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Enhanced activation of bound plasminogen on *Staphylococcus aureus* by staphylokinase

Tomi Mölkänen[a], Jaana Tyynelä[b], Jari Helin[c], Nisse Kalkkinen[c], Pentti Kuusela[a,d,*]

[a] *Department of Bacteriology and Immunology, The Haartman Institute, P.O. Box 21 (Haartmaninkatu 3), FIN-00014 University of Helsinki, Helsinki, Finland*
[b] *Protein Chemistry Unit and Institute of Biomedicine, Biomedicum, Helsinki, Finland*
[c] *Laboratory of Protein Chemistry, Institute of Biotechnology, University of Helsinki, Helsinki, Finland*
[d] *Division of Clinical Microbiology, HUCH Laboratory Diagnostics, Helsinki University Central Hospital, Helsinki, Finland*

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Abstract Activation of plasminogen (plg) to plasmin by the staphylococcal activator, staphylokinase (SAK), is effectively regulated by the circulating inhibitor, α_2-antiplasmin (α_2AP). Here it is demonstrated that intact *Staphylococcus aureus* cells and solubilized staphylococcal cell wall proteins not only protected SAK-promoted plg activation against the inhibitory effect of α_2AP but also enhanced the activation. The findings suggest that the surface-associated plg activation by SAK may have an important physiological function in helping staphylococci in tissue dissemination. Amino acid sequencing of tryptic peptides originating from the 59-, 56- and 43-kDa proteins, isolated as putative plg-binding proteins, identified them as staphylococcal inosine 5′-monophosphate dehydrogenase, α-enolase, and ribonucleotide reductase subunit 2, respectively. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Plasminogen; Staphylokinase; Activation of plasminogen; *Staphylococcus aureus*

1. Introduction

In order to invade host tissues, bacteria can utilize the plasminogen (plg)/plasmin system of the host by employing various mechanisms at various stages during infection. These include the synthesis and secretion of bacterial plg activators (PA) as well as stimulation of the host cells to secrete PAs and their inhibitors. Several invasive pathogens express plg-binding proteins or receptors which immobilize plg on the bacterial surface and enhance its activation by mammalian PAs. In essence, these receptors turn the bacteria into proteolytic organisms capable of degrading and invading extracellular matrix and basement membranes (for reviews see [1,2]). The staphylococcal PA staphylokinase (SAK) is a 136-amino acid protein, secreted by certain transduced *Staphylococcus aureus* strains (see for review [3]). Based on deduced amino acid sequences, there are so far four natural SAK variants differing from each other in only four amino acid residues [4–7]. Similar to streptokinase (SK), the streptococcal PA SAK lacks proteolytic activity but can activate plg in a mechanism resembling the activation by SK. The initial step of activation involves complex formation between SAK and the tiny amount of plasmin formed by a spontaneous conversion of plg to plasmin. The primary formation of the SAK–plasmin complex is efficiently inhibited by α_2-antiplasmin (α_2AP), the main plasmin inhibitor in circulation. The situation changes drastically when plg or SAK–plasmin complex is bound to fibrin, or to another target, through the lysine-binding sites of plg/plasmin. Most probably occupation of the sites prevents binding of α_2AP to the complex and leads to protected plasmin activity. Okada et al. [7] have shown that binding of plg to fibrinogen fragments enhances the SAK-mediated plg activation. The observation that SAK activates primarily plg bound to fibrin clots has created great enthusiasm about the role of SAK in thrombolytic therapy [3].

In the present work we have investigated the effect of intact *S. aureus* cells as well as of solubilized staphylococcal cell wall proteins on SAK-induced plg activation. Additionally we have purified plg-binding staphylococcal cell wall proteins using affinity chromatography on immobilized plg and identified them by amino acid sequencing.

2. Materials and methods

2.1. Chemicals

α_2AP, aprotinin, ethylmaleimide, phenylmethylsulfonyl fluoride, DNase I, RNase A, and 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluric acid (Iodogen) were purchased from Sigma and ε-aminocaproic acid (EACA), formic acid and acetonitrile were from Fluka Chemie AG. Lysostaphin was obtained from Ambi, New York, USA, chromogenic substrate S-2251 (H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide hydrochloride) from Chromogenix (formerly KabiVitrum) and sequencing-grade trypsin form Promega (Madison, WI, USA). Glu-plg was isolated from citrated outdated human plasma (Finnish Red Cross Blood Transfusion Center, Helsinki, Finland) using affinity chromatography on immobilized lysine as described [8]. SAK was a gift from the Medicine Department of Yakult Honsha, Tokyo, Japan.

