Crystal Structures Reveal the Multi-Ligand Binding Mechanism of *Staphylococcus aureus* ClfB

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Abstract

*Staphylococcus aureus* (*S. aureus*) pathogenesis is a complex process involving a diverse array of extracellular and cell wall components. ClfB, an MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) family surface protein, described as a fibrinogen-binding clumping factor, is a key determinant of *S. aureus* nasal colonization, but the molecular basis for ClfB-ligand recognition remains unknown. In this study, we solved the crystal structures of apo-ClfB and its complexes with fibrinogen α (Fg α) and cytokeratin 10 (CK10) peptides. Structural comparison revealed a conserved glycin-serine-rich (GSR) ClfB binding motif (GSSGKXGXXG) within the ligands, which was also found in other human proteins such as Engrailed protein, TCF20 and Dermokine proteins. Interaction between Dermokine and ClfB was confirmed by subsequent binding assays. The crystal structure of ClfB complexed with a 15-residue peptide derived from Dermokine revealed the same peptide binding mode of ClfB as identified in the crystal structures of ClfB-Fg α and ClfB-CK10. The results presented here highlight the multi-ligand binding property of ClfB, which is very distinct from other characterized MSCRAMMs to-date. The adherence of multiple peptides carrying the GSR motif into the same pocket in ClfB is reminiscent of MHC molecules. Our results provide a template for the identification of other molecules targeted by *S. aureus* during its colonization and infection. We propose that other MSCRAMMs like ClfA and SdrG also possess multi-ligand binding properties.

Citation: Xiang H, Feng Y, Wang J, Liu B, Chen Y, et al. (2012) Crystal Structures Reveal the Multi-Ligand Binding Mechanism of *Staphylococcus aureus* ClfB. PLoS Pathog 8(6): e1002751. doi:10.1371/journal.ppat.1002751

Editor: Mark A. Saper, University of Michigan, United States of America

Received November 29, 2011; Accepted April 30, 2012; Published June 14, 2012

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Funding: This work was supported by the Ministry of Science and Technology of China (2011CB910500 and 2012CB911100) and National Natural Science Foundation of China (31030020, 3110769 and 31101824 ). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

*Staphylococcus aureus* (*S. aureus*), an important opportunistic pathogen, is a major threat to humans and animals causing high morbidity and mortality worldwide. It is responsible for a variety of infections ranging from mild superficial infections to severe infections such as infective endocarditis, septicarthritis, osteomyelitis and sepsis [1]. Such infections are of growing concern because of the increasing antibiotic resistance of *S. aureus* infections ranging from mild superficial infections to severe infections such as infective endocarditis, septicarthritis, osteomyelitis and sepsis [1]. Approximately 80% of invasive *S. aureus* infections are autologous in that they are caused by strains attributed to two classes of virulence determinants: cell wall-associated proteins and extracellular protein toxins. The initial step in pathogenesis is often cell adhesion, mediated by surface adhesins called MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) [6,7]. To date, *S. aureus* is known to express more than 20 different potential MSCRAMMs [8,9].

SD-repeat-containing (Sdr) proteins are members of the MSCRAMM family, including clumping factor A (ClfA), ClfB, SdrC, SdrD and SdrE of *S. aureus* and SdrF and SdrG of *S. epidermidis*. The Sdr proteins are characterized by the presence of an R region composed largely of repeated SD dipeptides [10]. They exhibit a comparable structural organization including an N-terminal secretory signal sequence followed by a ligand-binding A region and a dipeptide repeat region (R) composed mainly of aspartate and serine residues. The LPXTG cell wall-anchoring motif (W) immediately follows the SD-repeat region and is followed by a hydrophobic membrane-spanning domain (M) and a short positively charged cytoplasmic tail (C). Despite their conserved structural organization, the Sdr proteins are not closely related in sequence, with only 20 to 30% identical amino acid residues in the ligand-binding A domain. This suggests that different Sdr proteins might play different roles in *S. aureus* pathogenesis [11].

ClfB is one of the best characterized surface proteins on *S. aureus* during the past decade [12–18]. The multi-functional character-
**Author Summary**

*Staphylococcus aureus* (S. aureus), an important opportunistic pathogen, is a major threat to humans and animals, causing high morbidity and mortality worldwide. It is responsible for a variety of infections ranging from mild superficial infections to severe infections such as infective endocarditis, septic arthritis, osteomyelitis and sepsis. Such infections are of growing concern due to the increasing antibiotic resistance of *S. aureus*. In order to understand the mechanism of the *S. aureus* pathogenesis, we studied one of the bacterial surface proteins clumping factor B (ClfB) bound by the fibrinogen α (Fg α) and cytokeratin 10 (CK10). From analyses of the high resolution crystal structures we found that the ClfB-binding peptides harbor a stretch with consensus sequence (GSSGXXGXG) that is also conserved in Engrailed protein, TCF20 and Dermokines. The interaction between ClfB and a dermokine-derived peptide was demonstrated using binding assays. Consistent with a role of ClfB in the inflammatory responses induced by *S. aureus*, expression of dermokines is predominant in epithelial tissues and upregulated in inflammatory diseases. The data presented in this study raise a possibility that multiple human proteins are targeted by ClfB during *S. aureus* infection. The multi-ligand binding feature of ClfB would be valuable for developing new therapeutic strategies.

