Cytokeratin 8 interacts with clumping factor B: a new possible virulence factor target

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Staphylococcus aureus is a human pathogen of growing clinical significance, owing to its increasing levels of resistance to most antibiotics. Infections range from mild wound infections to severe infections such as endocarditis, osteomyelitis and septic shock. Adherence of *S. aureus* to human host cells is an important step, leading to colonization and infection. Adherence is mediated by a multiplicity of proteins expressed on the bacterial surface, including clumping factor B. In this study, we aimed to identify new targets of clumping factor B in human keratinocytes by undertaking a genome-wide yeast two-hybrid screen of a human keratinocyte cDNA library. We show that clumping factor B is capable of binding cytokeratin 8 (CK8), a type II cytokeratin. Using a domain-mapping strategy we identified amino acids 437–464 as necessary for this interaction. Recombinantly expressed fragments of both proteins were used in pull-down experiments and confirmed the yeast two-hybrid studies. Analysis with *S. aureus* strain Newman deficient in clumping factor B showed the clumping factor B-dependence of the interaction with CK8. We postulate that the clumping factor B–CK8 interaction is a novel factor in *S. aureus* infections.

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INTRODUCTION

Staphylococcus aureus causes a variety of superficial and systemic infections. Furthermore, it is the major cause of nosocomial infections such as osteomyelitis, endocarditis, pneumonia and toxic shock syndrome (Ali *et al.*, 2007). The bacterium colonizes the moist squamous epithelium of the anterior nares as well as the skin of the axillae and the groin (Williams, 1963). Whereas earlier studies classified individuals with regard to *S. aureus* carriage into persistent carriers (20 %), intermittent carriers (30 %) and non-carriers (50 %) (Wertheim *et al.*, 2005), a recent study has defined only two types of nasal *S. aureus* carriage: persistent carriers and others (van Belkum *et al.*, 2009). In that study, the authors prepared a human colonization model and observed there-with that intermittent carriers and non-carriers have similar *S. aureus* nasal elimination kinetics, similar anti-staphylococcal antibody responses (van Belkum *et al.*, 2009), and the same risk of *S. aureus* infection (Nouwen *et al.*, 2005).

The pathogenic varieties of *S. aureus* express a large number of extracellular proteins on their surface, the so-called

‘microbial surface components recognizing adhesive matrix molecules’ (MSCRAMMs) (Foster, 2004; von Eiff *et al.*, 2001a). *S. aureus* also expresses specific virulence factors that thwart host defences and enable the pathogen to invade tissues. Additionally, *S. aureus* produces toxins and secretes an array of extracellular enzymes that cause damage to the host (Archer, 1998).

MSCRAMMs enable *S. aureus* to adhere to components of the human extracellular matrix (e.g. fibrinogen, fibronectin and collagen) and thereby initiate colonization of the host tissue, which is known to be a prerequisite for subsequent infection. *S. aureus* expresses up to 20 different MSCRAMMs, of which eight have been characterized in detail. These include the fibrinogen-binding proteins clumping factor A (ClfA) and clumping factor B (ClfB), the fibronectin-binding proteins Fnbp A and Fnbp B, the collagen-binding protein Cna, and Protein A (O’Brien *et al.*, 2002; Perkins *et al.*, 2001; Walsh *et al.*, 2008). All of these proteins are anchored to the cell wall by sortase-mediated cleavage of the C-terminal LPXTG motif (Navarre & Schneewind, 1994; Mazmanian *et al.*, 1999). The domain organization of ClfB is characterized by an N-terminal A region that is composed of three subdomains (N₁, N₂, N₃), and a dipeptide repeat region (R) that acts as a stalk (Deivanayagam *et al.*, 2002; O’Brien *et al.*, 2002; Perkins *et al.*, 2001). ClfA and ClfB are able to bind to human fibrinogen *in*

Abbreviations: CK8, cytokeratin 8; CK10, cytokeratin 10; GST, glutathione *S*-transferase; MSCRAMM, microbial surface component recognizing adhesive matrix molecules; RT, room temperature; RU, resonance units; SPR, surface plasmon resonance.

vitro (Ní Eidhin *et al.*, 1998). However, ClfA and ClfB recognize different parts of the fibrinogen molecule: whereas ClfB binds to the α -chain of human fibrinogen, ClfA interacts with the γ -chain (Walsh *et al.*, 2008). In addition to fibrinogen, ClfB has been shown to bind to cytokeratin 10 (CK10), which is a type I cytokeratin exposed on the surface of desquamated epithelial cells and keratinocytes. The keratin-binding domain of ClfB has been localized to within the N₂–N₃ domains (amino acids 197–542) of the A region of ClfB.

It is hypothesized that the interaction of ClfB with CK10 is an important step in nasal colonization (O'Brien *et al.*, 2002). The importance of ClfB as a major determinant in nasal carriage and persistence has been shown in several studies. In a human colonization model, human volunteers were artificially inoculated with an *S. aureus* wild-type strain or with its isogenic mutant defective in ClfB. The mutant strain was not able to survive in the nose and showed a faster elimination from the nares than the wild-type strain, indicating a crucial role for ClfB in nasal adherence (Wertheim *et al.*, 2008). Moreover, immunization studies in mice have also shown the relevance of ClfB in nasal colonization. It has been demonstrated in mice that immunization with recombinant vaccines of ClfB domain A reduces the level of colonization compared with that of control animals (Schaffer *et al.*, 2006).

In an earlier study, Tamura & Nittayajarn (2000) sought to identify new adhesion receptors that mediate the attachment of Group B streptococcus (GBS) to epithelial cells. They successfully demonstrated binding of GBS to cytokeratin 8 (CK8) in solution as well as GBS binding to immunoaffinity-purified CK8. Furthermore, they showed that other Gram-positive cocci are also able to bind to CK8. Interestingly, they demonstrated adherence of *S. aureus* to CK8 by incubating *S. aureus* cells with the protein lysate and eluting adherent proteins from bacteria (an *in vivo* pull-down). However, the authors did not identify the virulence factor that was responsible for the ability of the bacterium to bind to CK8.

