Fg, D-fragment or ClfA proteins were prepared on different sensor chips (CM5 for high density, CM3 for medium density and C1 for low density ligand surfaces). The ligands were covalently coupled to the chips using standard amine-coupling chemistry. Frozen Fg stock (about 10 mg/ml in 20 mM sodium citrate–HCl, pH 7.4) were thawed in a 37 °C water bath without any agitation. After equilibrating to room temperature, Fg was diluted in 10 mM sodium acetate (pH 5.5) to 10 or 20 μg/ml and injected into an EDC/NHS activated flow cell. The surface was deactivated with ethanolamine. Fg D-fragment (5 μM/ml), ClfA229–545 and ClfA221–559 (~20 μg/ml) were prepared in 10 mM sodium acetate (pH 5.0), ClfACC was prepared in 10 mM sodium acetate (pH 4.5) at 40 μg/ml for immobilization. A reference surface was made with activation and deactivation steps but with no protein coupled. To prepare a capturing surface for tefbazumab, about 1400 RU of F(ab’2)-goat anti-human IgG Fc gamma antibody (Thermo Fisher Scientific Catalog #31163) were immobilized on CM5 chip using 5 μg/ml of F(ab’2) in 10 mM sodium acetate pH 5.5. Tefbazumab was diluted in PBS-T and captured by F(ab’2), and another flow cell with only immobilized F(ab’)2 served as reference surface. For capturing the GST fusion protein, approximately 11,000 RU of goat anti-GST antibody (GE Healthcare/Biacore) was immobilized on a CM5 chip. The GST-tagged Fg γ peptide was captured by the antibody and created a GST-γ ligand surface. Another flow cell with immobilized anti-GST antibody and captured GST was used a reference surface. To regenerate the ligand surfaces, bound proteins were removed by a 1 min injection of 1 M NaCl for the Fg surface, 10 mM glycine pH 2.6 for tefbazumab surface, and 0.01% SDS for the GST-γ surface.

All SPR responses were baseline corrected by subtracting the response generated from the corresponding reference surface. Double-referenced SPR response curves (with the buffer blank run further subtracted) were used for affinity determination. For steady-state interaction, the equilibrium response of each injection was collected and plotted against the concentration of injected protein. A one-site binding (hyperbola) model was fitted to the data (GraphPad Prism 4, GraphPad Software, Inc., La Jolla, CA, USA) to obtain the equilibrium dissociation constant KD. Non-equilibrium data were globally fitted to a 1:1 Langmuir binding model using BIevaluation software (Version 4.1). Association and dissociation rate constants k1 and k2 were obtained from the fitting, and the dissociation constant KD was derived (k2 = k4 / k3). Errors are reported as standard error of mean from two or more experiments.

3. Results

3.1. Tefbazumab Binds to Different Forms of ClfA and Partially Inhibits the Binding of ClfA to Fibrinogen

The mAb 12-9 (also called aurexis) was raised in mice using ClfA221–559 from S. aureus strain Newman as the antigen and was shown via SPR analysis to bind strongly to the A domain ClfA50–559 with a reported dissociation constant KD of 0.21 nM (rate constants k1 = 1.99 × 106 M−1 s−1 and k2 = 4.18 × 10−2 s−1) (Hall et al., 2003). We recorded very similar binding parameters for 12-9’s interaction with ClfA221–559 with a KD of 0.25 nM (rate constants k1 = 2.28 × 106 M−1 s−1 and k2 = 5.71 × 10−4 s−1, Fig. S1A). Earlier structural and biochemical studies showed that two shorter variants of ClfA covering residues 229–545 called ClfA229–545 and ClfA221–559, respectively, also effectively bound to Fg (Ganesh et al., 2008). In ClfA two Cys residues (D327C/K541C) have been introduced to form a disulfide bond and keep the latch in the latching trench. Since the N2N3 subdomain orientation of ClfA221–559 and ClfA221–559, respectively, also effectively bound to Fg (Ganesh et al., 2008). In ClfA two Cys residues (D327C/K541C) have been introduced to form a disulfide bond and keep the latch in the latching trench. Since the N2N3 subdomain orientation of ClfA221–559 and ClfA221–559, respectively, also effectively bound to Fg (Ganesh et al., 2008). In ClfA two Cys residues (D327C/K541C) have been introduced to form a disulfide bond and keep the latch in the latching trench. Since the N2N3 subdomain orientation of ClfA221–559 and ClfA221–559, respectively, also effectively bound to Fg (Ganesh et al., 2008). In ClfA two Cys residues (D327C/K541C) have been introduced to form a disulfide bond and keep the latch in the latching trench. Since the N2N3 subdomain orientation of ClfA221–559 and ClfA221–559, respectively, also effectively bound to Fg (Ganesh et al., 2008). In ClfA two Cys residues (D327C/K541C) have been introduced to form a disulfide bond and keep the latch in the latching trench.
mAb. Furthermore, the data demonstrate that the epitope for teffbazumab is confined within the N2N3 subdomain and is present in all our recombinant forms of this region.