**Results**

**Structure of apo-ClfB(197–542)**

Previous studies indicated that a segment of ClfB containing N2 and N3 regions (Figure 1A) is sufficient for recognition of Fg α and CK10 [18,27,29]. We therefore cloned the segment encoding the two regions (amino acids 197 to 542) of the ClfB protein from *S. aureus* and purified the protein from *E. coli* for our structural studies. The structure of the ClfBapo_197–540–Fg α,CK10 complex was solved by a Se-Met derived protein and was used as a starting model for determination of the other structures by the molecular replacement method (Table 1).

The apo-ClfB(197–542) structure was solved at 2.5 Å resolution, consisting of residues Ser197-Ala534 (Figure 1B). No electron density was observed for the C-terminal eight residues in the apo-ClfB structure. The polypeptide chain of apo-ClfB(197–542) is composed of two distinct domains N2 and N3, as previously described for other MSCRAMMs in *S. aureus* (Figure 1A) [30]. The N-terminal N2-domain contains 146 residues (amino acids 213–358) and the N3 domain 170 residues (amino acids 359–528). In the crystal structure, both N2 and N3 have two layers of β-sheets that pack tightly against each other (Figure 1B). In contrast, packing between the two domains is much looser, resulting in the formation of a large groove between them where presumably ligands bind. In N3 domain, strands A, B, E, and D form one of the two principal sheets, while strands D’, D**, C, F, and G on the opposite face present the other. Similar to the structures of other Fg-binding MSCRAMMs [21,28,29,31], the structures of N2 and N3 display a typical Dev-IgG fold featured by the existence of the additional strands D’ and D** as compared to the C-type IgG fold [30]. The structures of the N2 and N3 domains can be well superposed with an rms deviation of 0.98 Å for all Cα atoms. One structural difference between them, however, is the three-stranded β-sheet (A, B and E) on one side of N2 in comparison with a four-stranded β-sheet (D, F, C and G*) on its corresponding side in N3, as described in the structures of ClfA, SdrG and ClfB [21,28,29].

ClfBSer197 and ClfBE218D or even a short N-terminally extended segment such as the unrelated His-tag were shown to be necessary to maintain the Fg binding activity of ClfB [16], though the mechanism of how the N-terminal segment of N2 participates in substrate binding is unclear. In the crystal structure of apo-ClfB, the N-terminus (Ser197-Ala201) of one ClfB-542 molecule binds to the N3 domain of a symmetry-related ClfB molecule, forming a β-sheet together with the strand G (Figure 1C and Figure S1) mediated by 2 pairs of main-chain hydrogen bonds. Additional hydrogen bonds involving ClfBSer197 and ClfBE218D further contribute to the N-terminus-mediated interaction between ClfBs (Figure 1D). These interactions may act together to stabilize the G-strand of the N3 domain, thus maintaining its Dev-IgG fold and mimicking the transition state of ligand binding.

[All structural figures in this paper were generated with PyMOL [32].]
ClfB is a key adhesin mediating *S. aureus* adherence by binding to CK10 and Fg [18,27]. To study the molecular mechanisms underlying ClfB-ligand recognition, we solved the crystal structures of ClfB(208–542) in complex with CK10 (amino acids 499–512, referred as CK10(499–512)) or Fg α (amino acids 316–328, referred as Fg α(316–328)) at 2.3 Å and 1.92 Å, respectively (Figure 2A). The electron density unambiguously defines the existence of the peptides in the structures (Figure S2). In both complexes, the peptides adopt an extended conformation and are inserted into the tunnel formed between N2 and N3. Structure comparison revealed that the peptide binding induces an extension of β-strand G at its C-terminal side, which covers the bound peptides (Figure 2A and Figure S2). Similar structural features have also been observed in the structures of ClfA and SdrG complexed with their respective ligands (Figure S3) [21,28]. Tight contacts between the peptide and the two domains in each complex result in extensive interactions, with a buried surface area of 966.6 Å² in ClfB-Fg α(316–328) and 1002.6 Å² in ClfB-CK10(499–512).