In the present study we searched for new host adhesion receptors for *S. aureus* ClfB, given the important role of the molecule in staphylococcal colonization. We performed a genome-wide protein interaction screen by using ClfB as the bait protein and prey proteins expressed from a human keratinocyte cDNA library. We demonstrate that CK8 is a novel host target of ClfB and propose that ClfB–CK8 binding mediates the known interaction of *S. aureus* with CK8, a type II cytokeratin. We verified this interaction both *in vitro* and *in vivo*, measured an apparent K_d of 12 nM and additionally demonstrated the ClfB-dependent *in vitro* affinity of *S. aureus* for CK8 by using ClfB-deficient and control strains.

METHODS

Bacterial strains, yeast strain and growth conditions. A non-MRSA isolate from a hospital patient, which was a kind gift of Dr Markus Hell (Department of Hospital Epidemiology and Infection

Control, SALK and Paracelsus Medical University, Salzburg, Austria), and *S. aureus* strain Newman and its isogenic ClfB mutant (*clfB*::Tc^r; DU5943), a generous gift of Professor Timothy J. Foster (Department of Microbiology, Trinity College, Dublin, Ireland), were used in this study. The bacteria were grown in trypticase soy broth (BD) or on agar at 37 °C. For several investigations, an overnight culture of *S. aureus* was used, which was grown at 37 °C in a shaking incubator.

Escherichia coli strain DH5 α (Invitrogen) was used for plasmid DNA amplification. *E. coli* strain BL21 Star (Invitrogen) was used for recombinant protein expression, induced with IPTG. Both *E. coli* strains were grown in L-broth or on agar supplemented with the following antibiotics, as appropriate: 100 mg ampicillin l⁻¹, 50 mg kanamycin l⁻¹.

Saccharomyces cerevisiae strain AH109 (Clontech) was used for the yeast two-hybrid assay. AH109 was grown on yeast extract peptone dextrose (YPD) plates at 28 °C on a rotary shaker.

Human cell culture. The spontaneously immortalized human HaCaT cell line, which has been employed for similar studies, is known to behave phenotypically like normal basal epidermal keratinocytes in terms of differentiation as well as growth. This line develops in the absence of serum or other exogenous growth factors, which makes it a widely used cell culture system and a crucial model for the highly proliferative epidermis (Lehmann, 1997).

HaCaT cells were cultured at 37 °C in 5.5 % CO₂. Cells were passaged every 4–5 days in EpiLife medium (Cascade Biologics) supplemented with Human Keratinocyte Growth Supplement (HKGS; Cascade Biologics), 60 μ M calcium chloride and 0.5 % penicillin/streptomycin.

Plasmids. The yeast two-hybrid vectors pGADT7 and pGBKT7 (Clontech) were used for the yeast two-hybrid work. For recombinant protein expression, the pET160 Directional TOPO Expression system (Invitrogen) and pDEST15 Gateway destination vector (Invitrogen) were used.

Yeast two-hybrid system. A keratinocyte cDNA library from human keratinocyte RNA was cloned in-frame with the GAL4 activation domain of the pGADT7 prey vector for protein–protein interaction screening. The ClfB N_{2–3} domain was amplified with specific primers (Table 1) from *S. aureus* genomic DNA and subcloned into the *EcoRI*/*Bam*HI sites of the pGBKT7 bait vector, creating a fusion protein to the DNA-binding domain of the yeast transcriptional activator GAL4. A library screen was performed by introducing both constructs into *S. cerevisiae* strain AH109 by a large-scale lithium acetate transformation procedure according to the Matchmaker Two-Hybrid Assay kit manual (Clontech). A fraction of the transformed cells was plated onto agar plates lacking leucine (Leu) and tryptophan (Trp) (SD/-Leu/-Trp medium, Q-BIOgene) to test the transformation efficiency of the screen. The remaining transformants were plated onto interaction-selective medium lacking Leu, Trp, adenine (Ade) and histidine (His) (SD/-Leu/-Trp/-Ade/-His) to test for putative interactions, indicated by activation of the *ADE2* and *HIS3* reporter genes. Plates were incubated at 28 °C for several days. Plasmids were isolated from the resulting yeast colonies, propagated in *E. coli* DH5 α and used to transform, together with the bait ClfB, strain AH109 in order to reproduce the interaction. As a control for potential auto-activators, which do not require ClfB for growth on interaction-selective plates, we used the isolated prey plasmids together with an empty bait vector to transform AH109. The transformants were plated on SD/-Leu/-Trp plates; growing colonies were picked and dispersed in H₂O, and the cells were dropped onto SD/-Leu/-Trp and SD/-Leu/-Trp/-Ade/-His media in parallel.

Clones that were excluded as auto-activators and that were positive when retested for reporter activity were further analysed by

Table 1. Primers used in this study

Gene	Direction of transcription	Sequence
CK8 full-length	Forward	5'-ATGTCCATCAGGGTGACCCAG-3'
	Reverse	5'-TCACTTGGGCAGGACGTCAG-3'
CK8-F2	Forward	5'-ACCTACAGGAAGCTGCTGGA-3'
	Reverse	5'-GGATCCCGTATCGATGCCC-3'
CK8-F3	Forward	5'-TACAGCCTGGGCTCCAGCTTT-3'
	Reverse	5'-GGATCCCGTATCGATGCCC-3'
CK8-F4	Forward	5'-TACAGCCTGGGCTCCAGCTTT-3'
	Reverse	5'-TCACTTGGGCAGGACGTCAG-3'
CK8-F5	Forward	5'-TACAGCCTGGGCTCCAGCTTT-3'
	Reverse	5'-TCTTCACAACCAACGGCCCTG-3'
CK8-F6	Forward	5'-CAGGGCCGTGGTTGTGAAGA-3'
	Reverse	5'-TCACTTGGGCAGGACGTCAG-3'
ClfB N ₂ -N ₃	Forward	5'-CTAGTTTAGCTGTTGCTGAACCGG-3'
	Reverse	5'-ATTTACTGCTGAATCACCATCAGC-3'
Y2H-5F	Forward	5'-CTATTCGATGATGAAGATACCCCA-3'
Y2H-6R	Reverse	5'-GTGAACCTGCGGGGTTTTCAG-3'
Y2H-7F	Forward	5'-TCATCGGAAGAGAGTAGTAAC-3'
Y2H-8R	Reverse	5'-GAGTCACTTTAAATTTGTATACAC-3'

