More than one tandem repeat domain of Extracellular Adherence Protein (Eap) of *Staphylococcus aureus* is required for aggregation, adherence and host cell invasion, but not for leucocyte activation

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Running Title: Functional characteristics of Eap tandem repeat domains

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Abstract

Eap is a multifunctional *Staphylococcus aureus* protein and broad spectrum adhesin for several host matrix and plasma proteins. We investigated the interactions of full length Eap and five recombinant tandem repeat domains with host proteins using surface plasmon resonance (SPR; BIAcore®) and ligand overlay assays. In addition, agglutination and host cell interaction, namely adherence, invasion, and stimulation of proliferation was determined.

In plasmon resonance, interaction of full length Eap isoforms (from strains Newman and Wood 46) with fibrinogen, fibronectin, vitronectin and thrombospondin 1 was found to be specific but with different affinities for the ligands tested. In ligand overlay assay, interaction of five single tandem repeat domains (D1 to D5) of Eap-7 (from strain CI-7) to fibronectin, fibrinogen, vitronectin, thrombospondin-1, and collagen I, differed substantially. Most prominently, D3 bound strongest to fibronectin and fibrinogen. Full-length Eap, but none of the single tandem repeat domains, agglutinated *S. aureus* and enhanced adherence to and invasion of host cells by *S. aureus*. D3-4 and D1-3 (*in cis*) increased adherence and invasiveness compared to single Eap tandem repeat domains. By contrast, single Eap tandem repeat domains and full-length Eap similarly modulated proliferation of PBMCs: low concentrations stimulated, whereas high concentrations inhibited proliferation.

Taken together, Eap tandem repeat domains appear to have distinct characteristics for binding of soluble ligands, despite a high degree of sequence similarity. In addition, more than one Eap tandem repeat domain is required for *S. aureus* agglutination, adherence and cellular invasion, but not for stimulation of PBMC proliferation.
Introduction

Staphylococcus aureus continues to be a major human pathogen responsible for superficial skin infections as well as for serious invasive infections, such as endocarditis, osteomyelitis, and sepsis (24). An important step in the initiation of invasive staphylococcal disease is adherence to host tissues and plasma proteins. S. aureus may interact with adhesive surface sites consisting of exposed or immobilized extracellular matrix (ECM) proteins such as fibronectin (Fn), fibrinogen (Fg), vitronectin (Vn), thrombospondin-1 (Tsp-1), collagen (Cn), bone sialoprotein, elastin, and several other proteins (30). Evidence from in vitro and ex vivo studies has suggested a role for these interactions in clinical disease (13,23,27,29,33,34).

S. aureus adhesins are either anchored to the cell wall via an LPXTG motif, or bind to the surface after their secretion by non-covalent interactions. S. aureus adhesins such as fibronectin-binding proteins (FnBPs), collagen adhesin (Cna) and clumping factor A (ClfA) have been well characterized on biochemical and molecular level (1,4,5,26,31). A number of secreted adhesins (Secreted Expanded Repertoire Adhesive Molecules; SERAMs), such as Eap (15-17), Emp, and Efb, play important roles in the establishment of disease. Eap shows a broad binding spectrum and at least seven plasma proteins have been found to bind to Eap (28).

The specific bacterial interaction with these adhesive proteins not only allows for adhesion and colonization of tissues, but interaction with Fn is also pivotal for invasion of non-professional phagocytes such as epithelial or endothelial cells by S. aureus (36-38). The importance of Eap in adherence of S. aureus to eukaryotic cells has been demonstrated by a decreased adherence of an eap mutant to both fibroblasts and epithelial cells. Addition of exogenous Eap increases adherence of both wild-type and an eap-negative mutant to fibroblasts. Anti-Eap antibodies significantly decrease adherence to and invasion of epithelial cells and fibroblasts (9,17,21). This scenario is important if fibronectin-binding proteins (FnBPs) which are major S. aureus invasins are absent, as in strain Newman, or produced only at a low level.
Binding of Eap to ICAM-1 inhibits leukocyte adhesion to endothelial cells and as a result prevents leukocyte extravasation (3). Eap also inhibits neutrophil recruitment during peritonitis, suggesting that Eap may be functioning as an anti-inflammatory agent (3,8). Therefore, the effects of blocking ICAM-1 interactions with leukocytes and the inhibition of neutrophil recruitment and the resulting dampening of the immune response may be important factors that determine the outcome of *S. aureus* infection. Hence, Eap is a critical factor in *S. aureus* adhesion and for development of infection. Adherence of *S. aureus* to matrix suprastructures via Eap can be supported by inflammatory reactions (12). Eap binding to extracellular matrix ligands is promiscuous at the molecular level but not indiscriminate with respect to supramolecular structures containing the same macromolecules (12).