2.2. Bacterial strains and cultivation

S. aureus strains included in studies were clinical isolates (Laboratory of Bacteriology, Division of Clinical Microbiology, HUCH Laboratory Diagnostics, Helsinki University Central Hospital, Helsinki, Finland) originating from septicemia patients (five strains), from patients with superficial infections (five strains), and one laboratory strain, strain Newman. Bacteria were maintained on blood agar plates at 4°C and transferred every 4–6 weeks. For experiments bacteria were grown in Todd–Hewitt broth overnight at 37°C, pelleted by centrifugation at 2700×*g* for 10 min at room temperature, washed twice with phosphate-buffered saline (PBS) containing 0.02% (w/v) sodium azide and finally suspended in the same buffer containing

*Corresponding author. Fax: (358)-9-191 26382.
E-mail address: pentti.kuusela@helsinki.fi (P. Kuusela).

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1% (w/v) bovine serum albumin (BSA) as a carrier (PBS–BSA 1%). Bacterial concentrations were measured spectrophotometrically using the Klett-OD 80 for 2×10^8 bacterial cells/ml and kept in an ice bath to be used within 24 h.

Phenotypic SAK producer status of staphylococcal strains was recorded by incubating Glu-plg, spent culture medium from overnight cultures, and S-2251 at 37°C and monitoring the cleavage of the chromogenic substrate spectrophotometrically at 405 nm (Ultrospec II™, LKB/Wallac).

2.3. Binding of Glu-plg to S. aureus

For binding experiments, Glu-plg was labelled with carrier-free $Na^{125}I$ (specific activity 100 mCi/ml; Amersham) with the Iodogen method as described [9,10]. Binding was determined by incubating 2×10^9 bacteria with 10 ng of [^{125}I]Glu-plg in 500 μl of PBS–BSA 1% at room temperature for 2 h under continuous mixing. Subsequently, the bacterial cells were pelleted and washed twice with 1.0 ml of PBS–BSA 1% and radioactivity associated with bacteria was quantified with an 1270 Rackgamma II™ γ-counter (LKB/Wallac). Specificity of binding was determined using 1000-fold molar excess of unlabeled Glu-plg to inhibit the binding of [^{125}I]Glu-plg.

2.4. Isolation of S. aureus cell wall proteins interacting with Glu-plg

For isolation of staphylococcal surface proteins, *S. aureus* strain Newman was treated with lysostaphin as described [11]. For the purification, 60 mg of Glu-plg was coupled to Sepharose beads (Pharmacia) according to the manufacturer's instructions. Proteins interacting with plg were isolated by passing 120 mg of lysostaphin digest through a plg–Sepharose column (10 ml) at 4°C, by washing the column with 200 ml of PBS and by finally eluting the bound proteins with 0.2 M EACA in PBS. In order to inactivate possible trace amounts of plasmin originating from the Sepharose–plg, 100 μl of aprotinin, 0.24 TIU/ml, was mixed with the lysostaphin digest prior to adding to the column. Aprotinin was removed from fractions by gel filtration on PD-10 columns (Pharmacia) before use in further experiments. Protein concentrations in various fractions were determined according to Lowry [12].

2.5. Enhancement of SAK-induced plg activation by S. aureus cells or by solubilized cell wall proteins

Generation of plasmin activity was monitored by incubating Glu-plg (2–4 μg), SAK (10 ng), α_2AP (0.1–5 μg) and S-2251 (58 μg) in the presence of various concentrations of intact bacteria in 500 μl of PBS containing 0.02–0.16% (w/v) BSA. Alternatively, solubilized *S. aureus* cell wall proteins, proteins adsorbed or purified by affinity chromatography on Sepharose–plg were used instead of intact bacteria. After incubation for 90–100 min at room temperature bacteria were pelleted by centrifugation and formation of *p*-nitroanilide in supernatants was measured spectrophotometrically at 405 nm.

2.6. Activation of S. aureus-bound and soluble plg by SAK

In order to compare the efficiency of SAK to activate soluble and receptor-bound plg, a stock solution was prepared by mixing [^{125}I]Glu-plg (5 μg/ml) with unlabelled Glu-plg (100 μg/ml) giving a specific activity of 72 000 cpm/μg protein. The amount of Glu-plg bound to bacteria was then determined by incubating 9.1 μg of [^{125}I]plg stock with 2×10^9 *S. aureus* cells, either strain Newman or strain 274, in 500 μl of PBS–BSA 1% for 2 h at room temperature under continuous shaking. Subsequently, cells were washed twice with 1 ml of PBS–BSA 1% and transferred into new tubes for counting the radioactivity associated with bacteria. Activation was finally performed by incubating equal amounts of bacteria-bound or soluble plg, as judged by the radioactivity, with SAK in a mass proportion of 1:200 in the presence of 0.08 μM S-2251. After incubation for 100 min at room temperature bacteria were pelleted by centrifugation and formation of *p*-nitroanilide in supernatants was measured as described above.