sequencing with pGADT7-specific vector primers (see Table 1, Y2H-5F, Y2H-6R), followed by identification of the clones using alignment search tools (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Plasmid isolation. Prey plasmids of putative positive transformants were prepared by scraping up a portion of the yeast cells, which were then resuspended in 50 µl 1 × Tris-EDTA buffer. Ten microlitres of lyticase (5 U µl⁻¹) solution was added to the yeast mixture, which was then resuspended by vortexing. To promote lysis, the mixture was incubated for 30 min at 37 °C. Afterwards, 10 µl 10 % SDS was added to the mixture and the solution was vortexed for 1 min. The samples were put through one freeze-thaw cycle to complete the lysis and the plasmid DNA was purified from the crude cell lysate by the GenElute PCR Clean-Up kit (Sigma-Aldrich). Prepared plasmids were introduced into *E. coli* DH5α for amplification and were analysed by DNA sequencing. Sequencing work was carried out by MWG using pGADT7-specific primers (see Table 1, Y2H-5F, Y2H-6R).

Domain mapping. Several primers were designed to systematically narrow down the CK8 fragment that can interact with ClfB (see Table 1). Each CK8 subdomain was separately cloned into the *Bam*HI/*Eco*RI site of the pGADT7 prey vector in-frame with the GAL4 activation domain. Each CK8 prey construct was individually introduced into yeast strain AH109 with the bait vector pGBKT7 containing ClfB fused to the DNA-binding domain, and was dropped out on SD/-Leu/-Trp and SD/-Leu/-Trp/-Ade/-His media. Putative positive clones were prepared as mentioned above and were sequenced for identification. To test for auto-activation of the subdomain prey, plasmids were co-transformed with empty pGBKT7 into the yeast strain AH109.

Protein expression. For recombinant protein expression, ClfB was cloned into the expression vector pET160 (Invitrogen) and fused to a His-tag by using the pET Directional TOPO Cloning system (Invitrogen). The bacterial strain BL21 Star (Invitrogen), transformed with ClfB in pET160, was grown to an optical density of 0.6 at 37 °C and induced with 1 mM IPTG for protein production for 3 h.

In addition, CK8-F4 was cloned into the pDEST15 expression vector containing a glutathione *S*-transferase (GST) tag, via an LR reaction using the Gateway LR Clonase II enzyme mix (Invitrogen) for the

analysis of protein expression. Protein was prepared as described above.

Protein purification. The crude cell lysates of the recombinantly expressed proteins were purified for further investigations. We made use of the respective tags of the vectors. ClfB was purified according to the MagneHis protein purification system protocol (Promega), whereas CK8-F4 was purified according to the MagneGST protein purification system protocol (Promega). Crude cell lysates as well as the purified proteins were analysed by Western blotting. Alternatively, ClfB was purified by large-scale purification via immobilized-metal affinity chromatography (Chelating Sepharose Fast Flow, Amersham).

Western blot analysis. Proteins were resuspended in NuPAGE sample buffer (Invitrogen) and heated at 95 °C for 5 min. Proteins were separated by SDS-PAGE on a 10 % NuPAGE BisTris acrylamide gel (Invitrogen) for 90 min at 160 V. Proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham) at 80 mA for 1 h. The membrane was blocked for 1 h in Tris-buffered saline (TBS) with 3 % non-fat milk (Sigma) containing 0.5 % Tween 20. His-ClfB was detected with primary anti-His biotin-conjugated antibody (dilution 1:1000, Santa Cruz) for 1 h at room temperature (RT) or overnight at 4 °C, and streptavidin conjugated with alkaline phosphatase (Promega) (1:10 000 dilution) as the secondary binding protein (1 h at RT).

Alternatively, ClfB was detected with a specifically produced rabbit anti-ClfB antibody (dilution 1:1000) from Biogenes (1 h at RT or overnight at 4 °C). Anti-rabbit IgG was conjugated with alkaline phosphatase (Sigma) (1:4000 dilution) and used to detect the primary bound antibody. The enzymic reaction of alkaline phosphatase was detected with CDP-Star reagent (New England Biolabs) or with Western Blue Stabilized Substrate (Promega).

GST-CK8-F4 was analysed in the same manner by using the anti-GST biotin-conjugated antibody as the primary antibody and alkaline phosphatase-conjugated streptavidin as the secondary binding protein.

In vitro His-tag pull-down assay. A pull-down assay was performed by using the MagneHis protein purification system (Promega). GST-

CK8-F4 was purified by the MagneGST purification system as described in the manual (Promega). The crude cell lysate of ClfB was treated according to the manual of the MagneHis protein purification system (Promega) until the binding step. After discarding the supernatant, ClfB bound to the magnetic beads was blocked with 2 % BSA for 1 h at 4 °C followed by one wash step with the wash buffer provided. Purified GST-CK8-F4 was incubated with the immobilized ClfB for 2 h at 4 °C with inversion. After incubation, GST-CK8-F4 proteins bound to ClfB were collected by magnetic attraction. The beads were washed twice with the wash buffer provided and once with RIPA buffer (Sigma) to increase the stringency of the wash steps.