It has been suggested that the combined anti-inflammatory and anti-angiogenic properties of Eap render this bacterial protein not only an important virulence factor during *S. aureus* infection but opens new perspectives for therapeutic applications in pathological neo-vascularization (40). Eap has been shown to block metastasis formation *in vivo* and Eap-derived agents may represent an attractive novel treatment for the prevention of breast cancer bone metastasis (35). Furthermore, Eap has been indicated as an attractive treatment for autoimmune neuro-inflammatory disorders such as multiple sclerosis (41).

PCR analysis detected the *eap* gene in all of 597 *S. aureus* isolates tested but not in *S. epidermidis* isolates or other Gram-positive cocci (n=216) (15). Based on PCR amplification, three different analogous genes of 1.8, 2.0 and 2.4 kb have been identified, consisting of five, six and seven tandem repeat domains of 93-110 amino acid (16). However, on protein level only two analogues of 65 and 72 kDa have been identified, consisting of five and six tandem repeat domains. Eap analogues consisting of six tandem repeat domains from strain FDA574 (20) and strain Wood 46 have also been designated as Map and p70, respectively (19). Recently, three further homologues have been described in strain Mu50, comprising four Eap tandem repeat domains (50 kDa), and one Eap tandem repeat domain, only. Based on this homologue, the three-dimensional
crystal structures of three different Eap domains have been resolved. Eap domains showed homology with C-terminal domains of bacterial superantigen SEC. Examination of the crystal structure of the superantigen SEC bound to TCR β–chain suggests a potential ligand binding site within Eap (6). However, Eap does not block major histo-compatibility complex-T cell receptor interactions and is not a super-antigen. Instead, it has nonspecific cross-linking activity that is dependent upon having at least two of its six 110-amino-acid tandem repeat domains (25). The structure of Eap in solution has been revealed recently, and has shown that Eap adopts an elongated conformation in aqueous solution (11).

The purpose of this study was to investigate binding of Eap and individual tandem repeat domains to Fn, Fg, Vn, Tsp-1, and Cn I, using surface plasmon resonance and ligand overlay assays. A further aim was to compare full length Eap and individual tandem repeat domains with regard to known functions, such as agglutination of staphylococci, and interactions with host cells.
Materials and Methods

**Bacterial strains and media.** *S. aureus* strains include Newman (kindly provided by T. Foster, Dublin, Ireland), clinical isolate 7 (CI7) (16) and Wood 46 (ATCC 10832). *E. coli* TG1 was used to express recombinant Eap and tandem repeat domains of Eap. For cultivation of staphylococci, TSB (tryptic soy broth; Difco, Detroit, USA) or agar; BHI (brain heart infusion; Merck, Darmstadt, Germany) broth or agar; MH (Muller Hinton; Mast, Merseyside, UK) broth or agar and LB (Luria-Bertani; Difco) broth or agar were used, as appropriate. For cultivation of *E. coli* LB broth or agar was used.

**Solubilization of staphylococcal cell surface proteins.** To prepare cell surface proteins, staphylococci were grown in 5 ml BHI broth at 37°C for 18 h, then centrifuged at 10,000 x g for 2 min. The pellet was resuspended in extraction buffer (125 mM Tris/HCl pH 7.0 + 2% sodium dodecyl sulfate [SDS, Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany]), heated at 95°C for 3 min, then centrifuged at 10,000 x g for 3 min. The supernatant was passed through a Nap-10 column (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany) containing Sephadex G-25 to remove SDS. The eluate was stored at –20°C.