Structural comparison of the apo-ClfB and the two complexes shows that the RMSDs of the Cα atoms in ClfB are 0.46 Å and 0.49 Å respectively, indicating that the overall ClfB remains unchanged upon binding of the ligands (Figure 2A). Marked conformational changes, however, occur to the C-terminus of ClfB(499–512) in both complexes. In ClfB-Fg α(316–328), the residues ClfB Arg529–Ser542 that are disordered in the structure of apo-ClfB become well defined following Fg α(316–328) binding. The distal C-
terminus of ClfB(197–542) forms a short β-strand G, which forms a parallel β-sheet with the β-strand E from the N2 domain. The formation of the β-sheet is mediated by several main chain and side chain hydrogen bonds (Figure 2B). The ligand-induced stabilization of the C-terminal peptide of ClfB allows it to run across Fga(316–328) on the top. This binding mode is consistent with the DLL model as demonstrated in SdrG-Fga complex [21,28]. In contrast with Fga(316–328), the peptide CK10(499–512) did not induce formation of the β-strand G in ClfB (Figure 2A). Nonetheless, the C-terminal portion of strand G that interacts with Fga(316–328) also becomes well defined and caps on the CK10 (499–512) peptide.

While we were preparing this manuscript, the structures of apo- and ligand binding ClfB were reported by V.Ganesh et. al [29]. Interestingly, the structural features we observed here are noticeably distinct from those of Fig α/CK10-ClfB complexes solved by them [29]. In both of their structures, particularly in the Fig α-ClfB complex, although the peptide adopts a conserved conformation as that in our structure, the C-terminus of the G-strand exhibits a different orientation and is not inserted into the N2 domain to form an extra strand G with the strand E, and thus the peptide is not locked in the groove between N2 and N3 (Figure 2C). In this way, their structures do not support the DLL model proposed based on the SdrG protein structure [21]. In addition, on peptide binding no rearrangement occurs to the loop between D and D9 in N2 (Figure 2C). Although the C-terminus of ClfB in the CK10-ClfB complexes has similar conformation as that in our structure, the D D9 loop in N2 domain shows no rearrangement, either (Figure 2D). The differences in the peptide conformations observed between our and Ganesh et al. works, could be attributed to the methodologies adopted in crystallization. While we co-purified the ClfB with the peptides to form a complex prior to crystallization, Ganesh et al. reported that they soaked the peptides into the apo-ClfB crystals [29]. In their structures, the conformational changes observed in our study to accommodate the peptide and then to lock it in place could have been hindered by crystal packing within the crystals.

| Parameter             | Fg α bound | CK10 bound | Dermokine bound | Peptide free |
|-----------------------|------------|------------|-----------------|-------------|
| Data collection       | Se-SAD     | Native     | Native          | Native      |
| Space Group           | P3 2,2    | P3 2,2     | P3 2,2          | P 4 1, 2, 2 |
| Unit Cell (Å)         | 70.98, 70.98, 174.91 | 70.33, 70.33, 177.15 | 70.08, 70.08, 175.76 | 94.42, 94.42, 86.71 |
| Wavelength (Å)        | 0.979      | 0.979      | 0.919           | 0.979       |
| Resolution (Å)        | 1.92 (1.99-1.92) | 2.3 (2.34-2.3) | 2.5 (2.59-2.5) | 2.51 (2.6-2.51) |
| Rsym* (%)             | 9.6(68)    | 8.6(50)    | 7.8(54)         | 7.5(53.1)   |
| I/σ (Å)               | 56.5(1.75) | 28(2.5)    | 22.9(2.1)       | 45.6(10.1)  |
| Completeness (%)      | 99.5(98.1) | 99.6(99.5) | 98.3(88.1)      | 100(100)    |
| Redundancy            | 8.2(7.6)   | 10.2(7.9)  | 8.5(5.9)        | 14.4(9.9)   |
| Wilson B factor (Å²)  | 40.6       | 38.7       | 61.3            | 52.1        |

SAD phasing
- Anomalous scatterers: 3 Se
- Figure-of-merit (FOM): 0.387
- FOM after DM: 0.687
- FOM after phase combination: 0.788

Refinement
- R factor*: 0.1792
- R free: 0.2145
- No. of atoms: 2899 protein atoms+
H2O+2 Mg²⁺
- B factors:
  - Overall: 38.53
  - Main chain: 36.45
  - Side chain: 40.6
  - RMSD bond lengths: 0.006
  - RMSD bond angles: 0.998
- Ramachandran plot statistics (%)
  - Preferred regions: 95.4
  - Allowed regions: 4.4
  - Outliers: 0.3
- PDB code: 4F27

Table 1. Statistics of data collection and structure refinement.

*Values in parentheses are for the highest resolution shell.