Elution was carried out by the addition of 4 × NuPAGE sample buffer (Invitrogen) and heating of the mixture. To exclude non-specific binding of GST-CK8-F4 to the magnetic beads, the protein was incubated without previously immobilizing ClfB. The supernatant was subjected to SDS-PAGE for protein separation, and the GST-CK8-F4 protein was detected by Western blotting using anti-GST biotin-conjugated antibody as the primary antibody and alkaline phosphatase-conjugated streptavidin as the secondary binding protein.

Direct yeast two-hybrid tests. Several plasmid combinations were introduced into the yeast strain AH109 as described above. To test for self-activation and to exclude non-specific binding, bait and prey combinations were tested as described in Fig. 3. From the resulting colonies, five randomly selected clones were dispersed in sterile H₂O and 10 µl of this cell suspension was dropped onto SD/-Leu/-Trp and SD/-Leu/-Trp/-Ade/-His media. Pictures of the plates were taken after 2 days of incubation at 28 °C.

Determination of the binding affinity by Biacore X surface plasmon resonance (SPR). The binding affinity between ClfB and CK8 was determined by SPR, using a Biacore X system. ClfB (N₂₋₃ domain), which was N-terminally fused to a 6 × His-tag, was recombinantly produced in *E. coli* and purified as described above (39 kDa, 215 ng µl⁻¹ in Tris-HCl, pH 8). For the affinity measurement, ClfB, as the ligand, was diluted 1:1 in 10 mM sodium acetate, pH 3.7, and covalently coupled to a Biacore CM5 sensor chip, according to the manufacturer's instructions. The flow cell is physically divided into two parts. Flow cell 1 was coupled with approximately 840 resonance units (RU) ClfB. Flow cell 2 served as the reference. Recombinant CK8 protein produced in *E. coli* was obtained from RayBiotech. Protein purity was determined by that company by reverse-phase HPLC and was 95 %. CK8 was delivered lyophilized and was resolved in HBS-EP buffer (Biacore) for the SPR measurement. CK8, as the analyte, was injected at different concentrations (92.5 nM–12 µM) in HBS-EP buffer. The SPR data were recorded and analysed with BIAevaluation software (Biacore) according to Feichtner *et al.* (2008).

CK8 protein isolation from HaCaT cells and primary keratinocytes. HaCaT cells and primary keratinocytes were grown to reach a confluency of 80 % in cell culture flasks. The EpiLife medium was removed and cells were lysed with 5 M urea, which enables efficient solubilization of the protein. Five micrograms of the total protein extract was used for Western blotting using the mouse anti-CK8 antibody Ks8.7 (1:500, Progen Biotechnik) as the primary antibody and anti-mouse alkaline phosphatase-conjugated streptavidin (1:1000, Sigma) as the secondary binding protein. The Western blot was prepared as described above.

Immunofluorescence. HaCaT cells were confluent grown in Lab-Tek chamber slides (Nunc). After two wash steps with PBS, one chamber slide was treated with 4 % formaldehyde containing 0.2 % Triton X-100, to combine the fixation and permeabilization steps, for 30 min at RT. The other chamber slide was treated with 4 % formaldehyde alone to fix the cells. HaCaT cells were blocked with 3 % BSA for 1 h at RT followed by incubation with the primary

antibody mouse anti-CK8, Ks8.7 (Progen Biotechnik) (1:200 in 3 % BSA) overnight at 4 °C. After three wash steps with PBS to remove unbound antibody, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen) (1:1000 in 3 % BSA) for 1 h. Additional washes removed any unbound antibody. Immunofluorescence staining was evaluated with a Zeiss Axioskop MC100 microscope (Carl Zeiss MicroImaging).

Bacterial adherence to immobilized proteins. The assay for *S. aureus* adherence to immobilized proteins was performed as described elsewhere (Hartford *et al.*, 1997). Nunc 96-well Immobilizer Amino plates were coated with 5 µg ml⁻¹ human aortic elastin (Elastin Products), 5 µg ml⁻¹ human fibrinogen (Enzyme Research Laboratories) and 5 µg ml⁻¹ recombinantly expressed and MagneGST-purified CK8-F4 in a coating solution (0.02 % sodium carbonate buffer, pH 9.6). To exclude non-specific binding of *S. aureus*, 5 µg ml⁻¹ recombinantly expressed methionine sulfoxide reductase MsrB peptide (UniProtKB/Swiss-Prot accession no. P65451) from *S. aureus* Mu50, 5 µg ml⁻¹ recombinantly expressed NADPH-dependent 7-cyano-7-deazaguanine reductase (UniProtKB/Swiss-Prot accession no. Q99VP5) from *S. aureus* Mu50, and 5 µg BSA ml⁻¹ as a non-specific control, were coated in sodium carbonate buffer. To determine the background signal, six wells were coated with the coating buffer alone. The plates were incubated overnight at 4 °C. After removing the supernatant, the plate was blocked with 2 mg BSA ml⁻¹ for 2 h at 37 °C. The plate was washed three times with PBS. A 300 µl volume of *S. aureus* cell suspension (overnight culture was inoculated into fresh medium and grown to OD₆₀₀ 0.5) was added to each well and incubated at 37 °C for 2 h. After incubation, unbound cells were washed four times with PBS (200 µl). Bound *S. aureus* cells were fixed with formaldehyde (25 %, v/v) for 5 min at 37 °C and dried for 5 min at RT. *S. aureus* cells were stained with crystal violet (0.5 %, v/v) for detection after 30 min at RT. After several wash steps with H₂O, the dye was dissolved with 10 % acetic acid by shaking for 30 min at RT. A₅₉₅ was measured in an ELISA plate reader (Tecan).

