Plasminogen capture by the cell wall-anchored protein FnBPB of *Staphylococcus aureus*

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INTRODUCTION

Stahylococcus aureus **is the causative agent of potentially harmful diseases, like sepsis endocarditis and necrotizing pneumonia and it is also responsible of less severe clinical manifestations such as epithelial and mucosal-associated infections. Nowadays, the emergence of methicillin (MRSA) as well as vancomycin-resistant (VRSA) strains is of great concern. In spite of the advance in antibiotic development, treating these infections remains a huge challenge. Gram-positive pathogenic bacteria, including *S. aureus*, display proteins on their surface that play important roles during infection. Among the multitude of interactions with host proteins *S. aureus* binds the human plasma protein plasminogen (PLG) and expresses a PLG activator, staphylokinase, that converts PLG to the serine protease plasmin. Plasmin controls several processes such as fibrinolysis, wound healing and tissue remodelling. Besides these activities plasmin can cleave native C3 leading to the formation of the C3b, which is subsequently degraded and inactivated to iC3b. Thus, binding of PLG to the surface of *S. aureus* and its activation to plasmin is an important pathogenetic mechanism of this Gram-positive bacterium.**

Several staphylococcal proteins have been reported to interact with PLG. For example, the surface immunoglobulin-binding protein (Sbi) and extracellular fibrinogen-binding protein (Efb) bound C3/C3b simultaneously with PLG (Plos One 2012; 7(10) e47638). Moreover, manganese transport protein C (MntC) takes part in this infectious scenario as an ion-scavenging factor and as a PLG-binding protein (Plos One. 2014; 9(11) e112730). However, the molecular aspects of these interactions have not been investigated in detail. In this study we screened several recombinant cell-wall associated staphylococcal proteins (CWA) for binding to PLG and found that some CWA interact with PLG and report on the binding of PLG to FnBPB, a fibronectin/fibrinogen binding protein, and provide evidence on the molecular aspects of this interaction.

RESULTS

Binding of PLG to *S. aureus* cells is mediated by cell wall-associated proteins.

In a preliminary approach we found that clinical isolates and laboratory strains of *S. aureus* strongly attached to surface-coated human PLG. In *S. aureus*, at least 20 surface proteins are covalently anchored to the cell wall by two sortase enzymes, SrtA and SrtB, that recognize specific surface protein sorting signals. Deletion of the *srtA* gene in *S. aureus* Newman and USA300 LAC strains abolished almost completely adherence of bacteria to PLG, suggesting that cell wall-associated proteins are the most important components involved in PLG binding (data not shown). To identify potential staphylococcal proteins with PLG-binding activity, a collection of recombinant cell wall-associated proteins were subjected to SDS/PAGE, transblotted onto a nitrocellulose membrane and probed with PLG. In these conditions, region A of FnBPA/B proteins interacted with PLG (Fig. 1). FnBPB contains a secretory signal sequence at the N-terminus and a C-terminal LPETG motif required for sortase-mediated anchoring of the protein to the cell wall peptidoglycan (Fig. 2A). The N-terminal A domain of FnBPB is predicted to fold into three sub-domains N1, N2 and N3 and promotes binding to fibrinogen through the dynamic "*dock, lock, latch*" mechanism (Fig. 2B).

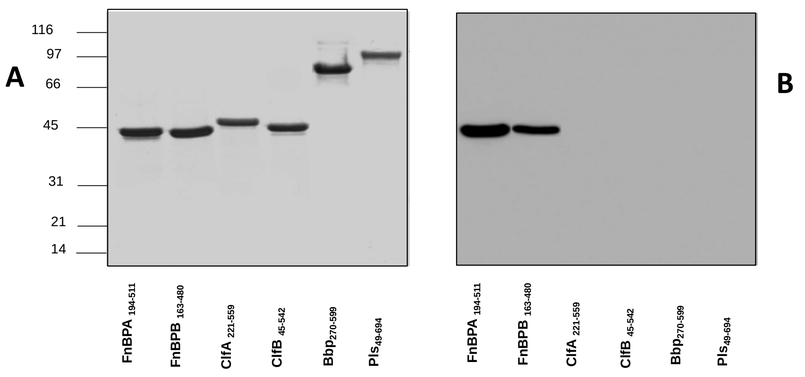


Fig.1- Binding of PLG to CWA staphylococcal proteins. SDS/PAGE (A) or Western blot (B). The membrane was then probed with PLG.