We then examined teffbazumab’s ability to inhibit ClfA binding to Fg. Even though teffbazumab binds ClfA229–545 with ~800-fold higher affinity (lower $K_d$) than Fg ($K_d$ values of 0.79 ± 0.03 nM vs. 0.61 ± 0.05 μM, Fig. 1A & B), the mAb’s ability to inhibit ClfA229–545 binding to immobilized Fg is relatively weak, with an IC$_{50}$ of 0.33 ± 0.06 μM (Fig. 1C & E), only marginally better than that observed for soluble Fg’s inhibition of ClfA binding to immobilized Fg (IC$_{50}$ = 0.39 μM and the fitted minimum is 27%, the Fg IC$_{50}$ is 0.48 μM and the fitted minimum is 0%). The binding of the inhibitors (at the highest concentration of 4 μM) to the Fg surface were very small (~10 RU), compared to the Fg response of ~550 RU, so they were not subtracted from the total responses.

### 3.2. Overall Structure of the ClfA/Teffbazumab Fab Complex

To uncover the structural basis for the ability of teffbazumab to inhibit ClfA binding to Fg and to determine the epitope on ClfA recognized by the mAb we attempted to crystallize a Fab fragment in complex with ClfA$_{559}$ (which is more stable than our other recombinant forms of ClfA N2N3). The Fab fragment of teffbazumab was generated by digesting the mAb with immobilized papain and cleared by passing the digest through an immobilized protein A column to remove undigested Abs and Fc containing antibody fragments. Different crystallization screens were initially performed for the MSCRAMM/Fab fragment. The conditions described in Materials and Methods were used to generate relatively large crystals suitable for X-ray analyses. The crystals diffracted X-rays to a 2.4 Å resolution and the structure of the complex was solved by the molecular replacement method. The data collection and refinement statistics are summarized in Table 1.

The overall structure of the complex is shown in Fig. 2A. As expected, ClfA$_{559}$ was found in the latched, closed form due to the presence of the engineered disulfide bond. The overall structure of ClfA in the complex is similar to the structure observed in the ClfA$_{559}$/Fg γ-peptide complex (Ganesh et al., 2008) with an rms deviation of 0.62 Å for 302 C$_\alpha$ atoms.

The teffbazumab Fab binds only to the ClfA N3 domain making it unlikely that the mAb could affect N2–N3 subdomain orientations (Fig. 2A) consistent with the SPR data showing that teffbazumab (Fig. S1B) binds bazumab’s ability to inhibit ClfA binding to Fg. Further, while soluble Fg could completely inhibit ClfA229–545 binding to immobilized Fg, there was about 25% of the Fg/ClfA$_{229–545}$ interaction that teffbazumab could not block (Fig. 1 C, D & E). These data indicate that teffbazumab binds ClfA strongly but does not efficiently or completely neutralize the Fg binding activity of ClfA.