**SDS-PAGE and ligand overlay analysis.** To 20 µl of cell surface extract, 5 µl of 5x sample buffer (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue [Merck]) was added, heated at 95°C for 3 min, then separated in a SDS-PAGE minigel. For Western ligand blot analysis, proteins separated by SDS-PAGE were electrophoretically transferred (Trans-blot SD, Bio-Rad, Munich, Germany) onto a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany), then the membrane was blocked with 3% BSA (fraction V, Sigma). For probing blocked blots, either Fn (Chemicon, Temecula CA, USA), Fg (Calbiochem, San Diego CA, USA), collagen type I (Cn I) (Sigma; Sigma product #7774) or Vn purified by the method of Yatohgo et al. (42) and thrombospondin 1 (Tsp-1, gift from Beate E. Kehrel, Department of Anaesthesiology and Intensive Care, Experimental and Clinical Haemostasis, University Hospital of Muenster, Germany) were used. Fn, Fg, Cn, Vn, and Tsp-
I were labeled with biotin according to instructions of the supplier (Roche, Mannheim, Germany). Blotted proteins on nitrocellulose were exposed with biotinylated ligands and subsequently detected using an avidin alkaline phosphatase color reaction (Bio-Rad). Alternatively, Fn, Vn, Fg Tsp-1, and Cn I were labeled with DIG (digoxigenin-3-O-methyl-carbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester, Roche), blotted S. aureus surface proteins were incubated with DIG-labeled ligands, and blots were subsequently exposed to anti-DIG-antibodies (Roche) and developed with a color reaction (Roche).

Cloning, expression and purification of recombinant proteins and rEap tandem repeat domains. Eap-N, Eap-7, and Eap-W (originating from strains Newman, clinical isolate 7, and Wood, respectively), all lacking the signal peptide, were expressed and purified as described earlier (16). Five domains of eap (eap1-5) of S. aureus clinical isolate 7 were amplified by PCR with a set of primers (Tab. 1). The PCR products were ligated into the plasmid pQ30-UA (Qiagen, Hilden, Germany). The ligation mixture was transformed into freshly prepared competent cells of E. coli TG1 and the transformation mixture was plated on LB plates containing ampicillin. Representative plasmids containing the eap fragments were designated as pQeap1-5. 6x-His-tagged rEap fusion proteins were expressed and purified according to the protocol provided by the manufacturer (Qiagen). The expression of His-tagged recombinant Eap using vector pQE30 in E. coli M15 allows single-step purification using Ni-nitrilotriacetic acid affinity resin. Strain E. coli with plasmid was grown in Luria-Broth containing ampicillin (100 µg/ml) overnight with shaking at 37 °C. One liter LB medium with ampicillin was inoculated with 50 ml of overnight culture. The culture was grown at 37 °C to an OD₆₀₀ of 0.5. IPTG (final concentration 1 mM) was added and the culture was incubated for 4h at 37 °C with shaking. The culture was centrifuged and the pellet was resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM Imidazol) pH8, then 1 mg/ml lysozyme was added to lyse the cell wall. After incubation for 1 h in cold, RNAse 4 U/ml and DNAse 24 U/ml in 1 mM MgCl₂ were added, and incubated for 30 min. The bacterial lysate (after centrifugation to remove cellular debris) was run through a Ni-nitrilotriacetic (Ni-NTA) (Qiagen) to
purify the 6xHis-tagged proteins. Bound proteins were eluted with elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl and 300 mM Imidazole) pH8. The eluted proteins were dialyzed against PBS overnight, and analyzed by SDS-PAGE.