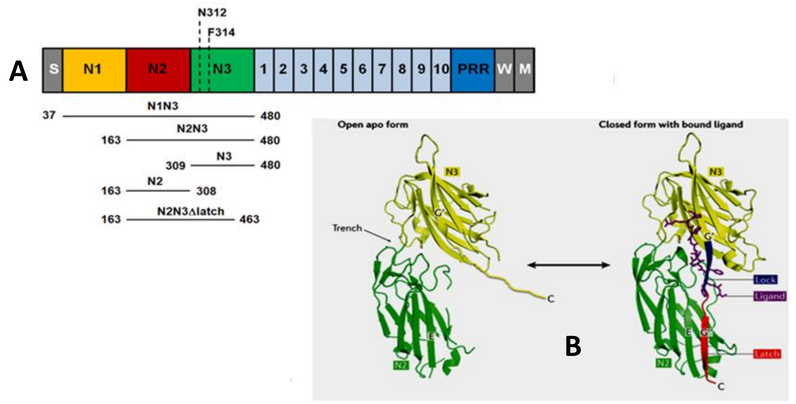


Fig.2 (A), schematic diagram of FnBPB. (B), the dynamic "dock, lock, latch" mechanism predicted for fibrinogen binding to the A domain of ClfA.

PLG binds to FnBPB with high affinity

PLG binding by recombinant FnBPB N2N3 (FnBPB$_{163-480}$) was assessed by ELISA-based solid phase assay. The protein bound to immobilized N2-N3 in a dose-dependent manner. To verify further these results, the affinity of N2N3 for PLG was measured using Surface Plasmon Resonance. FnBPBN2N3 was immobilized onto the surface of dextran chips and PLG was passed over the surface in concentrations ranging from 0.40 µM to 400 µM. The K_D for the interaction of FnBPB N2-N3 with PLG was 0.532 ± 0.028 µM (Fig.3A).

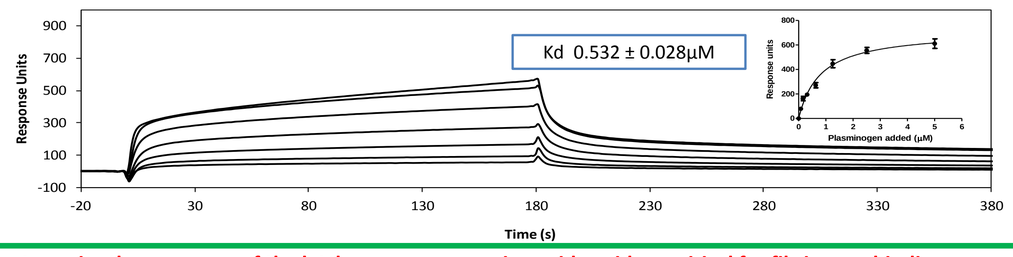


Fig.3

Recognition of N2-N3 by PLG does not require the presence of the latch sequence or amino acid residues critical for fibrinogen binding

In order to investigate the role of the C-terminal residues of the shortened FnBPB$_{163-480}$ N2N3 construct in ligand binding, a C-terminally truncated derivative FnBPB$_{163-463}$ (Δlatch mutated protein) was constructed and tested for fibrinogen and PLG binding by Western Blot. As expected, the mutated protein did not bind to fibrinogen (Fig.4A). On the contrary, FnBPB$_{163-463}$ kept intact the ability to interact with PLG (Fig.4B). To further examine FnBPB binding to PLG, a substitution was introduced in FnBPB$_{163-480}$ in residues predicted to be located in the trench between subdomains N2 and N3 that might be involved in ligand binding (FnBPB N312A/F314A). The mutated form of N2N3 completely lost the potential to bind to fibrinogen (Fig.5A), while it showed an interaction with PLG (Fig.5B)

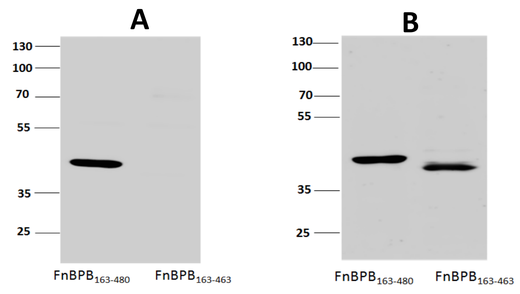


Fig.4- Interaction of PLG with the C-terminally truncated derivative FnBPB$_{163-463}$. Western immunoblotting with fibrinogen (panel A) or PLG (panel B).

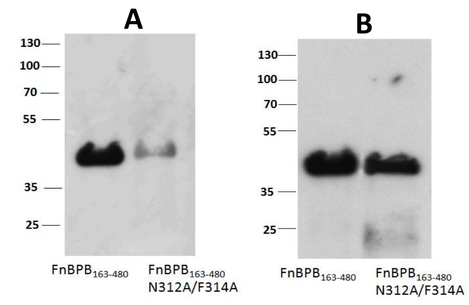


Fig.5- Binding of fibrinogen and PLG to the mutated form FnBPB$_{163-480}$ N312A/F314A. Western immunoblotting with fibrinogen (panel A) or PLG (panel B).