### 3.3. ClfA$_{559}$/Fg γ-peptide complex

The overall structure of the ClfA$_{559}$/Fg γ-peptide complex shows that the Fab fragment binds to the N3 subdomain in the Fab/ClfA$_{559}$ complex to induce a major conformational change in the MSCRAMM. Furthermore, teffbazumab binds on “top” of the N3 subdomain of ClfA where
The interaction of CIFA with the light chain of tefibuzumab is primarily hydrophilic in nature and is shown in Fig. 2B. In total nine hydrogen bonds (≤3.2 Å cut off distance) help stabilize the light chain/CIFA ACC interactions (Fig. 2B). These hydrogen bonds are listed in Supplementary Table ST3. The long CDR1 of the light chain makes extensive contact with CIFA and involves eight hydrogen bond interactions with Tyr315(OH) (CDR1) in the D-fragment (Pdb id: 2H43 (Doolittle et al., 2006)) and ClfACC respectively. In addition, Lys345(NZ) and Asn460(OD1) of ClfA contact the backbone carbonyl oxygens of Asn460 and Thr464 in ClfA, respectively. In the N3 domain, CIFA residues Tyr512 and Asp481 of CIFA form hydrogen bonds with residues in the CDR1 targeting the backbone carbonyl oxygen “O” of Ser30 and the side chain of Arg31(NH1), respectively. The Trp518(NE1) and Asn468(ND2) form hydrogen bonds with the CDR2 residues Asn568(OD1) and Asn468(ND2). In addition, Asn477 and Asn463 participate in hydrogen bonds with the backbone oxygen (O) of Phe101 and Gly104, respectively. Surface exposed Trp518 of CIFA stacks with the backbone of Gly54 of the heavy chain. Trp52 of CDR2 docks in a hydrophobic pocket formed by Val411, Trp518 and Tyr510 of CIFA. In addition, a significant number of hydrophobic residues that are surface exposed in the apo structure of CIFA are masked by the interaction with the Fab. Masking of this large patch of a hydrophobic surface could be responsible for the high affinity that tefibuzumab shows for CIFA.

3.5. Biochemical Characterization of the Tefibuzumab Epitope

To confirm the structural model of the CIFA/tefibuzumab complex derived from the diffraction data we made several amino acid substitutions in the CIFA N3 domain at the tefibuzumab epitope (Fig. 3A) and evaluated the effects of the substitutions on the overall CIFA-tefibuzumab interaction. We found that changing Tyr512 in the F-strand of the N3 subdomain to Ala (Y512A) resulted in a slightly reduced binding to tefibuzumab, while a triple mutant (CIFAαvβ3) comprising P467A, Y512A and W518A substitutions almost completely abolished tefibuzumab binding to CIFAαvβ3 (Fig. 3B). Since all these three residues are surface exposed their substitutions should not cause gross structural changes to the protein. Circular dichroism spectroscopy (Fig. S2) indicates that CIFAαvβ3 is properly folded with a similar secondary structure composition as that of CIFAαvβ3.

3.6. The Tefibuzumab/CIFA Complex Structure Implicates a Second Site Required for High Affinity Fibrinogen Binding to the MSCRAMM

Superposition of the ClfACC-Fg γ-peptide ligand complex and the CIFA/tefibuzumab Fab inhibitor complex is shown in Fig. 3D. The peptide binding site is located between the N2 and N3 subdomains and extends along the “C” strand of N3 while the tefibuzumab epitope is found on “top” of N3 (Fig. 3D and E) and does not overlap with the peptide binding site. Thus we are left with an apparent paradox where tefibuzumab effectively inhibits the CIFA/Fg interaction but the Fg γ-peptide binding site and the tefibuzumab epitope do not overlap. Is it possible that the interaction of CIFA with Fg extends beyond the C-terminal segment of the Fg γ-chain and that CIFA makes additional contacts with Fg? In support of a more complex Fg/CIFA binding mechanism we have found that a synthetic Fg γ-peptide, even at high concentrations, can only reduce Fg binding to the MSCRAMM by a maximum of ~50% (Geoghegan et al., 2010). Tefibuzumab may inhibit CIFA binding to Fg by blocking a potential second binding site. Four additional pieces of evidence support a binding model with at least two contact sites between Fg and CIFA.

3.6.1. Molecular Modeling of CIFA/Fg D-fragment

The possibility of multiple contacts between CIFA and Fg was first evaluated by molecular modeling studies. As tefibuzumab inhibits CIFA binding to both Fg and the D-fragment in an almost identical way (Fig. 4C), and the affinity of CIFA for Fg and the D-fragment are very similar (Fig. 4C), it is likely that all the contacts between CIFA and Fg involve only the D-fragment. We therefore used the crystal structures of the Fg D-fragment (Pdb id: 2H43 (Doolittle et al., 2006)) and ClfACC (Ganesh et al., 2008) for the modeling studies. The 17-residue C-terminal segment of the Fg γ-chain, which binds to CIFA in the N23N3 trench, corresponds to a disordered region and cannot be detected in the crystal structure of the ~80 kDa Fg D-fragment. Rigid body docking of the Fab D-fragment on ClfACC resulted in a model of the CIFA/D-fragment complex where the orientation of the C-terminal residue (His400) of the Fg γ-
chain in the D-fragment structure is placed close to the N-terminal residue (Leu392) of the Fg γ-peptide in the ClfA/peptide structure. Molecular modeling showed that the docking of the C-terminal of the Fg γ-chain in the trench between the N2 and N3 subdomains could place the 30 kDa γ-globular module of the D-fragment close to the top face of the N3 domain of ClfA (Fig. 3F). In this model there is a substantial contact area between the D-fragment and the N3 domain of ClfA. Thus it is clear from the model that a second binding site located on "top" of the N3 domain of ClfA is sterically possible when the Fg γ-peptide is docked in the N2N3 trench.