2.7. Ligand blotting of S. aureus surface proteins with plg

In order to identify plg-binding proteins of *S. aureus*, strain Newman, lysostaphin digest or proteins eluted from Sepharose–plg affinity column were first run on 10% SDS–PAGE under non-reducing conditions, transferred electrophoretically to nitrocellulose membrane (Amersham, Hybond-C extra) and stained with Ponceau S to visualize the transferred proteins. Subsequently, the membrane was destained, saturated with 5% (w/v) defatted milk powder in PBS for 2 h at room temperature, and treated with Glu-plg at a concentration of 10 μg/ml in TEN (0.05 M Tris, 0.025 M EDTA, 0.15 M NaCl, pH 7.5) containing 0.05% (v/v) Tween (TEN-Tween) for 12 h at +4°C in the presence or absence of 200 mM EACA. After four washes with TEN-Tween buffer the membrane was incubated in a predetermined dilution of rabbit anti-human plg (Dako) for 2 h at room temperature, followed by four washes with TEN-Tween and by treatment with affinity-purified antibodies to rabbit IgG (Jackson Immunoresearch Laboratories, Westgrove, PA, USA) in TEN-Tween. Finally, after four washes in TEN-Tween, bound plg was visualized by treating the membranes with 0.5 μM 3,3′-diaminobenzidine tetrahydrochloride dihydrate (Fluka) in PBS containing 0.5% (v/v) H_2O_2.

2.8. Digestion, separation and amino acid sequencing of the proteins interacting with plg

The cell wall proteins isolated by the affinity chromatography on Sepharose–plg were separated by electrophoresis using 12% SDS–PAGE gels [13] and stained with Coomassie brilliant blue R. The 59-, 56-, 45-, 43-, and 39-kDa protein bands were excised from the gel, washed, reduced, and digested with sequencing-grade trypsin as described previously [14]. The resulting peptides were solubilized in 300 μl of 0.1% formic acid and filtered through a 0.2-μm filter (Millipore, Bedford, MA, USA). The peptides were separated in a reversed phase HPLC system using a microscale reversed phase Pepmap column (RP18, LC packings, 0.3 mm×15 cm) and a short concentrating column of the same material (0.8 mm×2 mm) preceding the separating column. The peptides were eluted by a linear gradient of acetonitrile (4–40% in 100 min) in 0.07% formic acid using a flow of 2 μl/min. The eluting peptides were detected by an on-line UV detector at 214 nm and collected manually in 25% acetonitrile/0.07% formic acid. Individual peptides were subjected to N-terminal amino acid sequencing by an automatic Procise 494A amino acid sequencer (Perkin Elmer, Applied Biosystems Division, Foster City, CA, USA) using Edman chemistry.

3. Results

3.1. Enhancement of SAK-induced plg activation by intact S. aureus cells

In order to study the effect of *S. aureus* on SAK-induced plg activation, increasing numbers of intact bacterial cells (*S. aureus*, strain 274) were added into the reaction solution containing Glu-plg, SAK and α_2AP. Under these conditions the plasmin formation was effectively inhibited by α_2AP (see Fig. 1A, open bars). The presence of bacteria led to a dose-dependent increase of plasmin activity as judged by cleavage of a chromogenic substrate, S-2251 (data not shown). The effect was observed with all tested bacterial strains: addition of 2×10^8 *S. aureus* cells, representing either SAK non-producers or SAK producers to the reaction solution enhanced the formation of plasmin, as compared to the control containing only Glu-plg and SAK (Fig. 1A). Further, it was tested whether there was a correlation between the ability of various *S. aureus* strains to enhance SAK-induced plg activation and the plg-binding capacity of the strains. As seen in the inset of Fig. 1A, the more [^{125}I]Glu-plg the *S. aureus* strain was able to bind, the more effective it was in enhancing the SAK-induced plg activation.

The ability of SAK to activate *S. aureus*-bound or soluble plg was studied by incubating SAK with equal amounts of the two plg forms. As seen in Fig. 1B, at a 1:200 molar ratio SAK was able to generate detectable plasmin activity only when plg was bound to *S. aureus* surface, either strain Newman or strain 247. The presence of exogenous SAK was important for the activation since in the absence of SAK no plasmin was formed from either plg forms.