The N3 module of region A is involved in PLG binding.

To test whether single subdomains of region A of FnBPB maintain the ability to bind PLG, recombinant N2 (FnBPB$_{163-308}$) and N3 (FnBPB$_{309-480}$) modules were produced. The purified proteins were examined for fibrinogen (Fig.6A) and PLG (Fig.6B) binding by Western Blot. In both the experimental conditions, the individual modules were unable to bind fibrinogen. Conversely, N3 module, but not N2 module, kept intact the ability to interact with PLG. Together these data suggest that PLG and fibrinogen recognize different sites in region A of FnBPB (Fig.6).

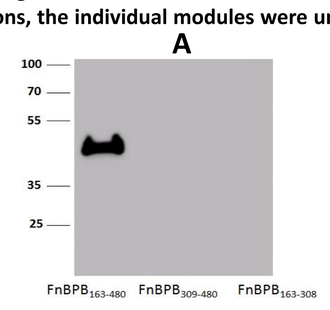
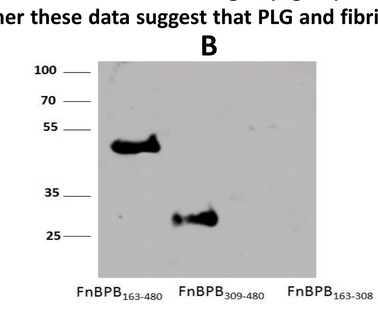


Fig.6- Binding of N2 and N3 modules to fibrinogen and PLG. Western immunoblotting with fibrinogen (panel A) or PLG (panel B)



Capture of PLG from human plasma by *S. aureus.*

To further determine the importance of PLG/Staphylococcus interaction, cells of *S. aureus* USA 300 LAC were tested for their ability to sequester PLG from human plasma. Staphylococcal cells were incubated with increasing amounts of human plasma and bacteria-bound plasma proteins were eluted at low pH, separated by SDS-PAGE under non reducing conditions and subsequently subjected to Western blot and probed with a rabbit anti-PLG antibody (Fig.7). The antibody reacted with a 90-kDa band, a component of similar size of purified PLG, suggesting that PLG binding by *S. aureus* can occur in also *in vivo*. The *srtA* mutant of USA 300 LAC showed a significantly reduced ability to capture PLG from plasma.

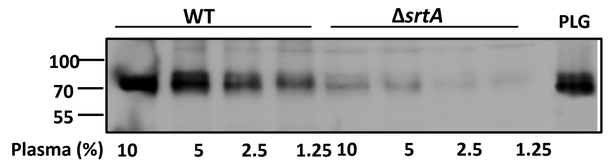


Fig.7- Capture of PLG from human plasma by *S. aureus*. *S. aureus strain LAC and LAC srtA were mixed with different concentrations of human plasma*

PLG captured by *S. aureus LAC* is functionally active.

Cells of *S. aureus* USA 300 (5x10^7) immobilized onto microtiter wells were incubated for 1h with human PLG (10 µg/well). After washing the plates, bacteria-bound PLG was activated by addition of tPA (Fig.8 A) or rSAK (Fig.8B) and incubated with 10 µg/well fibrinogen for the indicated times. In the negative controls PLG or tPA or bacteria were not included in the assay. The mixtures were subjected to 12% SDS-PAGE and degradation of FBG was evaluated by Coomassie blue staining.

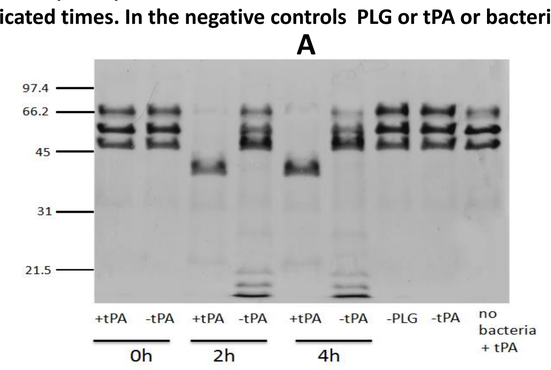
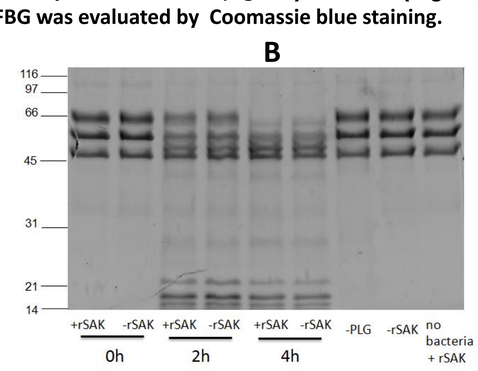


Fig .8- *S.aureus LAC wild-type cells were also immobilized on microtitre* plates and incubated with human PLG. It was activated by addition of tPA(A) or rSAK (B). Samples were incubated for 2h or 4h with FBG.