Fast Fourier Transform (FFT)
- FOM after DM: 0.687
- FOM after phase combination: 0.788

Refinement
- R factor*: 0.2204
- R free: 0.2546
- No. of atoms: 2593 protein atoms+
H2O+2 Mg²⁺
- B factors:
  - Overall: 39.47
  - Main chain: 37.11
  - Side chain: 41.84
  - RMSD bond lengths: 0.008
  - RMSD bond angles: 1.168
- Ramachandran plot statistics (%)
  - Preferred regions: 93.4
  - Allowed regions: 4.4
  - Outliers: 0.3
- PDB code: 4F1Z

Values in parentheses are for the highest resolution shell.

Rsym = \frac{\sum_{i=1}^{N} |I_{h,i}| - \bar{I}_{h,i}}{\sum_{i=1}^{N} \bar{I}_{h,i}}

R = \frac{\sum_{h \neq h_{obs}} |F_{h_{obs}} - F_{h_{calc}}|}{\sum_{h \neq h_{obs}} F_{h_{obs}}}

Values in parentheses are for the highest resolution shell.

Rfree was calculated with 5% of the reflections.

doi:10.1371/journal.ppat.1002751.t001

PDB code: 4F27
In all, our structures strongly support the DLL model for ClfB-ligand binding. Briefly, “Dock” of the peptide triggers the rearrangement of the C-terminus of the N3 domain, allowing ClfB\textsuperscript{Arg529} to form a hydrogen bond with the ClfB\textsuperscript{Asn238} from N2 domain. This would result in “Lock” of the peptide into the substrate binding groove, whereas the strong interaction between G’ and the E strand of N2 can “Latch” the peptide (Figure 2B).

Structural comparison of ClfB with ClfA and SdrG

In spite of the low identities in the amino acid sequences, the structures of ClfB, ClfA and SdrG exhibit high similarities (Figure 3). The most conserved residues are mainly located in the loop region of them (Figure 3B). Although the adherence domain organizations of ClfB, ClfA and SdrG and their ligand binding sites are conserved, the ligand binding specificities of the
three MSCRAMMSs vary (Figure 3D) [18,21,28]. All the bound peptides form into a β-strand paired with the G-strand and pass through the tunnel formed by the N2, N3 and the end of the G-strand (Figure S3). In the ClfB-Fgα(316–328)/CK10(499–512) structures, one peptide is bound to one ClfB, in the same orientation as the Fgγ-chain peptide in ClfA and a reverse orientation compared to the Fgβ-chain peptide in SdrG (Figure 3D) [21,28].

In both ClfA-Fgγ and SdrG-Fgβ structures, the C-terminus of the N3 domain forms a β-strand G′ (Figure 3D). ClfA Tyr338 that is conserved in the structures of SdrE and SdrD (data not shown), forms a hydrogen bond with the amino acid at the end of the G′ strand (Asn530 in ClfA), thus stabilizing the conformation of the G′ strand (Figure S4). In ClfB, the amino acid at the corresponding position is substituted with phenylalanine (ClfBPhe328) (Figure 3A). Comparison of the apo- and ligand-bound form structures of ClfB indicates that the interactions between the ligands and the G strand of N3 play a vital role in the redirection of the C-terminus of N3. ClfBArg529, the last residue in the C-terminus of the G strand in ClfB, interacts with the ligand peptides in both complex structures. ClfBAsn238 and ClfBArg529 form a stable hydrogen bond to lock the peptides into the GG′ covered tunnel. Interestingly, although in the ClfB-CK10 structure the G′ strand appears disordered, the ClfB Asn238-Arg522 hydrogen bond also exists (Figure 2B), consistent with the DLL model. Taken together, our structures strongly support the DLL model for ClfB-ligand binding.

Peptides recognition of ClfB

In the crystal structures of the ClfB-Fgα(316–328)/CK10(499–512) complexes, both peptides lie down into a tunnel between N2 and N3. The peptides are covered by the amino acid at the end of the G′ strand (Asn530 in ClfA), thus stabilizing the conformation of the G′ strand (Figure S4). In ClfB, the amino acid at the corresponding position is substituted with phenylalanine (ClfBPhe328) (Figure 3A). Comparison of the apo- and ligand-bound form structures of ClfB indicates that the interactions between the ligands and the G strand of N3 play a vital role in the redirection of the C-terminus of N3. ClfBArg529, the last residue in the C-terminus of the G strand in ClfB, interacts with the ligand peptides in both complex structures. ClfBAsn238 and ClfBArg529 form a stable hydrogen bond to lock the peptides into the GG′ covered tunnel. Interestingly, although in the ClfB-CK10 structure the G′ strand appears disordered, the ClfB Asn238-Arg522 hydrogen bond also exists (Figure 2B), consistent with the DLL model. Taken together, our structures strongly support the DLL model for ClfB-ligand binding.