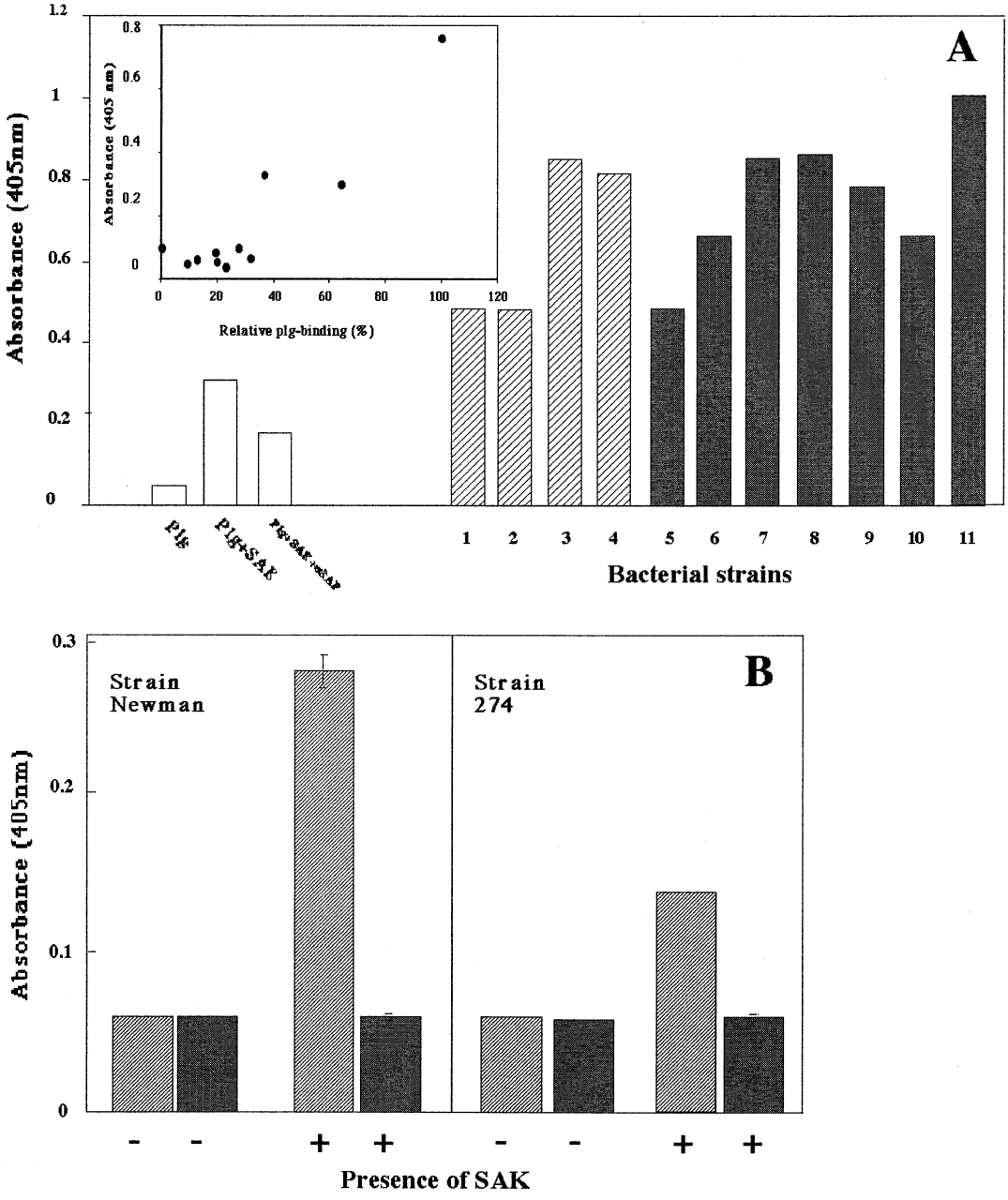

Fig. 1. Enhancement of SAK-induced plg activation by intact *S. aureus* cells as judged by cleavage of the chromogenic substrate S-2251. A: Plasmin formation in the presence of various combinations of plg, SAK and α_2AP (white bars) and enhancement of plasmin formation when various SAK-non-producing (hatched bars) and SAK-producing (gray bars) staphylococcal strains were incubated together with plg, SAK and α_2AP. Inset: Correlation of plg-binding capacity and enhancement of SAK-induced plg activation by various *S. aureus* strains. Plg activation is indicated as optical density at 405 nm after cleavage of chromogenic substrate S-2251, and relative plg binding as percentages of binding of [^{125}I]Glu-plg to *S. aureus*, strain Newman, which is expressed as 100%. B: SAK-induced activation of free soluble plg (gray bars) and plg bound to plg-binding structures (hatched bars) on *S. aureus*, strain Newman and strain 274. Presence or absence of SAK in the incubation medium is indicated as + or −, respectively. For details, see Section 2.