Captured PLG can be activated by endogenously expressed SAK.

To determine if endogenously expressed SAK is responsible for activating captured PLG wild type and LAC mutant for *sak* gene (LAC *sak)* were compared. Wild-type cells capture and activate PLG and cleave FBG whereas the *sak mutant was* defective (Fig.9A). *Culture supernatants* and cell surface extracts were examined by SDS-PAGE and Western immunoblotting using anti-SAK IgG. SAK was present both in the supernatant and is associated with the cell surface of LAC and that no SAK was detected in fractions from LAC *sak* (Fig.9B) .

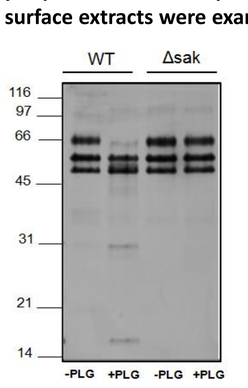
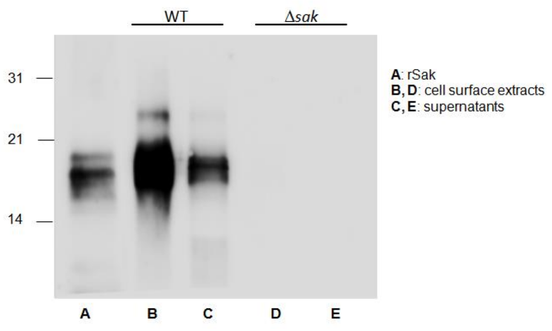


Fig.9- *S. aureus cells-bound PLG can be activated by endogenous SAK.* A) *S. aureus LAC wild-type and sak mutant cells were immobilized on* microtitre plates and incubated with human PLG . Samples were incubated for 4h with FBG. *B) TCAprecipitated* culture supernatants and proteins extracted from the cell surface were subjected to SDS-PAGE and Western immunoblotting probing with mouse anti-rSAK IgG.



DISCUSSION AND CONCLUSIONS

Here we have shown for the first time that sortase A-anchored cell wall-associated proteins are the dominant PLG binding proteins on the *S. aureus cell surface. A* sortase A mutant of *S. aureus LAC bound at* least a 10-fold lower level of PLG than the wild-type. Two CWA proteins, FnBPA and FnBPB, were shown to bind PLG *in vitro and in the case of* FnBPB, when expressed on the surface of the surrogate host *L. lactis. However, a deletion* mutant of *S. aureus USA300 LAC lacking* FnBPs bound the same level of PLG as the parental strain while a mutant of BH1CC showed a ~50% reduction in PLG binding, indicating that at least one other CWA protein is also important. The A domain of FnBPB was chosen fordetailed analysis of PLG binding. This domain can bind to FBG by the dock, lock, and latch mechanism We have shown that PLG and FBG bind FnBPB at distinct non-overlapping sites and that PLG binding does not involve the dock, lock, and latch mechanism. This is important since PLG and FBG are both components of blood plasma. FnBPB can simultaneously capture both PLG and FBG. The PLG binding site is confined to subdomain N3. Our data indicates that kringle 4 comprises the only binding domain within PLG for FnBPB and that this binds the two lysine-rich patches within subdomain N3 of FnBPB. PLG captured from human plasma could be activated in plasmin by exogenously added tissue PLG activator t-PA and SAK .The presence of active plasmin on the surface of *S. aureus* cells most likely contributes to the athogenesis of different infections. For example, degradation of host- or staphylocoagulase- promoted fibrin clots could help bacteria to spread . Damage to subcutaneous tissue could contribute to the pathogenesis of skin soft tissue infections (SSTIs) caused by CA-MRSA . Degradation of opsonins could contribute to the avoidance of neutrophil-mediated phagocytosis and killing . To investigate this further requires the employment of appropriate animal models, and to test the role of staphylokinase in these processes requires the use of a transgenic mouse expressing human PLG. In summary we have shown that CWA proteins are the dominant bacterial receptors for capturing host PLG by *S. aureus and we* have investigated in detail the mechanism of PLG binding by one such CWA protein, FnBPB.

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