Adherence of the knockout ClfB mutant to CK8. Nunc 96-well Immobilizer Amino plates were coated with 5 µg ml⁻¹ human aortic elastin (Elastin Products) and with 5 µg ml⁻¹ recombinant CK8 full-length (RayBiotech) in a coating solution (0.02 % sodium carbonate buffer, pH 9.6) overnight at 4 °C. The plate was washed three times with PBS and blocked with BSA (2 mg ml⁻¹) for 1.5 h at 37 °C. Overnight cultures of *S. aureus* strain Newman and the ClfB mutant strain were freshly inoculated and grown for 1.5 h at 37 °C. From each culture, 3.5 × 10⁸ cells per well were added and the plate was incubated for 2 h at 37 °C. Subsequent steps of the assay were performed as described above.

In vivo pull-down analysis. HaCaT cells were lysed with FastBreak Cell Lysis reagent (Promega) for 30 min. Insoluble material was pelleted and the supernatant was stored at -20 °C for later use. A 1 ml volume of an overnight culture of *S. aureus* was pelleted and washed twice with 1 ml PBS to remove the culture medium. The *S. aureus* pellet was resuspended in 3 % BSA for 60 min to prevent non-specific binding. Bacteria were pelleted and resuspended in 500 µl HaCaT lysate and incubated on a rocking platform for 2 h. Bacteria were pelleted and washed three times with 1 ml PBS. Bound proteins were eluted with 50 µl 1 × NuPAGE LDS Sample buffer at 95 °C for 5 min. Eluted proteins were analysed by Western blotting using a specific anti-CK8 antibody.

RESULTS

CK8 is a ClfB-interacting protein

The region of ClfB known to interact with CK10 is located within the N₂₋₃ domain (amino acids 197–542) (O'Brien

et al., 2002); therefore, this region of ClfB was used as bait in a yeast two-hybrid screen of a cDNA library obtained from human keratinocytes. From an initial 70 putative interactors, 54 were again able to grow on selective medium, confirming the putative interactions. By sequencing the candidates, we identified CK8 multiple times as a novel ClfB-interacting protein (data not shown), and CK8-F1 was the smallest subdomain of CK8 found in the screen. Testing the putative interactors of ClfB by co-expressing them with empty pGADT7 showed no auto-activating capacities.

Domain mapping of CK8 identifies the region containing amino acid residues 437–464 as interacting with ClfB

A consecutive screening procedure of CK8 subdomains against ClfB enabled us to delineate the domain that is potentially involved in the CK8–ClfB interaction. To this end, the CK8 gene was split further into five subdomains (Fig. 1a), which were inserted into the prey vector. The subdomains were tested for their ability to interact with ClfB in the two-hybrid system by co-transforming yeast with each of the subdomains and ClfB bait (Fig. 1b, A). Testing each subdomain for self-activation ability by introducing them into yeast along with the empty pGBKT7 vector revealed no false-positive interactions due to transcriptional self-activation (Fig. 1b, B).

Subdomains CK8-F2 to -F5 were all able to interact with ClfB, resulting in yeast growth on selective medium. Only the CK8-F6 domain (amino acids 457–484) exhibited no yeast growth on SD/-Trp/-Leu/-Ade/-His medium, indicating that this subdomain is not critical for binding of CK8 to ClfB. Thus, CK8-F5 was mapped as the minimal interacting domain, localizing the ClfB-binding site to within a 28 aa region at the C-terminal end of CK8 (amino acids 437–464) encoded by exon 8 (Fig. 1c).

Verification of the CK8–ClfB interaction by a pull-down assay using bacterially expressed CK8 protein

To validate the specificity of this novel protein interaction, we conducted *in vitro* His pull-down experiments. The ClfB N_{2–3} domain (39 kDa), fused to a His-tag, was recombinantly expressed in *E. coli* (Fig. 2a), whereas a 46 aa part of CK8 (CK8-F4) that harbours the interaction domain was recombinantly expressed fused to a GST tag. ClfB protein immobilized on paramagnetic pre-charged nickel particles (MagneHis Ni-Particles, Promega) was able to specifically pull down the CK8-F4 protein from the crude cell lysate. Elution of the pull-down was analysed by Western blotting, and the expected 31 kDa CK8 fragment fused to the GST tag was successfully detected with an anti-GST antibody. To exclude non-specific binding between GST–CK8 protein and the immobilizer material, CK8 was incubated without previous ClfB immobilization. Because we did not detect CK8 protein in the negative control of

the pull-down by Western blotting, we can exclude non-specific CK8 binding to the nickel particles (Fig. 2b).

The ClfB–CK8 interaction is highly specific, as shown by direct yeast two-hybrid tests

Several yeast two-hybrid tests were performed to exclude non-specific binding and to confirm the interaction between CK8 and ClfB (Fig. 3). We demonstrated that CK8 is not able to interact with the yeast GAL4-binding domain expressed from a bait control vector. In the same manner, we could also show that ClfB does not interact non-specifically with the GAL4 yeast activation domain. Furthermore, CK8-F5 is not able to homodimerize. The CK8 protein interacts strongly with ClfB in the yeast two-hybrid system, and this was further confirmed by swapping the proteins between the bait and the prey vectors.

Determination of the binding affinity of ClfB to CK8

The association between the recombinantly expressed ClfB and CK8 was analysed by SPR using a Biacore X device. Flow cell 1 of a CM5 chip was coupled with 840 RU recombinant ClfB. Flow cell 2 remained uncoupled and served as a reference. CK8 protein was injected at different concentrations (92.5 nM–12 µM) and surface resonance was recorded. The K_d of 12 nM, determined by Scatchard plot analysis from the respective curves according to Feichtner *et al.* (2008), shows the high affinity of CK8 for the ClfB substrate (Fig. 4).

Expression studies identify CK8 protein in HaCaT cells and primary keratinocytes

To investigate CK8 protein expression in HaCaT cells as well as in primary keratinocytes at the protein level, total proteins were isolated and analysed by SDS-PAGE. On a Western blot using 5 µg of isolated total protein and a CK8-specific antibody, we detected endogenous CK8 (53.7 kDa) in protein extracts isolated from human primary keratinocytes as well as from HaCaT cells (data not shown).