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mediated through a number of hydrogen bonds. The conserved hydrogen bonds are observed between ClfB and the middle region of the two peptides (Figure 4). Hydrophobic contacts of the middle region of both peptides with the G strand of ClfB (316–328), the loop between β-sheet A, B and the loop between β-sheet C, D of N2 domain also contribute to peptide-protein interactions.

In the ClfB(328-331)-CK10(499-512) complex structure, four pairs of hydrogen bonds were observed between the main chains of the peptide and the G strand of N3 domain, resulting in the formation of a parallel β-sheet. Polar groups in side chains of ClfB (Trp522, and Asn524, Gly269, Val271, and Phe328). Modeling studies (data not shown) indicated that any residue with a side chain would generate steric hindrance and cannot be accommodated in the pocket defined by the above four ClfB residues (Figure 4B and Figures S6C,D).

Importance of the GSR motif in recognition by ClfB

After carefully analyzing the sequences and the peptide binding specificities of ClfB, we propose that a small motif G-S-S-G-S/T-G-S-X-G (Figure 5A). The Fg α C-terminal domain (amino acids 221–610) of human Fg contains ten 13-residue tandem repeats, within which up to eight residues are glycines or serines [34]. Despite the similar sequences among the repeats, only Fg α5 was shown to be recognized by ClfB [18].

Figure 4: Detailed interactions between the ligand binding of ClfB in the ClfB-CK10/Fg α complexes. A. Detailed interactions between the ligand and ClfB in the ClfB-CK10 complex. The ClfB and CK10 peptides are shown as sticks, colored in magenta and yellow, respectively. The hydrogen bonds are indicated by red dashed lines. The amino acids of ClfB and CK10 are marked with black and red characters, respectively. B. Detailed interactions between the ligand and ClfB in the Fg α complex. The ClfB and Fg α peptides are shown as sticks, colored in cyan and slate, respectively. The hydrogen bonds are indicated by red dashed lines. The amino acids of ClfB and Fg α are marked with black and red characters, respectively.

doi:10.1371/journal.ppat.1002751.g004
example, within this motif, the G4 is limited by the side chain of ClfB(253–255) with the limitation of the space and is also required for the Fg α peptide making a turn thus exiting the tunnel. The S2 is the most critical residue because it not only forms two hydrogen bonds with the side chains of ClfB(252–255) and ClfB(250–253) but also binds to the main chain of ClfB(258–260). Similar to the S2, the S3 forms two hydrogen bonds with the side chain of ClfB(253–255) and ClfB(250–253) in the N2 domain, which could be replaced by a smaller residue such as alanine. The following residues, especially the G4, G/S/T5 of the Fg α, CK10 (type I cytokeratin 10, residues 473–485 and residues 499–511), K10 (Keratin 10, type I cytoskeletal isoform-1 from Pan troglodytes, residues 501–513), Derm (Dermokine, residues 250–264), TCF20 (TCF20, residues 49–57), EN (Engrailed protein, residues 37–45) and the derived peptide 9. The conserved amino acids are shown in red and the consensus sequence is designated below the sequences. The repeat 2, 3 and 4 of the Fg α which have been proved to bind to ClfB are indicated in skyblue.

doi:10.1371/journal.ppat.1002751.g005

**Figure 5. Mechanisms of specifically recognizing repeat 5 of Fg α.** A. Superimposition of the Fg α and CK10 peptides. The Fg α and CK10 peptides are shown as sticks, colored in yellow and slate, respectively. Residues highlighted within the boundaries of the red dashed line constitute the segment important for ClfB binding. The consensus amino acids are shown above the peptides. B. Sequence alignment of the repeat 2, 3, 4 and 5 of the Fg α. CK10 (type I cytokeratin 10, residues 473–485 and residues 499–511), K10 (Keratin 10, type I cytoskeletal isoform-1 from Pan troglodytes, residues 501–513), Derm (Dermokine, residues 250–264), TCF20 (TCF20, residues 49–57), EN (Engrailed protein, residues 37–45) and the derived peptide 9. The conserved amino acids are shown in red and the consensus sequence is designated below the sequences. The repeat 2, 3 and 4 of the Fg α which have been proved to bind to ClfB are indicated in skyblue.

Dermokine is a potential ligand of ClfB

Our results suggest that proteins carrying the GSR motif are able to bind to ClfB. To find other potential ligands of ClfB, we searched the NCBI protein database for additional proteins containing the sequence of G1-S2-G3-G4-G5/S-T6-G7-X8-G9. Three proteins, TCF20, Engrailed protein and Dermokine (Derm) were found to be hits, out of which Dermokine was evaluated more in detail in this study (Figures 5 and 6). Dermokine is expressed in many epithelial tissues, localized to intracellular or pericellular spaces and overexpressed in inflammatory diseases. The two major isoforms α and β are transcribed from different promoters at the same locus. Recently, additional transcript variants γ, δ and ε have been identified [35,36].