3.6.2. Recombinant ClfA Domains Bind to Intact Fg or the Fg D-fragment With Significantly Higher Affinities Than to the Fg γ-peptide

A binding mechanism involving multiple contact sites in Fg with ClfA should result in a significantly higher affinity of the MSCRAMM for full-length Fg compared to the Fg γ-peptide. An ITC experiment where ClfACC was titrated into a cell containing full-length Fg gave a $K_D$ of 0.3 μM (Fig. 4A) whereas titrating a synthetic C-terminal γ-chain peptide into a cell containing ClfACC gave a $K_D$ of 6.2 μM (Fig. 4B). The 20-fold higher affinity observed for full-length Fg is consistent with a model involving additional contacts between ClfA N2N3 and intact Fg beyond the Fg γ-peptide region and that these second site interactions contribute to the overall higher affinity.

SPR experiments where ClfA229–545 was run over chips containing immobilized intact Fg, Fg D-fragment or GST-γ-globular module showed about a 50-fold difference in $K_D$ for binding of the MSCRAMM to the Fg γ-peptide and the Fg or Fg D-fragment, respectively (Fig. 4C, Supplementary Table ST5). On the other hand, the $K_D$ values for ClfA binding to intact Fg or Fg D-fragment are very similar; 0.56 μM for Fg compared to 0.75 μM for the Fg D-fragment (Fig. 4C). Since most of the known and putative Fg interactive sites in ClfA are located within the N3 subdomain we examined the interactions of a recombinant form of N3 (ClfA370–559) to the different forms of immobilized Fg. ClfA N3 bound to all forms of Fg (Fig. 4D, Supplementary Table ST5). The $K_D$ values for ClfA N3 binding to intact Fg or the Fg D-fragment were similar and about 10 fold higher than the $K_D$ values measured for ClfA229–545 binding to the same proteins. The Fg...
γ-peptide bound to CIfA N3 with about 10-fold lower affinity than those for intact Fg or the Fg D-fragment. Taken together, the results of these binding studies are consistent with our model proposing a second Fg binding site in ClfA and further suggest that all sites in Fg targeting the MSCRAMM are located in the D-fragment.

3.6.3. Amino Acid Substitutions in the Tefibazumab Epitope in CIfA Affect Fg but not Fg γ-peptide Binding to the MSCRAMM

We propose that tefibazumab inhibits the binding of CIfA to Fg by competing for the second Fg binding site on the MSCRAMM. If this model is correct, substitution of residues in the tefibazumab epitope may affect Fg binding to CIfA. Consequently, we examined the Fg binding of CIfA proteins (CIfA\textsubscript{Y512A} and CIfA\textsubscript{PWW}) that were affected in mAb binding (Fig. 3A). SPR analysis using immobilized human Fg showed that the CIfA\textsubscript{Y512A} exhibited reduced binding to Fg (Fig. 3B). The effect of the Y512A substitution was much more pronounced when Fg rather than tefibazumab was the ligand indicating that Tyr512 plays a more important role for Fg binding than for mAb binding. The triple mutant CIfA\textsubscript{PWW} completely lost its ability to bind tefibazumab and showed minimal binding to Fg (Fig. 3B).

Furthermore, our binding model predicts that substitution of the residues located on top of the N3 domain would not affect the binding of CIfA to the Fg γ-peptide. We used ITC to explore if the CIfA\textsubscript{PWW} mutant has retained the ability to bind the Fg γ-peptide P16 (Fig. 3C). P16 is a 17 amino acid long Fg γ-peptide that contains one residue substitution (Asp to Ala at the 16th position of the γ-peptide) and binds CIfA with a higher affinity compared to a corresponding peptide with a wild-type sequence (Ganesh et al., 2008). P16 bound CIfA\textsubscript{PWW} with a similar affinity (\(K_D = 5.8 \mu M\)) to that previously recorded for P16 binding to CIfA\textsubscript{Y512A} (\(K_D = 3.0 \mu M\)) (Ganesh et al., 2008). In addition, SPR analysis of CIfA\textsubscript{PWW} binding to Fg and the D-fragment, respectively, indicated \(K_D\) values in the low micromolar range (10 \(\mu M\) and 17 \(\mu M\), respectively, data not shown) comparable to the Fg γ-peptide binding to CIfA (4.3 \(\mu M\), Fig. 3F). These results suggest that the Dock, Lock and Latch mechanism of Fg binding remains intact in the CIfA\textsubscript{PWW} mutant and that CIfA\textsubscript{PWW} specifically lost the second Fg binding site.