**Biologic interaction analysis (BIA).** Surface plasmon resonance measurements were performed using the BIAcore® 2000 instrument from Biacore AB (Uppsala, Sweden). Sensor chip C1 (research grade), amine coupling kit, surfactant P20, sample tubes and caps were also obtained from Biacore AB. Immobilization of proteins and analysis of the interaction was carried out with an automatic method in BIAcore® 2000. The protein Eap was covalently coupled to sensor chip C1 via primary amine groups. After activation of the carboxylated matrix of C1 sensor chip with a single injection of 50 µl of 0.1 M N-hydroxysuccinimide / 0.4 M N-ethyl-N’-(3-dimethylaminopropyl)-carbodiimide (NHS/EDC), 100 µl Eap (Eap was 1:10 diluted in 10 mM NaAc pH 4.5) were injected over the activated surface. Excess activated esters were blocked by injection of 55 µl 1 M ethanolamine pH 8.5. The immobilized amount of Eap was in the range of 92 pg - 166 pg. Binding experiments were performed at 25°C in buffer of pH 7.4 containing 10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P-20. Sequential injection of Fn, Fg, Vn, Tsp-1, and Cn I allowed for determination of the respective binding kinetics to Eap. The sensor chip was regenerated between each run with a pulse of 100 mM NaOH. The association and dissociation rate constants, $k_{on}$ and $k_{off}$, were analyzed using BIA-evaluation 3.1 software from Biacore AB as described.

**Agglutination of bacteria by Eap or rEap tandem repeat domains.** *S. aureus* Newman was grown in LB broth overnight at 37°C. The bacteria were washed and suspended in PBS. A 40µl bacterial suspension containing $2.3 \times 10^9$ CFU/ml was placed on glass slides together with Eap or tandem repeat domains of Eap at final concentrations ranging from 0 µg/ml to 150 µg/ml. Agglutination was visible within 15 to 20 min at room temperature and was scored as follows: - no agglutination; + weak agglutination and ++ strong agglutination.
Adherence and internalization of *S. aureus* strain Newman to fibroblast and endothelial cells in the presence of Eap or rEap tandem repeat domains. Fibroblasts (human fetal lung) cell were cultured in Dulbecco’s modified Eagle’s medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone), hepes buffer, α-glutamine, penicillin (100U/ml), sodium pyruvate, glucose and pyridoxine. Human aortic endothelial cells (HAECs; Clonetics, Walkersville, MD) were cultured using EBM-2 medium supplemented according to the supplier (Clonetics). The cells were seeded (4 x 10⁴ cells/ml) in 24 well culture plates (Costar) and incubated at 37°C under 5% CO₂. The following standard procedure was followed. Upon reaching confluency, the cells were washed with the standard medium (Eagles medium without supplements), and 900 µl of the standard medium was added to the cells. A mixture of 50 µl of strain Newman (2.3 x 10⁹ CFU/ml) and 50 µl of Eap tandem repeat domains or full-length Eap protein (30 µg/ml final concentration) was pre-incubated for 30 min at 37°C. Bacteria and PBS were used as control. The mixture was then added to the cells in the wells and incubated for 2 h at 37°C. After incubation wells were washed 3 times with PBS to remove non adherent cells. A 200 µl volume of 10% trypsin was added to the wells to detach the cells, which were subsequently lysed by the addition of 800 µl of sterile water. Bacteria were then serially diluted and plated onto blood agar plates to determine viable counts. For the internalization assay, lysostaphin (final concentration 20 µg/ml) was added for 20 min to kill extracellular bacteria before the trypsin step was performed.

**Preparation of peripheral blood mononuclear cells (PBMC) and proliferation assay.** PBMC were isolated from heparinized blood of healthy donors by Ficoll-Hypaque gradient centrifugation. The PBMC were cultured in RPMI 1640 medium supplemented with 25 mM Hepes, 4mM L-glutamine, 100 U/ml penicillin/streptomycin, and 5% heat-inactivated fetal calf serum (FCS). PBMC (2x10⁶ cells/ml) were cultured for 72h after which they were pulsed for 6h with 1 µCi per well of [³H]-thymidine (specific activity, 5.0 Ci/mmol; Amersham Pharmacia Biotech, UK). Phytohemmagglutinin-L (PHA) (Sigma, St. Louis, MO) was used as a positive control for stimulation at a concentration of 2 µg/ml. All samples were assayed in triplicates, and the data are presented as
counts per minute (cpm). The experiments were performed twice using cells from different individuals. For stimulation of proliferation, PBMC were cultured with Eap or Eap tandem repeat domains at concentrations ranging from 0 to 81 µg/ml (final concentration). After 72h, cells were pulsed for 6h with 1µCi per well of [³H]-thymidine (specific activity, 5.0 Ci/mmol).