3.2. Enhancement of SAK-induced plg activation by S. aureus cell wall proteins

Similar to the intact *S. aureus* cells also the solubilized staphylococcal cell wall protein pool abolished the α_2AP-mediated inhibition of plasmin and enhanced SAK-induced plg activation in a dose-dependent way at concentrations of 50–100 μg/ml (Fig. 2). The ability of the cell wall protein pool to enhance SAK-induced plg activation was abolished when it was passed through the plg affinity column (Fig. 2). However, the proteins bound to the column retained the ability to protect against α_2AP-mediated inhibition and to enhance SAK-induced plg activation; a reaction solution containing 25 μg/ml of plg-binding proteins generated plasmin formation corresponding to that produced in the presence of 100–200 μg/ml of whole lysostaphin digest (Fig. 2).

3.3. Identification of the plg binding proteins

SDS–PAGE analysis of solubilized *S. aureus* cell wall pro-

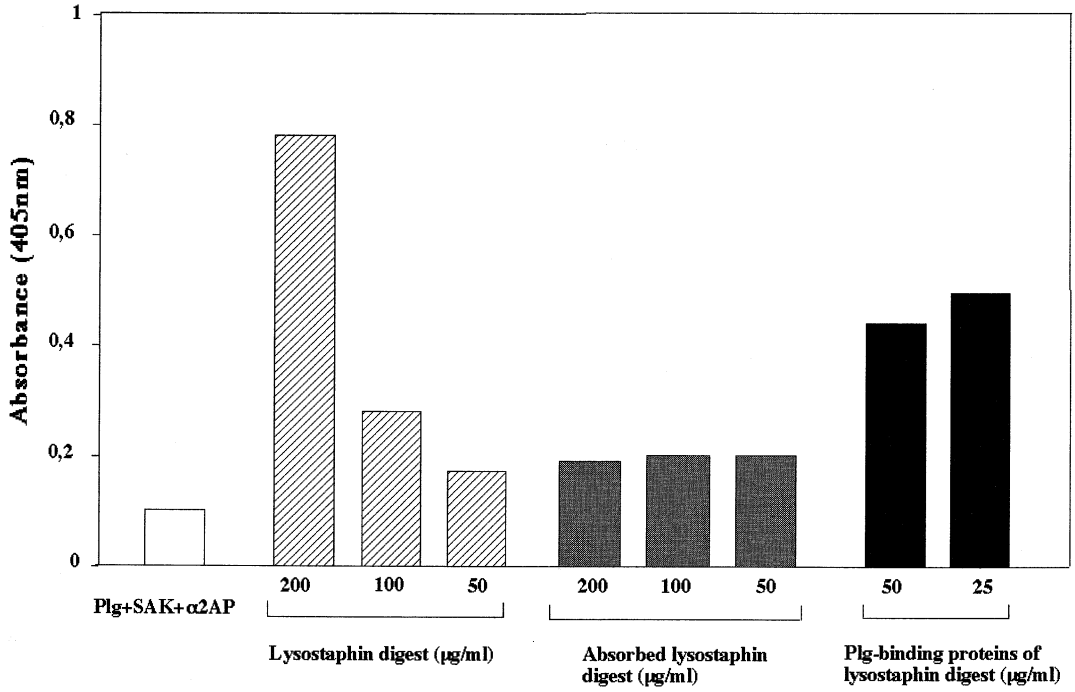

Fig. 2. Enhancement of SAK-induced plg activation by various amounts of lysostaphin released cell wall proteins (strain Newman). Plasmin formation, as judged by cleavage of the chromogenic substrate S-2251, when indicated concentrations of lysostaphin released cell wall proteins (hatched bars), proteins passed through the plg affinity column (gray bars) and proteins eluted from the affinity column (black bars) were incubated together with plg, SAK and α_2AP (white bar).

teins, isolated by affinity chromatography on Sepharose–plg, revealed major 59-, 56- and 39-kDa proteins as well as minor 45- and 43-kDa protein bands (Fig. 3A). Ligand blotting with Glu-plg showed that the 59-, 56-, and 43-kDa proteins bound very efficiently to soluble plg, whereas the 39-, and 45-kDa proteins showed weaker plg-binding ability (Fig. 3B). In the presence of 0.2 M EACA ligand blotting with plg showed no immunoreactive bands (data not shown).