Intracellular CK8 expression in the HaCaT cell line

To clarify the extent of CK8 distribution in HaCaT cells, we undertook immunofluorescence investigations. HaCaT cells were grown on Lab-Tek chamber slides. To localize CK8 expression intracellularly, HaCaT cells on one chamber slide were pre-treated with Triton X-100, resulting in permeabilization of the HaCaT cells. For extracellular CK8 determination, HaCaT cells on the other chamber slide remained untreated prior to antibody incubation.

As expected, CK8 was predominantly found throughout the cytoplasm of Triton X-100-treated HaCaT cells, giving characteristic cyokeratin expression patterns (data not

Fig. 1. Yeast two-hybrid domain mapping of CK8 subdomains. (a) Schematic representation of the CK8 gene and the six subdomains of CK8 fused to the GAL4 activation domain. (b) (A) Each fragment was tested with the pGBKT7 bait vector expressing ClfB. (B) To rule out auto-activation, each fragment was tested with the empty bait vector. Transformed AH109 strains were plated onto SD/-Leu/-Trp/-Ade/-His medium, upon which growth depends on the interaction between the bait and prey. The arrow points to the non-interacting CK8 subdomain. To test for the correct transformation of both plasmids, cells were dropped out onto SD/-Leu/-Trp plates (data not shown). (c) Protein sequence of each subdomain. The boxed amino acid residues represent the minimal ClfB-interaction domain of CK8.

shown). Untreated HaCaT cells also demonstrated CK8 staining, although it was much weaker than that observed in permeabilized HaCaT cells, indicating intracellular rather than extracellular CK8 expression. In this case, we

can exclude non-specific binding of the FITC-labelled secondary antibody, because HaCaT staining with secondary antibody alone showed no fluorescence staining (data not shown).

Fig. 2. Western blot of recombinant ClfB, CK8-F4 and pull-down assay. (a) Recombinant His-ClfB (39 kDa) expressed in *E. coli* and analysed with anti-His antibody. Lanes: 1, recombinantly expressed ClfB, crude cell lysate; 2, MagneHis-purified ClfB. (b) GST-CK8-F4 protein eluate (31 kDa) of the pull-down assay detected with anti-GST antibody. Lanes: 1, MagneGST-purified CK8-F4 protein; 2, immobilized ClfB pull-down CK8-F4 protein, eluate of the pull-down, the arrow indicates CK8 protein; 3, protein eluate of the control pull-down; CK8-F4 was incubated without previous ClfB immobilization. +, Immobilization of ClfB; -, no immobilization of ClfB.

CK8 shows an affinity for live *S. aureus* cells

An adhesion assay was performed to demonstrate the binding efficiency of *S. aureus* cells to CK8 (Fig. 5). Recombinant CK8 protein expressed in *E. coli* was coated onto ELISA plates and incubated with an overnight culture of *S. aureus*. In parallel, known host target proteins of *S. aureus*, fibrinogen and human aortic elastin, were coated onto the plates as positive controls. To exclude non-specific binding, control wells were coated with BSA or recombinant *S. aureus* proteins expressed in *E. coli*. We demonstrated strong binding of the clinical *S. aureus* isolate to CK8. Furthermore, we could not detect non-specific binding of *S. aureus* to BSA or to randomly selected *S. aureus* proteins.

ClfB seems to be the major adhesin for the *S. aureus* interaction with CK8

To assess the specific role of ClfB for binding to immobilized CK8 we prepared an adherence assay using the characterized *S. aureus* wild-type strain Newman and its isogenic mutant defective in ClfB. We clearly observed that the ClfB mutant strain adheres very poorly to the recombinant CK8 protein. The wild-type Newman strain adheres 4.3-fold better to immobilized CK8 than does its isogenic mutant. In comparison with these observations, the wild-type Newman strain binds 1.9-fold better to immobilized elastin than the mutant Newman strain (Fig. 6). Because there are differences in elastin binding as well

as in CK8 binding between the strains, we hypothesize that there is a basic adherence defect in the mutant strain as a consequence of the ClfB gene knock-out. Based on these observations, we suggest that *S. aureus*-CK8 adherence is predominantly mediated by ClfB.

Adherence of *S. aureus* to CK8 protein from HaCaT cell lysates, as shown by an *in vivo* pull-down assay

Next, we investigated whether *S. aureus* cells are able to adhere to endogenous CK8 protein from a HaCaT cell lysate. For this purpose we prepared an *in vivo* pull-down assay (Fig. 7). HaCaT cell lysate, containing CK8 protein, was incubated with an overnight culture of *S. aureus* cells. After several wash steps by centrifugation of *S. aureus* cells and discarding the supernatant, unbound proteins were removed. Proteins bound to *S. aureus* were eluted by heating the solution with SDS sample buffer and then analysed by Western blotting using a CK8-specific antibody. Our data clearly revealed that endogenous CK8 protein was pulled down and purified from HaCaT cell lysate by *S. aureus* cells *in vivo*. We could also exclude the possibility that the estimated protein bands at 53.7 kDa derived from *S. aureus* proteins, because the bacterial lysate gave a different protein pattern on Western blot analysis.

A ClfB-interacting non-regular secondary structure (Ω -loop) in CK8 is questionable

Interestingly, the tail region of CK10, which is reported to be responsible for ClfB binding, consists of quasi-repeats of tyrosine-(glycine/serine)_m representing Ω -loops (Walsh *et al.*, 2004). Further studies showed strong binding of ClfB to a Y-Y loop peptide (YGGSSGGSSGGGY) corresponding to the largest glycine loop in the tail region of CK10, indicating the binding site within this loop. We analysed the residues differing in the CK8-F5 sequence in comparison with the reported Y-Y loop and evaluated whether the changed amino acids were favourable to the formation of Ω -loops. This investigation scored amino acids according to their frequency of occurrence in Ω -loops and was based on amino acid frequencies determined from 979 Ω -loops (Fetrow, 1995). Principally, amino acids that differed between the CK8-F5 and Y-Y loops were evaluated with respect to the frequency found in 979 Ω -loops from 191 non-redundant proteins. We scored Ω -loops by assigning a plus or minus sign to specific amino acid residues. As shown in Table 2, almost all the variant amino acid residues of CK8-F5 are unfavourable to an Ω -loop. Only the tyrosine to serine change (Y→S) and the serine to glycine change (S→G) provide an advantage for CK8-F5.