Firstly, Derm was tested for its interaction with ClfB. To this end, we synthesized a 15-amino-acid-peptide (250–264; GQGSSSGSGSGNGDNN, designated as Derm15 hereafter) derived from Derm and then characterized its binding to ClfB using the SPR (Surface Plasmon Resonance) assay. In the assay, ClfB bound to the peptide with a dissociation constant of 2.37 μM (Figure 7A). Interestingly, the results also showed that the Derm peptide interacted with ClfB with slow kinetics, further supporting the DLL model (Figure 7A). To understand the molecular mechanism underlying this interaction, we solved the crystal structure of ClfB(208–542) bound to the peptide at 2.5 Å resolution. As expected, Derm15 interacts with ClfB in a nearly identical manner with Fg α(316–328) (Figure 7 B). The ClfB-Derm15 forms a hydrogen bond with ClfB(320–322) and the C-terminus of N3 forms an extra strand, which is similar as that in the ClfB-Fg α(316–328) and ClfB-CK10(499–512) complexes (Figure 7 C). Mutagenesis studies were conducted to further verify the binding of Derm15 to ClfB. We replaced the residues ClfB(250–256, W252) that participate in interactions with the peptide with alanine respectively. The mutant proteins were purified to homogeneity and tested for their interaction with the Derm peptide using SPR. While the wild type ClfB bound tightly to Derm15, the mutant proteins ClfB(197–542, S236A) or W522A exhibited much lower binding affinities with the peptide in mM range (Figure S8). Interestingly, besides the low binding affinities, both mutant proteins exhibited rapid association and dissociation behaviors in the experiments, as compared to the slow association and scarcely any dissociation behaviors observed for the wild type protein. These results indicated that the residues ClfB(250–256, W252) are not only involved in the binding with ClfB, but also participate in stabilizing or “locking” the peptide...
in place. Collectively, our results strongly support the interaction between ClfB and Derm in vitro and suggest that Derm may involve in the infection process and pathogenesis caused by S. aureus in vivo.

Discussion

The colonization of the host nares by the Gram-positive bacterium S. aureus is mediated by a family of cell surface proteins which promote its adhesion to the extracellular matrix, that is, the MSCRAMMs [13,18]. ClfB, as a component of this family protein, has been studied for the past decade and was unique in its multi-functional characteristics, as compared to ClfA and SdrG that only bind to fibrinogens [18,19,21,29].

Consistent with the studies of the SdrG-fibrinogen complex [21], data from this study support the DLL binding mechanism of ClfB with the Fg α/CK10-derived peptides, but not the mechanism suggested in the previous study by V. Ganesh et al. [29]. In their work, due to the absence of the “Latch” procedure observed in the crystal structure, the binding mechanism was ascribed to the “DL” model. However, the structures of ClfB-peptide complexes solved in this study, together with the SPR data, indicate that the DLL model should be the mechanism utilized by ClfB to bind to its ligands. Our results also indicate that the DLL model may be the principal mechanism of MSCRAMM-ligand complexes.

In V. Ganesh et al.’s studies of the ClfB complexes, they proposed a common GSSGXG motif constituting the ClfB binding site [29], which is inconsistent with the previous studies on ClfB. For example, within the ten tandem Fg α repeats, repeat 2, 3, 4 and 5 all contain the GSSGXG motif but only the repeat 5 can bind to ClfB (Figure 5B) [18]. Our structural and the alanine screening analyses demonstrate that a 9-residue peptide G1-S2-S3-G4-G/S/T5-G6-X7-X8-G9 is necessary and sufficient for binding to ClfB in vitro. It is therefore predicted that a protein incorporating such a motif is able to interact with ClfB. Indeed, our biochemical assays showed that a Dermokine-derived peptide containing the ClfB binding motif interacted with ClfB (Figures 7B, 7C). Further supporting this prediction, our structural studies revealed that the
binding mode of the Dermokine-derived peptide to ClfB is nearly identical with that of the Fgα/CK10-derived peptide (Figure 7B). Collectively, these findings raise a provocative possibility that ClfB might act on multiple targets during S. aureus infections. Given the fact that ClfB acts as a key determinant of S. aureus nasal colonization, this may not be totally surprising.