Statistical method: Unpaired two sided Student’s t test was used, with a threshold of statistical significance assumed at p < 0.05 and p < 0.01 (labeled by * and **, respectively).
Results

Interaction of rEap and rEap tandem repeat domains with extracellular matrix ligands.

(i) rEap. Coomassie Blue-stained SDS-PAGE of recombinant Eap-N Eap-7, Eap-W (originating from strains Newman, CI-7, and Wood 46, respectively), revealed proteins of similar size compared to the Eap analogues extracted with 2% SDS from the respective S. aureus strains Newman, CI-7 and Wood 46, respectively. In ligand overlay assays the recombinant proteins also showed binding to biotin-labelled Fn, Fg, Vn, Tsp-1, and Cn I (data not shown). The specific interactions between rEap and either Fn, Fg, Vn, and Tsp-1 was evaluated by using surface plasmon resonance (BIAcore®). For this purpose, recombinant Eap (rEap) isolated from E. coli was attached to a C1 sensor chip, a solution containing either Fn, Fg, Vn, and Tsp-1, respectively was perfused over the surface, and the interaction was analysed (Fig. 1). All four ligands interacted with immobilized rEap-N; however, the affinity of ligand interaction varied greatly. For Fg, the mean Kd value was 173 nM. For Vn, (assuming a mean molecular mass of the native [i.e., polymeric] Vn molecule of 1.200 kDa) the Kd value was 3.2 nM. The Kd value for Tsp-1 was 0.36 nM. rEap-W expressed in E. coli interacted with Fg, Tsp-1 and Vn in surface plasmon resonance experiments at a Kd of 2.36 nM, 0.504 nM, and 2.98 nM, respectively. There was a specific binding of Fn to immobilized Eap-N and Eap-W. However, a quantitative evaluation of this binding was not possible due to the fact that no available model in the BIAevaluation software fitted the obtained curves well enough. The Kd values suggest that Eap-W binds stronger to Fg and Vn than Eap-N. Eap-N and Eap-W did not show binding to bovine serum albumin, used as a control protein.

(ii) Characterization of rEap tandem repeat domains. Five tandem repeat domains of rEap of S. aureus strain CI-7 were expressed as 6xHis-tagged recombinant protein and each tandem repeat domain was purified in a single step on Ni-NTA resin. Coomassie Blue-stained SDS-PAGE confirmed purity (Fig. 2) and the expected deduced sizes of each tandem repeat domain (Tab. 1). The five tandem repeat domains also showed binding to biotin-labelled Fn, Fg, Vn, Tsp-1, and Cn I (Fig. 2). Tandem repeat domains applied in equal amounts on a gel (SDS-PAGE) stained alike with
Coomasie Blue. Tandem repeat domain 5 (D5) showed weaker binding with all tested ligands, whereas domain D3 showed strong binding with Fg, Tsp-1, and Cn I. Tandem repeat domain 1 (D1) exhibited strong binding of Fn and Vn but weak binding of Fg, Tsp-1 and Cn I. Results of interaction of all 5 tandem repeat domains of Eap7 with Fn, and Fg in surface plasmon resonance is summarized in Tab. 2. Tandem repeat domains D1 to D3 showed binding to Fg in increasing orders with $K_d$ values of 61.9 nM, 27 nM, and 6.78 nM for D1, D2, and D3, respectively. Tandem repeat domains D4 and D5 did not show binding to Fg. Binding of D1, D2, and D3 to Fn was specific but a quantitative evaluation of this binding was not possible due to the fact that no appropriate model in the BIAevaluation software was available, similar to the full length proteins. D4 did not bind to Fn, and Fn binding of D5 was inconclusive, and thus recorded as absent. However, D4 showed binding to biotinylated Fn and Fg, and weak binding of D5 to Fn and Fg corresponded to ligand overlay assays.