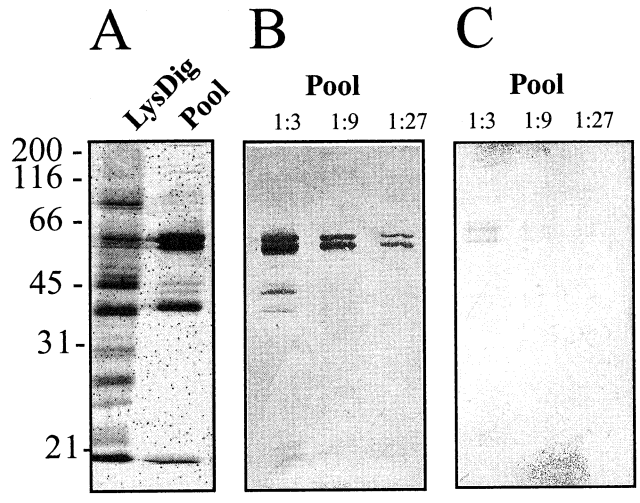

Fig. 3. Cell wall proteins of *S. aureus* interacting with plg. A: Coomassie blue staining of SDS–PAGE analysis of cell wall proteins of *S. aureus*, strain Newman, released by lysostaphin treatment (LysDig; 60 μg) as well as of the pool of cell wall plg-binding proteins isolated by affinity chromatography on Sepharose–plg (Pool; 2 μg). B: Pool of plg-binding proteins diluted 1:3, 1:9 and 1:27 (corresponding approximately to 630 ng, 210 ng and 70 ng of protein, respectively) visualized by ligand blotting employing plg and anti-plg antibodies. C: Control of ligand blotting without plg. Migrations of molecular weight markers are indicated on the left. For details, see Section 2.

Peptide sequences from the plg-binding proteins were matched against protein databases [15]. These comparisons revealed high homologies between the 59-, 56-, and 43-kDa proteins with inosine 5′-monophosphate dehydrogenase (IMPDH), α-enolase and ribonucleotide reductase of different species, respectively. When the obtained amino acid sequences were compared with IMPDH (unfinished genomic sequence: gn1ıTIGR 1280ı S. aureus 8087), α-enolase (accession number T47276), and ribonucleotide reductase β chain (accession number AJ133495) of *S. aureus*, all peptides corresponded to the candidate proteins thus providing a reliable identification of the three major plg-binding proteins (Fig. 4). The quantity of the 45-kDa protein was too low to allow for identification, and the sequences originating from the 39-kDa protein band did not match completely any single protein, suggesting unsuccessful purification of the protein using preparative gel electrophoresis.

4. Discussion

The present study shows that intact *S. aureus* and plg-binding components isolated from the staphylococcal cell not only protected plasmin against the inhibition by α_2AP but also enhanced the SAK-induced plg activation. In addition, identification of the three staphylococcal proteins, IMPDH, α-enolase and ribonucleotide reductase, which were shown to to bind plg, suggests a role for these proteins as putative plg-binding structures of *S. aureus* surface.

Our results are in accordance with the earlier observation showing that plasmin bound to *S. aureus* surface is protected