Interestingly, Dermokine was first identified as a gene expressed in the suprabasal layers of the epidermis, and more recently, other isoforms of this gene besides its α and β isoforms have also been found. This gene is expressed in various cells and epithelial tissues and over-expressed in inflammatory conditions [35,36], suggesting that Dermokine might play a role in inflammatory processes since the over-expression of the mediators in immune cell activation characterizes many inflammatory diseases. ClfB is involved not only in the S. aureus colonization of human nares but also in the diseases caused by this bacterium. Additionally, S. aureus has also been implicated in several inflammation processes including corneal inflammation. ClfB’s binding to Dermokine raises the possibility that ClfB might play a role in the S. aureus caused inflammation and the Dermokine gene’s over-expression might serve as biological markers whose products could bind to ClfB and participate in this process. Obviously more investigations are needed to verify ClfB-Dermokine interaction during S. aureus infections as well as the biological significance of the interaction.

The characterization of ClfB as a multi-ligand binding protein will be meaningful for the identification of putative substrates and for furthering our understanding of the S. aureus infection pathway. Our findings also provide important leads towards the development of new therapeutic agents capable of eradicating S. aureus carriage in individuals and efficiently interfering with staphylococcal infection. This is particularly important since new antibacterial strategies are in urgent need to combat the drug resistant bacteria that continuing to emerge [37,38].
Materials and Methods

Cloning, expression and purification of the recombinant proteins

The fragment of the ClfB gene (corresponding 197–542 aa) was amplified by PCR from the *S. aureus* Newman genomic DNA. After digestion with BamHI and HindIII (NEB), the amplified genes were cloned into the prokaryotic expression vector pQE32 (GE Healthcare Life Sciences) to produce His-tagged fusion protein and were confirmed by DNA sequencing. The expected protein was expressed in *E. coli* strain BL21 with a high yield. Recombinant His-tagged protein was purified by Ni-affinity column chromatography and ion exchange chromatography. For the purification of protein-peptide complexes, the synthesized peptides were added into the concentrated protein samples at a 10:1 ratio and further subjected to gel filtration chromatography (Superdex-75 column) using buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM DTT) on the FPLC system (GE Healthcare Life Sciences). The proteins from different stages of purification (i.e. affinity and gel filtration chromatography) were monitored by SDS-PAGE. The Se-SAD data were solved with molecular replacement methods in CCP4 and all ClfB molecules in COOT [44]. The structures with other peptides were applied to the electron density map in DM [43]. The resulting map was of sufficient quality for model building of the protein and the peptide are designated, respectively.

Cryocrystallography and structure determination

The apo-ClfB and its complexes with different peptides were concentrated to 30 mg/ml in 10 mM Tris-HCl pH 8.0, 150 mM NaCl and 2 mM DTT. Crystals were produced by the hanging-drop vapor diffusion method [39] using sparse-matrix screen kits from Hampton Research (Crystal Screen reagent kits I and II), followed by a refinement of the conditions through the variation of precipitants, pH, protein concentrations and additives. Crystals were grown at 18°C by mixing 1.1 μl of protein with 1.1 μl of reservoir solution and equilibrating against 200 μl of reservoir solution. The apo-ClfB crystals are grown in 0.2 M LiSO₄, 0.1 M Tris-HCl pH 8.5, 30% polyethylene glycol 4000 and all the complexes with peptides are grown in 0.1 M sodium citrate tribasic dehydrate pH 5.6, 20% 2-propanol and 20% polyethylene glycol 4000. Similar conditions were used for generation of the crystals of Se-Met-substituted ClfB. Native and Se-SAD data were collected at Shanghai Synchrotron Radiation Facility (SSRF) at a wavelength of 0.919 Å and 0.979 Å respectively using a MAR225 [31] and processed with HKL2000 [40]. Further processing was carried out using programs from the CCP4 suite (Collaborative Computational Project, 1994).

The selenium sites were located using SHELXs [41] from the Bijvoet differences in the Se-SAD data. Heavy atom positions were determined by PHASER’s SAD Bijvoet differences in the Se-SAD data. Heavy atom positions were applied to the electron density map in DM [43]. The resulting map was of sufficient quality for model building of the ClfB molecules in COOT [44]. The structures with other peptides were solved with molecular replacement methods in CCP4 and all the structures were refined with the PHENIX [45] packages. Data collection and structure statistics are summarized in table 1.

Synthetic peptides

The synthesis and purification of the peptides were described previously [18,27]. For the following peptides, the amino acid residue numbers are given and the sequences are as follows: peptide from repeat 5 of the C terminus of Fg (SGSGSSGTGSGNQ); a peptide in the tail region of CK10 (YGGGSSGGGGSSG); peptide 15 from Dermokine protein (SQSGSSGSGNQ); and the peptide 9 of GSR motif (GSSGSSGNG) and its mutated forms by alanine scan; The six-amino-acid peptide (GSSGSG).