3.6.4. Tefibazumab Does Not Inhibit the Fg γ-peptide Binding to CIfA

If tefibazumab affect Fg binding only at the second site, but not in the N2N3 trench, we would not expect binding of the Fg γ-peptide to CIfA to...
be inhibited by the mAb. To evaluate this hypothesis, we used SPR analysis of tefibazumab-mediated inhibition of ClfA221–545 binding to immobilized GST-γ-peptide (GST-γ). ClfA221–545 could still bind to GST-γ in the presence of tefibazumab (Fig. 5). In fact the response is higher in the presence of the mAb, which likely reflects that a tefibazumab: ClfA221–545 complex is formed and bind to the immobilized GST-γ. To demonstrate that ClfA can simultaneously bind to the Fg-γ-peptide and the mAb we took advantage of the relatively stable interaction between ClfACC and GST-γ, shown by a slower dissociation phase in the SPR curve (Fig. 5B). In this system the GST-γ bound

Fig. 5. Effect of tefibazumab on ClfA binding to immobilized GST-γ. (A) SPR experiments for 5 μM ClfA229–545, binding to GST-γ surface (700 RU, captured by anti-GST pAb), in the presence (dashed line, with the tefibazumab background response subtracted) or absence (solid line) of 2 μM tefibazumab. (B) Taking advantage of the slow off-rate of ClfACC (5 μM) binding to GST-γ (black line), strong binding of 2 μM of tefibazumab to the GST-γ bound ClfACC was shown (in green), compared to non-specific binding of tefibazumab (2 μM, in blue) to the GST-γ.

Fig. 6. ClfA229–545 and some natural variants binding to tefibazumab and Fg. (A) Sensorgrams generated by binding of each ClfA protein at 32 nM concentration to the surface of tefibazumab (~200 RU captured through goat anti-human IgG (Fc) polyclonal F(ab’)2). Dissociation constants $K_D$ for each ClfA/tefibazumab interaction are listed, and sensorgrams with fitting are shown in Supplementary Figure S3. (B) Binding of 320 nM of each protein to the immobilized Fg (about 600 RU) are overlaid and the $K_D$ for each of the ClfA/Fg interactions is listed. Standard errors for the $K_D$ measurements from different experiments ($n$ ≥ 2).
ClfA was still able to bind tefibazumab. Thus tefibazumab did not inhibit ClfA binding to GST-γ, while the mAb could inhibit ClfA binding to Fg (about 70%, Fig. 1D&E) and D-fragment (about 80%, Fig. S1C).

Taken together these results demonstrate that tefibazumab inhibits Fg binding by blocking a second binding site located at the top of the N3 domain which is distinct from the Fg γ-chain binding site located in the trench between the N2N3 subdomains. A schematic diagram representing the regions of Fg and tefibazumab binding to ClfA and the mechanism of tefibazumab’s partial inhibition of Fg binding to the MSCRAMM is shown in Fig. 7.

4. Discussion

The crystal structure of ClfA N2N3 in complex with the Fab fragment generated from tefibazumab defines the epitope for this inhibiting mAb, which to our surprise is located on top of the N3 subdomain. Through biochemical studies we demonstrate that the tefibazumab epitope partially overlaps with a second Fg binding site, which is required for high affinity binding of Fg to ClfA. A substantial variation in the amino acid sequence of the identified tefibazumab epitope is apparent from examining ClfA sequences available in the public domain (Supplementary Table S1). To evaluate the significance of these variations we expressed four of the more common tefibazumab epitope isoforms and determined their relative binding to immobilized tefibazumab by SPR. One of these isoforms (ClfAN463R) where Asn is replaced by the bulky charged residue Arg, showed a 60-fold reduced affinity for the mAb (K_D = 0.7 nM (ClfA229-545) to K_D = 45 nM (ClfAN463R) (Fig. 6A). Interestingly, this variant appears to have maintained its high affinity for Fg (Fig 6B). These results demonstrate that tefibazumab recognizes some but not all naturally occurring ClfA variants of S. aureus. In addition, tefibazumab cannot completely block Fg binding to ClfA and although the recorded K_D for the mAb’s binding to ClfA is low it is not quite as low as that demonstrated for the corresponding mouse mAb before being “humanized” (Fig. 1). Furthermore, the observed IC_50 is rather high (Fig. 1), which may be a consequence of the complex Fg binding mechanism employed by ClfA. Taken together these properties may explain the modest effect of tefibazumab as an anti-staphylococcal therapy in humans (Weems et al., 2006).