A. S. AUREUS INOSITOLE-5-MONOPHOSPHATE DEHYDROGENASE (unfinished genomic sequnce: gn1|TIGR 1280|S.aureus 8087)

1 MWESKFAKES LTFDDVLLIP AQSDILPKDV DLSVQLSDKV KLNIPVISAG MDTVTESKMA

61 IAMARQGGLG VIHKNMGVEE QADEVQKVKR SENGVISNPF FLTPEESVYE AEALMGKYRI

fr 49.58

121 **SGVPIVDNKE DRNL**VGILTN RDLRFIEDFS IKIVDVMTQE NLITAPVNTT LEEAEKILQK

fr 45.86

181 HKIEKLPLVK **DGRLEGLITI** KDIEKVIEFP NAAKDEHGRL LVAAAIGISK DTDIRAQKLV

241 EAGVDVLVID TAHGHSKGVI DQVKHIKKTY PEITLVAGNV ATAEATKDLF EAGADIVKVG

301 IGPGSICTTR VVAGVGVPQI TAIYDCATEA RKHGKAIIAD GGIKFSGDII KALAAGGHAV

fr 24.08

361 MLGSLLAGTE ESPGATEIFQ GRQYKVYRGM GSLGAMEK**GS NDRYFQEDK**A PKKFVPEGIE

fr 39.35

421 GRTAYKGALQ DTIYQLMGGV R**AGMGYTGS**H DLRELREEAQ FTRMGPAGLA ESHPHNIQIT

481 KESPNYSF

B. S. AUREUS α-ENOLASE (T47276)

fr 21.61

1 M**PIITDVYAR** EVLDSRGNPT VEVEVLTESG AFGRALVPSG ASTGEHEAVE LRDGDKSRYL

61 GKGVTKAVEN VNEIIAPEII EGEFSVLDQV SIDKMMIALD GTPNKGKLGA NAILGVSIAV

fr 45.28

121 AR**AAADLLGQ PLYK**YLGGFN GKQLPVPMMN IVNGGSHSDA PIAFQEFMIL PVGATTFKES

fr 21.62

181 LRWGTEIFHN LKSILSQR**GL ETAVGDEGGF** APKFEGTEDA VETIIQAIEA AGYKPGEEVF

241 LGFDCASSEF YENGVYDYSK FEGEHGAKRT AAEQVDYLEQ LVDKYPIITI EDGMDENDWD

301 GWKQLTERIG DRVQLVGDDL FVTNTEILAK GIENGIGNSI LIKVNQIGTL TETFDAIEMA

361 QKAGYTAVVS HRSGETEDTT IADIAVATNA GQIKTGSLSR TDRIAKYNQL LRIEDELFET

421 AKYDGIKSFY NLDK

C. S. AUREUS RIBONUCLEOTIDE REDUCTASE MINOR SUBUNIT (AJ133495)

fr 50.36

1 MIAVNWNTQE DMTNMFWRQN ISQMWVETEF KVSKDIASWK TLSEAEQDTF KK**ALAGLTGL**

61 DTHQADDGMP LVMLHTTDLR KKAVYSFMAM MEQIHAKSYS HITTLLPSS ETNYLLDEWV

121 LEEPHLKYKS DKIVANYHKL WGKEASIYDQ YMARVTSVFL ETFLFFSGFY YPLYLAGQGK

181 MTTSGEIIRK ILLDESIHGV FTGLDAQHLR NELSESEKQK ADQEMYKLLN DLYLNEESYT

fr 57.91 fr 33.74

241 KMLYDDLGIT EDVLNYVKYN GNK**ALSNLGF EPYF**EEREFN PIIENALDTT TK**NHDFFSVK**

301 GDGYVLALNV EALQDDDFVF DNK

Fig. 4. Comparison of sequences of tryptic peptides of plg-binding proteins with sequences of *S. aureus* proteins. Alignment of peptides (expressed in bold) originating from the 59-kDa protein with IMPDH (A), peptides from the 56-kDa protein with α-enolase (B), and of peptides from the 43-kDa protein with ribonucleotide reductase β-chain (C). Accession numbers of the proteins are indicated in parentheses. Numbers above sequences shown in bold refer to fractions used for sequencing. For details, see Section 2.

against α_2AP [10]. Most probably the cell wall plg-binding structures occupy the lysine-binding sites of the plg molecule and thus prevent the interaction of α_2AP with active plasmin. Additionally, Christner and Boyle [16] demonstrated that plasmin is formed on the surface of SAK-producing *S. aureus* cells cultured in plasma milieu containing functional α_2AP. The present study shows that the plasmin formation on the bacterial surface takes place in an enhanced manner, and that there is a direct correlation between the capacities of a *S. aureus* cell to bind plg and to enhance the SAK-induced plg activation in the presence of α_2AP. These findings further emphasize the role of staphylococcal plg-binding proteins in SAK-induced activation of plg.

Binding of Glu-plg to staphylococcal plg-binding proteins made it more sensitive to SAK-mediated activation. This was clearly shown in experiments where the activation of *S. aureus*-bound plg by SAK resulted in active plasmin formation, whereas the same amount of SAK was unable to produce any measurable plasmin from the same amount of soluble plg. The enhanced activation efficiency may be due to a conformational change in the Glu-plg molecule induced by plg-binding proteins of *S. aureus* thus making bacteria-bound plg

more susceptible to activation by SAK. This type of activation mechanism has been proposed to other PAs, such as tissue-type plasminogen activator [10]. These findings are also in good agreement with the recent observation showing that SAK has a higher binding affinity towards plg bound to fibrin fragments than towards soluble plg [17], and that SAK induces strong activation of plg bound to fibrinogen fragments [7].