DISCUSSION

The pathogenicity of *S. aureus* infection is due to a multiplicity of factors. Numerous microbial surface

Fig. 3. Direct yeast two-hybrid assay, testing several bait and prey combinations for self-activation and to exclude non-specific binding. The transformed yeast strain AH109 was plated onto interaction-selective (SD/-Leu/-Trp/-Ade/-His) medium (a) to test for positive interactions and additionally on non-selective (SD/-Leu/-Trp) medium (b) to test for the correct transformation of both plasmids. (i) Prey vector expressing CK8-F5 tested with the empty bait vector, (ii) prey vector expressing CK8-F5 tested with bait vector expressing ClfB, (iii) prey vector expressing ClfB tested with bait vector expressing CK8-F5, (iv) prey vector expressing CK8-F5 tested with bait vector expressing CK8-F5, (v) prey vector expressing ClfB tested with the empty bait vector, (vi) prey vector expressing CK8-F1 tested with bait vector expressing ClfB. +, Positive interaction; –, no interaction.

components, termed MSCRAMMs, physically interact with adhesive matrix molecules, thereby enabling the pathogen to adhere to and colonize its host, and several surface proteins allow *S. aureus* to evade the immune system, leading to its virulence.

We turned our attention to the MSCRAMM ClfB to search for novel host targets. ClfB, originally described as a fibrinogen-binding protein, aroused our interest because earlier studies showed ClfB to be capable of adhering to the human type I cytokeratin CK10 (O'Brien *et al.*, 2002). CK10 and its partner cytokeratin 1 (CK1) are the major keratins expressed in differentiated cells of the suprabasal layer of the epidermis and other stratified epithelia (Kirfel *et al.*, 2003). We searched for additional MSCRAMM-interacting cytokeratins, in particular cytokeratins of undifferentiated keratinocytes, as potential novel targets for *S. aureus* ClfB.

The CK10-binding site in ClfB is located within the N₂₋₃ domain (amino acids 197–542) (O'Brien *et al.*, 2002), and this domain was also used in our screen. Surprisingly, we found another type of keratin, the type II cytokeratin CK8, to be the target of ClfB instead of the anticipated cytokeratins 5, 14 and 15, which are strongly expressed in

undifferentiated basal cell layers (Moll *et al.*, 2008). Because CK8 and its partner cytokeratin 18 are the primary keratin pair expressed in simple epithelial cells (Owens & Lane, 2003), this result was unexpected. In fact, however, our findings are in accordance with the results of Tamura & Nittayajarn (2000), who independently demonstrated binding of *S. aureus* cells to CK8. We propose ClfB as the putative mediator of this established adherence. Moreover, we were able to verify that CK8 is a potential interactor of ClfB *in vitro* and *in vivo*. Using an SPR analysis method we further confirmed the interaction of recombinant ClfB (amino acids 197–542) with full-length CK8 and measured an apparent K_d of 12 nM. The determined affinity is significantly higher than the reported K_d value of 50 nM for the binding of recombinant ClfB (amino acids 45–542) to recombinant human CK10 (Walsh *et al.*, 2004). Although the lower K_d determined would suggest a higher substrate specificity of ClfB for CK8 than for CK10, it is not advisable to directly compare and make final conclusions with K_d values from studies which employed different proteins and different methods (SPR compared with ELISA-type binding assays, different purities of proteins, different procedures for manipulating recombinant proteins).

Fig. 4. Affinity measurement of ClfB with CK8. SPR analysis was performed by coupling ClfB and injecting increasing amounts of CK8 protein. The Scatchard plot analysis (inset) was calculated with the data points of the relative response units. The slope of the deduced straight line equation was used to determine the binding affinity constant.

Fig. 5. *S. aureus* adherence assay. One microgram of each protein was coated onto 96-well plates (x axis) and incubated with *S. aureus* cells, which were stained with crystal violet. The A_{595} of the bound *S. aureus* cells (y axis) was measured in an ELISA plate reader. Non-specific binding to BSA and to control I and control II (peptide methionine sulfoxide reductase MsrB and NADPH-dependent 7-cyano-7-deazaguanine reductase, respectively, both from *S. aureus* Mu50) was not observed. Values are the means of triplicate samples. Error bars, SD.

Fig. 6. Adherence of wild-type strain Newman (grey bars) and ClfB mutant (white bars) to CK8. Coated proteins were incubated with *S. aureus* strains and adherence to elastin and CK8 was measured using crystal violet. The A_{595} of the bound *S. aureus* cells (y axis) was measured in an ELISA plate reader. Values are the means of triplicate samples. Error bars, SD.

Cytokeratins all share a common structural architecture (Fig. 8b), even CK8 and CK10, which are different types of cytokeratins. Therefore, it is possible that a general motif in both CK8 and CK10, or even in other cytokeratins, could mediate ClfB binding. Interestingly, the tail region of CK10, which is reported to be responsible for ClfB binding, consists of quasi-repeats of tyrosine-(glycine/serine)_m representing Ω -loops (Walsh *et al.*, 2004). Further studies showed strong binding of ClfB to a Y-Y loop peptide (YGGGSSGGSSGGGY) corresponding to the largest glycine loop in the tail region of CK10, indicating that the binding site was within this loop (Walsh *et al.*, 2004). Our experimentally mapped minimal interacting domain is also localized in the tail region of CK8 and possesses several glycine/serine repeats. We compared the sequence of the ClfB-binding site in CK8 with the reported binding site in CK10 and observed amino acid similarities between the Y-Y loop region and its counterpart in CK8-F5; however, only six of 15 residues are identical (Fig. 8a).