**Effect of Eap and rEap tandem repeat domains on agglutination, adherence and invasion of host cells, and proliferation of human leukocytes.**

In order to determine the minimum number of tandem repeat domains required for interaction of staphylococci and host cells, we examined three properties known to be at least partially mediated by Eap, namely staphylococcal agglutination, as well as adherence to and invasion of host cells.

(i) Agglutination. The ability of Eap to re-bind to *S. aureus* has been shown to cause bacterial aggregation (28). To investigate the effect of individual tandem repeat domains on agglutination of intact staphylococci, *S. aureus* strain Newman was incubated with Eap or rEap tandem repeat domains at various concentrations (0-150 µg/ml). *E. coli* was used as a negative control (data not shown). Control experiments performed with nEap and rEap did not show differences between the two preparations (data not shown). The addition of Eap, but not the addition of individual Eap tandem repeat domains, promoted agglutination of strain Newman. Agglutination by full-length Eap was observed starting from 20 µg/ml and was maximal at the highest concentration used (150 µg/ml). By contrast, no agglutination was visible for any of the single tandem repeat domains,
regardless of the concentration used (up to 150 µg/ml). The two-domain constructs D1-2 (tandem repeat domains 1 and 2) and D3-4 (tandem repeat domains 3 and 4) result in weak agglutination of strain Newman. The three domain construct D1-3 (tandem repeat domains 1, 2 and 3) causes agglutination of strain Newman (approx. 70 % of full length Eap). Thus, two domain constructs had a weak effect on agglutination, whereas the three domain construct led to a stronger agglutination, which was still surpassed by full length Eap.

(ii). Adherence and invasion. Externally added Eap can enhance the binding and internalization of S. aureus Newman into fibroblasts and epithelial cells (9,17). The role of Eap tandem repeat domains on fibroblast and endothelial cell adherence and invasion by S. aureus was examined. A confluent layer of human fibroblasts was inoculated with strain Newman after Eap or Eap tandem repeat domains had been added and incubated at 37°C for 2 hours. As expected, Eap enhanced S. aureus binding to (p<0.05; Fig. 3) and invasion of (p<0.05; Fig. 4) human fibroblasts and human aortic endothelial cells significantly. The presence of the monomeric Eap tandem repeat domains had no effect on either the binding to or invasion of fibroblasts by S. aureus. To determine if invasion is dependent on the number of tandem repeat domains, a confluent layer of human aortic endothelial cells was incubated with tandem repeat domains D1-3 (in cis), consisting of the first three domains of Eap, native Eap, and rEap domain monomers, respectively. Invasion of S. aureus in the presence of D1-3 was increased significantly when compared with control wells (endothelial cells in the presence of strain Newman but without any exogenous protein added) (Fig. 4B; p <0.05 versus controls).

We tested adherence of strain Newman to EA.hy 926 cells in the presence of single tandem repeat domains D1 to D5, D3-4, D1-3, and full-length Eap. Adherence of strain Newman to EA.hy 926 cells in the presence of single tandem repeat domains was only moderately enhanced (up to 10% of full length Eap). By contrast, the presence of construct D3-4 and D1-3 caused a substantial increase in adherence (up to 57% of full length Eap) (Fig. 3B).

(iii) Proliferation of human PBMCs. To assess the effect of Eap on human immune cells, PBMCs were cultured for 72 h in the presence of different concentrations of Eap or rEap tandem repeat
domains (0-81 µg/ml). As in a previous study (10), Eap showed a stimulatory effect at concentrations of 0-9 µg/ml and an inhibitory effect at higher concentrations, with regard to PBMC proliferation. Single tandem repeat domains D1 to D3 and D5 had a stimulatory effect at concentrations of 9-27 µg/ml (Fig. 5A). D1-3 (in cis) displayed a similar dose profile as full length native Eap (Fig. 5B).
Discussion

In this study, we have shown that single tandem repeat domains of Eap have different binding characteristics to a number of soluble host ligands, despite a relatively high sequence similarity. Two different methods, ligand overlay assays and surface plasmon resonance (SPR; BIAcore®) analysis yielded compatible results for most of the ligand-domain pairs tested. However, for D4 the interaction with Fn and Fg had to be interpreted as absent, whereas both ligands showed strong binding in ligand overlay assays. Inversely, D1 displayed only very weak binding of Fg in ligand overlay assays, whereas SPR indicated a moderate binding. Whereas agglutination of staphylococci, as well as adherence to and invasion of host cells, required more than one Eap tandem repeat domain, modulation of PBMC proliferation was possible with single Eap tandem repeat domains.