Our earlier study showed that ClfA binding to Fg involves a variant of the Dock, Lock and Latch mechanism where the C-terminus of the Fg γ-chain docks in a trench formed between the N2 and N3 subdomains of the MSCRAMM (Ganesh et al., 2008). In the current report we demonstrate that the high affinity interaction of ClfA with intact Fg involves additional contacts between the MSCRAMM and the ligand protein. These additional interactions increase the overall affinity of the interaction and appear to involve residues in the γ-globular domain of the γ-chain of Fg that can be brought into close contact and interact with the “top” of the N3 domain according to our modeling experiments.

Is it possible that this complex, multi-contact binding mechanism here shown for the ClfA/Fg interaction, also applies to other ligand/MSCRAMM interactions. The Fg binding MSCRAMMs FnBPA (Wann et al., 2000), FnBPB (Burke et al., 2011) of S. aureus and FnB of S. lugdunensis (Geoghegan et al., 2010) all target the C-terminus of the Fg γ-chain using N2N3 segments with similar subdomain organization to ClfA. Thus Fg binding to these MSCRAMMs could also involve additional binding sites. A synthetic peptide corresponding to the linear sequence in Fg targeted by the related protein Cfb binds to the MSCRAMM with rather low affinity and the peptide is a poor inhibitor of Fg binding to Cfb (unpublished data). To account for the much higher affinity seen

![Fig. 7. Schematic representation of the binding and inhibition mechanism of Fg and tefibazumab respectively to ClfA. (A) Domain organization of fibrinogen and ClfA. The inter chain disulphide bonds linking the individual α, β, and γ chains and the dimeric Fg molecule are shown as black lines. The α, β, and γ chain regions corresponding to D-fragment are shown in orange, light blue and red respectively. The N2 and the N3 subdomains in ClfA are colored green and yellow respectively. (B) A schematic model showing the Fg binding regions on ClfA and the tefibazumab epitope. Tefibazumab can only partially inhibit Fg binding to ClfA by competing for the second Fg binding site on the MSCRAMM.](image-url)
with intact Fg, CIB like ClfA, may provide additional interactive sites outside the N2N3 trench. The Fg-binding MSCRAMM SdrG binds to a 15 amino acid linear Fg sequence in a rather high affinity interaction (Ponnuraj et al., 2003). In this case it is possible that additional sites are not required for an overall high affinity, although preliminary modeling experiments suggest that additional contacts are possible. Thus, although the DLL binding mechanism seems to be involved in most MSCRAMM ligand interactions additional contacts may be required for high affinity interactions. Furthermore, it is possible that sequence variations in the MSCRAMM at the second binding site could affect the affinity for Fg and thus the virulence potential of the ClfA variant. It is also possible that amino acid variations in human Fg sequences targeting the second site may bind with different affinities to the MSCRAMM and consequently affect the susceptibility of an individual to staphylococcal infections.

It should be pointed out that the study reported here has been conducted with a segment of ClfA and it is unclear if Fg binding to the full-length MSCRAMM can involve even more extensive interactions outside the N2N3 sub-domains. Our studies with tefibazumab have also revealed a novel inhibitory mechanism for the binding of ClfA to Fg. It is clear that the mechanism by which ClfA binds Fg is much more complex than previously appreciated and this complexity can at least partly explain the difficulty in generating effective anti-ClfA therapeutic agents including mAbs that can efficiently recognize natural ClfA variants from different strains of S. aureus. However, further characterization of the second binding site(s) may provide the opportunity to design antibodies and other molecules that interfere with both Fg-binding sites on ClfA to effectively inhibit this interaction that appears to be critical and this complexity can at least partly explain the difficulty of platelet aggregation. Blood 109, 2178–1794.

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