Fig. 7. Pull-down assay. *S. aureus* cells were incubated with HaCaT cell lysate to pull down endogenous CK8 through binding to *S. aureus* ClfB. Bound proteins were analysed by Western blotting using anti-CK8-specific antibody. Lanes: 1, CK8 protein (53.7 kDa) from HaCaT cell lysate; 2, eluate of CK8 bound to *S. aureus* cells; 3, cell lysate of *S. aureus*. The arrow indicates eluted CK8 protein.

Table 2. Evaluation of CK8-F5 amino acids favoured in Ω -loops

Amino acids differing between CK8-F5 and Y-Y loops were evaluated with respect to the frequency found in 979 Ω -loops from 191 non-redundant proteins. The plus and minus signs indicate an advantage or a disadvantage related to an Ω -loop. –, Clear disadvantage related to Ω -loops; (–), slight disadvantage related to Ω -loops; (+), slight advantage related to Ω -loops.

Y-Y loop*	CK8-F5	Advantage
G→	S	(–)
G→	L	–
G→	F	–
G→	S	(–)
S→	G	(+)
S→	A	–
G→	S	(–)
G→	S	(–)
Y→	S	(+)

*Walsh *et al.* (2004).

Furthermore, the tail region of CK10 has six glycine loops, two of which are identical to the Y-Y loop sequence and four of which are very similar. Therefore, it is believed that CK10 contains multiple binding sites for ClfB (Walsh *et al.*, 2004). In contrast to CK10, we could not find multiple putative loops similar to the Y-Y loop sequence in the tail region of CK8 (Fig. 8b).

If CK8-F5 represents an Ω -loop, then the Y-Y loop of CK10 might be a better one (see Table 2), based on the greater similarity between the CK10 amino acid composition and the amino acid frequencies determined from 979 Ω -loops (Fetrow, 1995).

While we cannot firmly conclude which cytokeratin, CK10 or CK8, is the biologically more significant target, it is interesting that two strategically and methodologically different approaches for ClfB target research have identified ClfB-binding regions within the cytokeratin tail domains of both CK8 and CK10.

The mechanism of ClfB-mediated adherence of *S. aureus* to CK8 is still not clear. Adhesin receptors are present on the surface of host cells and therefore can be recognized by pathogens. However, cytokeratins are localized intracellularly, where they are responsible for the mechanical stability of cells. In general, CK8 protein is not expressed on the cell surface, so we wondered what could be the subcellular location of CK8 protein in HaCaT cells and where bacterial adherence can occur. We observed major intracellular and residual extracellular CK8 expression in HaCaT cells by immunofluorescence using anti-CK8 antibodies of detergent-treated and non-treated cells. According to our study, CK8 is likely to be localized intracellularly; however, whether our observed residual extracellular staining is a technical artefact or biologically relevant remains to be confirmed. It has been demonstrated by others that CK8 is present on the surface of established human mammary carcinoma cells (Godfroid *et al.*, 1991) as well as on the surface of hepatocytes (Hembrough *et al.*, 1996). Although these cells are different from keratinocytes, it appears that CK8 may be expressed on the surface of at least some cell types.

Alternatively, one can hypothesize that *S. aureus* could interact with CK8 through ClfB after internalization of *S. aureus* by host cells. *S. aureus* is an extracellular pathogen; however, it can be internalized by a variety of non-phagocytic host cells (Kintarak *et al.*, 2004). Such intracellular persistence of *S. aureus* in host cells would allow the bacterium to evade host humoral immunity (Foster, 2005; Mempel *et al.*, 2002) and drug treatment

Fig. 8. Sequence alignment of CK8-F5 with the Ω -loop of CK10. (a) Comparison of the Y-Y loop with the tail region of CK8 shows six identical amino acids overlapping the minimal interacting domain CK8-F5, mapped via the yeast two-hybrid method. (b) Schematic representation of the subdomain structure of keratins. The 6 $\times$ Ω -loops of CK10 and the 1 $\times$ Ω -loop of CK8 are localized in the tail region.

(von Eiff *et al.*, 2001b). Furthermore, it has been shown by Mempel *et al.* (2002) that *S. aureus* cells are able to invade HaCaT cells, leading to necrosis or apoptotic cell damage after the bacteria have persisted within the cytoplasm for a while. It is also possible that *S. aureus* adheres to cytoplasmic CK8 in damaged cells. Tamura & Nittayajarn (2000) have shown that damaged epithelial cells have exposed CK8 protein. Another possibility is that such cells are damaged by toxins secreted by *S. aureus*, which are known to damage the plasma membrane of host cells (Foster, 2005).

Even if all the evidence is considered together, the role of the interaction between *S. aureus* and CK8 via ClfB is still not clear. It is also not clear whether this interaction promotes bacterial virulence or represents a host defence mechanism, leading to apoptosis of the host cells, which may prevent further bacterial colonization. Previous studies have shown that *S. aureus* internalization causes the expression of certain cytokines [interleukin (IL)-1, IL-6] (Yao *et al.*, 1995). Therefore, we assume that the interaction between ClfB and CK8 is just one step in a more complex process, which may increase cytokine release, resulting in the activation of the immune system. However, it is possible that the CK8–ClfB interaction counteracts apoptosis and therefore enables *S. aureus* to persist within host cells, allowing the bacterium to withstand a number of different antimicrobial agents (Alexander & Hudson, 2001). Further potentially biologically significant functions for *S. aureus* adherence to CK8 have been described in detail by Tamura & Nittayajarn (2000).

In summary, we demonstrated ClfB as the missing link of the already known *S. aureus* adherence to CK8. The significance of this interaction in different layers of the human skin or skin-derived cells (primary keratinocytes, immortalized HaCaT cells) is not clear, and whether this interaction plays a role in the pathogenesis of *S. aureus* or is involved in host defence mechanisms deserves further investigation.

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