Eap has a broad binding spectrum and at least seven plasma proteins have been found to bind Eap (28). Results of the surface plasmon resonance study clearly indicate the specific nature of binding of Eap to most of the tested ligands. Eap did not show binding to BSA used as a control, and shows different affinities for different ligands. Therefore, the previous view that Eap can interact with almost every host ligand, due to the net positive charge, is no longer supported in the light of these data.

Eap genes from different strains revealed a high degree of overall similarity (74 to 96 %) at the nucleotide level (16). S. aureus may be able to produce shorter or longer forms of Eap depending on a point mutation in an adenine rich region of the eap gene (poly-A-stretch), which may cause premature translational termination. The PCR products of strains Newman D2C and Wood 46 were 2,056 and 2,364 bp resulting in predicted mature proteins of 77 and 85.55 kDa with six and seven tandem repeat domains, respectively. However, proteins of 65.5 and 74 kDa for Newman D2C and Wood 46 were observed instead, with five and six tandem repeat domains, respectively. The reason for this is a stop codon preceded by nine adenine bases (starting at nucleotide 1740 in Newman D2C and nucleotide 2049 in Wood 46). Such phase variation for Eap,
which has been described for several strains (2), might enable \textit{S. aureus} to differentially modulate the host immune system (16,22).

The Eap structure has been solved with single domain constructs (6). It has been shown that four domain constructs of Eap adopt an elongated conformation in solution, a situation in which the single domains appear to be only connected by the linker region between the tandem repeat domains (11). Most of the identified Eap homologues comprise 5 or 6 tandem repeat domains of 93-110 amino acids. In Eap-7, tandem repeat domain 1 exhibited the highest alignment score to other tandem repeat domains, and tandem repeat domain 5 showed a lower alignment score to the other four tandem repeat domains. The intra-Eap alignment score between D1 to D4 in Eap-7 ranged from 45%-73%. Surprisingly, D5 of Eap-7 did not show binding to Fn and Fg. This may be explained by the fact that D5 showed very little homology with the other four tandem repeat domains: an intra-Eap alignment score of D5 with D1 to D4 was 29%, 31%, 23% and 29%, respectively. This low homology explains the inability of D5 to bind to Fn and Fg in BIAcore® studies and the weak reactivity in ligand overlay assays. D3 of Eap-7 reacted strongly with Fg in ligand overlay assays and also showed the strongest binding to Fn and Fg in BIAcore® studies. These data suggest that the binding specificity for different soluble ligands is apparently not necessarily based on recognition by linear Eap epitopes, as presented e.g. by blotted Eap in ligand overlay assays. This difference might explain divergent results for SPR and ligand overlay assays, observed for a minority of the tandem repeat domains.

Eap is able to form oligomers and this direct Eap–Eap interactions cause bacterial aggregation due to the surface association of Eap (28). In the present study the full length Eap caused agglutination of bacteria but none of the rEap tandem repeat domain monomers (D1 to D5) were able to enhance agglutination of \textit{S. aureus}.