Surface Plasmon Resonance spectroscopy

Binding of ClfB197–542 to peptide 15 was assessed by SPR using the ProteOn XPR36 equipment (Bio-Rad Laboratories, Inc.). Each SPR experiment used multichannel detection. The system was equilibrated with buffer (10 mM HEPES pH 7.2, 150 mM NaCl). At each channel, peptide was captured to a ProteOn NLC Sensor Chip (BIO-RAD) at 25°C, using a flow rate of 100 μl/min. This resulted in peptide coupled at response levels of 460 RU. For binding measures, ClfB197–542 was injected simultaneously at different concentrations at a flow rate of 100 μl/min. The experiments were repeated three times.

The binding affinities between ClfB and the ten 9-amino-acid peptides and the 6-amino-acid peptide were determined by surface plasmon resonance (SPR) using BLAcore T200 instrument (GE Healthcare) at 10°C. The ClfB protein was immobilized to about 5300 Response Unit (RU) on a research-grade CM5 sensor chip in 10 mM sodium acetate, pH 5.0 by standard amine coupling method. The flow cell 1 was left blank as a reference. For the collection of data for affinity analyses, the 11 peptides in a buffer of 10 mM HEPES pH 7.4, and 150 mM NaCl, plus 0.005% (v/v) Tween 20, were injected over the flow cells at various concentrations at a 50 μl/min flow rate. The ligands were allowed to associate for 60 s and dissociate for 120 s. Data were analyzed with the BLAcore T200 evaluation software by fitting to a 1:1 Langmuir binding fitting model.

Supporting Information

Figure S1 The two symmetry-related molecules in the unit cell. A. Ribbon representation of the two symmetry-related molecules in the unit cell. The two molecules are shown in orange and cyan, respectively. B. Electron densities showing the interaction between N terminus of one molecule and the G strand from the other one in the unit cell. S197 and L198 of the N terminus, F and G strand from the other one are marked.

Figure S2 The electron density of Fg a and CK10 peptides. A. Ribbon representation of ClfB197–542-Fg S16–328 complex. The peptide is shown in sticks and the 2Fo-Fc map around the peptide contoured at 1.5σ is also shown. The color scheme is the same as in Figure 1B. The N and C-termini of both the protein and the peptide are designated, respectively. B. Ribbon representation of ClfB197–542-CK10199–512 complex. The peptide is shown in sticks and the 2Fo-Fc map around the peptide contoured at 1.5σ is also shown. The color scheme is the same as in Figure 1B. The N and C termini of both the protein and the peptide are designated, respectively.

Figure S3 Surface representation of ClfB197–542, ClfA29–542, and SdrG179–297 showing the peptide “locked” into the molecule. The surface is color-coded according to negative and positive charge residues that are represented as red and blue. The peptides are shown as sticks. (A), ClfB-Fg a (316–328); (B), ClfB-CK101499–512; (C), ClfA-Fg γ (395–411); (D), SdrG-Fg b (86–207).

Figure S4 Cartoon view showing the interactions between the Fg γ peptide with ClfA. The carbon, oxygen and nitrogen atoms are shown in cyan, red and blue, respectively. The residues of peptide are shown as sticks in magenta. The residues of...
ClfA are marked in black and those from Fg γ are shown in magenta. The hydrogen bonds are indicated in red dashed lines. (TIF)

Figure S5  Closer view of the ligand binding tunnel of ClfB. A. The N termini of the peptides. ClfB is represented as an electrostatic surface model with negative and positive charges indicated by red and blue, respectively. The Fg α peptide was superposed onto the CK10 peptide and they are shown as sticks in blue and yellow, respectively. B. The C-termini of the peptides. The color scheme is the same as in Figure S5A. (TIF)

Figure S6  Detail interaction of the ligands binding. A. Closer view of the ligand binding tunnel of ClfB in the ClfB-CIκ domain complex. ClfB is represented as an electrostatic surface model with negative and positive charges indicated by red and blue, respectively. The CK10 peptide is shown as sticks in yellow. The hydrogen bonds are indicated by red dashed lines. B. Schematic representation of the hydrogen bond interactions between ClfB and the CK10 peptide. Hydrogen bonds are shown as dashed lines. The interactions with the CK10 come from strand G in N3 domain, AB- and CD-loops from N2 domain in ClfB. C. Closer view of the ligand binding tunnel of ClfB in the ClfB-Fg α complex. The color scheme is the same as in Figure S6A. The hydrogen bonds are indicated by red dashed lines. D. Schematic representation of the hydrogen bond interactions between ClfB and the Fg α peptide. Hydrogen bonds are shown as dashed lines. The interactions with the Fg α come from strand G in N3 domain, AB- and CD-loops from N2 domain in ClfB. (TIF)

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