Recent evidence indicates that the SAK-induced activation starts with a complex formation between SAK and a small amount of plasmin which is probably formed in circulation as a result of endogenous activation. The active site exposed in the complex can subsequently convert SAK–plg complexes to SAK/plasmin and plg to plasmin (for a review, see [3]). In the present experimental setting, the purified Glu-plg was pretreated with insolubilized aprotinin in order to remove the possible tiny amount of plasmin. It is, however, not excluded that on *S. aureus* cells a proportion of the receptor-bound Glu-plg might be converted to plasmin and would be subsequently responsible for the enhanced plg activation.

Similar to intact bacterial cells, solubilized staphylococcal cell wall proteins could enhance SAK-induced plg activation in the presence of α_2AP. This effect could be abolished by adsorption with immobilized plg indicating that at least part of the plg-binding capacity of *S. aureus* is mediated by surface proteins. We were able to purify the proteins responsible for the enhancement of activation by using an affinity chromatography on immobilized Glu-plg. The 59-, 56-, and 43-kDa proteins, which exhibited strong plg-binding capacity in the ligand-blotting assay, were identified as staphylococcal IMPDH, α-enolase and ribonucleotide reductase, respectively. All these observations favor the idea that these proteins, as tentative plg-binding proteins, are also responsible for the enhanced SAK-induced plg activation.

IMPDH is a key enzyme in purine nucleotide biosynthesis by catalyzing NAD-dependent conversion of inosine 5′-monophosphate to xanthosine 5′-monophosphate. The importance of IMPDH in maintaining cellular guanylate pools has made it a target for a number of immunosuppressive agents (for a review, see [18]). Another tentative plg-binding molecule, ribonucleotide reductase, belongs to the group of enzymes providing the only mechanism by which nucleotides can be converted to deoxynucleotides. Both of these enzymes are intracellular and there is no previous evidence for their extracellular localization. In contrast, α-enolase (2-phospho-D-glycerate hydrolyase), an intracellular key enzyme of the glycolytic pathway, has been identified as a plg/plasmin-binding structure on the surface of several eukaryotic cells [19–21] and on hemolytic group A streptococci [22].

More recently, Modun and Williams [23] demonstrated that another intracellular glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), functions as a transferrin receptor on *S. aureus* surface. The purified staphylococcal GAPDH also bound plg but its role as a staphylococcal plg-binding protein has not been reported. In the present investigation we were not able to find GAPDH among proteins interacting with plg. This may be due to differences between various staphylococcal strains, or due to the fact that plg binding is a characteristic of the purified enzyme. GAPDH has been demonstrated, however, as a target for plg and plasmin on group A streptococci [24] albeit more recent observations based on mutational analysis have questioned this property [25].

The presence of intracellular proteins on the staphylococcal surface is intriguing albeit difficult to explain. The surface localization of α-enolase and GAPDH on eukaryotic or prokaryotic cells has been convincingly demonstrated [19–22,26], thus rendering the idea that also other intracellular proteins may appear on the bacterial surface quite feasible. The present results must, however, be considered with caution due to the possibility that during bacterial cultivation or lysostaphin treatment some staphylococcal cells break and release intracellular enzymes which then could adsorb to bacterial surface proteins and become solubilized from the surface as complexes with their carrier molecules. If this were the case, the proteins eluted from the Sepharose–plg column should also include the cell wall proteins associated with these putative plg-binding structures. As revealed by SDS–PAGE and ligand-blotting analysis this was not the case but rather all five proteins eluted were also capable of interacting with plg. Further, no differences could be observed in protein patterns of lysostaphin digests done in the absence or presence of 20% sucrose in order to prevent the break-down of possible staphylococcal protoplasts.

The present results indicate that staphylococcal plg-binding structures may have a physiologically important function and a possible role as staphylococcal virulence factors. Together with SAK production, they form a mechanism by which a staphylococcal cell can generate a protected protease on its surface which subsequently can assist bacterial spreading by destroying components of extracellular matrix directly, by activating matrix metalloproteinases, and by helping bacteria to penetrate basement membranes [1,27–29]. In this context, understanding of in vivo regulation of the expression of both plg-binding molecules and SAK becomes important. Albeit the function of α-enolase as plg-binding protein has been demonstrated for several eukaryotic cells and for group A streptococcus, further experimental evidence is required on the localization and roles of IMPDH, α-enolase and ribonucleotide reductase as staphylococcal plg-binding structures.

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