\textit{S. aureus} possesses the ability to adhere to and invade non-professional phagocytes (32,36,37,39). Previous studies by us (17) and others (21) showed a role of Eap in adherence of \textit{S. aureus} to eukaryotic cells. In these studies an \textit{eap}-negative mutant adhered less well to both
fibroblasts and epithelial cells. Addition of exogenous Eap increased the adherence of both the wild-type and the eap-negative mutant to fibroblasts (17). In the present study full length Eap but none of the rEap tandem repeat domain monomers (D1 to D5) were able to enhance adherence of \textit{S. aureus} to fibroblasts, demonstrating the need for at least two tandem repeat domains of Eap for host cell interaction with \textit{S. aureus}. We reported earlier that Eap plays an important role in the internalization of \textit{S. aureus} strain Newman (9). Addition of exogenous Eap increased the internalization of both the parent strain and the mutant strain by fibroblasts and addition of antibodies against Eap blocked this effect. Strain Newman is defective in FnBPs (7) resulting in poor invasiveness. In order to dissect the role of Eap in defined functions, strain Newman may be the best choice, due to this reason. In addition, it is \textit{cna}-negative and thus the role of Eap in adherence and invasion is easier to identify. The most straightforward approach would be to use heterologous expression, e.g. in \textit{S. carnosus}. Unfortunately, this has not been successful to date for any group working in this area, since \textit{S. carnosus}, unlike \textit{S. aureus}, appears not to be able to bind Eap on its surface. Hence, that use of strain Newman in this context is currently probably one of the best options available. This approach has been chosen in most of the studies regarding Eap. Additionally, we have studied invasion for different clinical strains and found a great variation between strains. Two selected strains have been further tested. Both, a weakly (L12) and a highly (U35) invasive strain could be enhanced by addition of external Eap (9).

Full length Eap but none of the rEap tandem repeat domain monomers (D1 to D5) were able to enhance invasion of fibroblasts by \textit{S. aureus}. On the other hand, D1-3 consisting of the first three tandem repeat domains of Eap as one polypeptide (\textit{in cis}) enhanced invasion of endothelial cells by \textit{S. aureus} strain Newman significantly. This is in accordance with the observation that Eap has a nonspecific cross-linking activity that is dependent upon having at least two of its six 110-amino-acid tandem repeat domains for blocking MHC-T-cell receptor interactions (25).

An Eap analogue designated p70 from \textit{S. aureus} Wood 46 is capable of inducing a time and dose-dependent increase in IgM and IgG synthesis in peripheral blood mononuclear cells (PBMC).
PBMCs stimulated with Eap display increased IL-4 (interleukin 4) synthesis (18). It is interesting to speculate that activation of IL-4 by Eap can modulate the immune response to S. aureus infection by interfering with the interactions of activated T cells and MHC class II-bearing antigen-presenting cells (APCs). However, the effect of IL-4 on the interactions between activated T cells and APCs is most likely additional to the effect of blockage of ICAM-1 interactions by Eap (3). We have shown that the proliferation of PBMCs had the same dose profile with single Eap tandem repeat domains as for the whole Eap protein; i.e. at low concentration, proliferation of PBMCs was stimulated whereas high concentrations inhibited PBMC proliferation.

For S. aureus aggregation, adherence and invasion, multiple binding sites are required on the protein. On the other hand, monomeric tandem repeat domains of Eap had the same effect on PBMC proliferation as full length Eap. Eap has a structural homology with the C-terminal domain of bacterial superantigens (toxins such as TSST-1, SEA, and SEB), both from S. aureus and SPE-C from Streptococcus pyogenes (6). However, Eap does not act as a superantigen (25). Nevertheless, TSST-1 has a similar activity profile as the one we present here with Eap tandem repeat domains: at low concentrations, TSST-1, like Eap, stimulates PBMC from normal subjects and at high concentrations TSST-1 induces B-cell apoptosis (10,14).

Taken together, our data indicate that at least some of the biological diverse functions of Eap have different structural requirements. Thus, the superficially non-discriminate appearance of Eap with regard to its broad spectrum of activities starts to give ways to a more differentiated view of different functions. This is in accordance with another surprisingly specific recognition of monomeric vs. aggregated Cn I, which we have shown earlier (12). In addition, it is tempting to speculate that for both TSST-1 and Eap, the effect elicited by them on PBMCs is due to the structural similarity that these proteins share with each other, although any other similarity between Eap and super-antigens remains to be demonstrated.
Acknowledgements

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References


Fig. 1. Sensorgrams (surface plasmon resonance, BIAcore® analysis) showing binding of Fn, Fg, Vn, and Tsp-1 to and dissociation from Eap-N. Eap (160 pg) was immobilized on sensor chip C1 as described in Materials and Methods. Sensor chip surfaces were subsequently exposed with various concentrations of purified extracellular matrix proteins, and surface plasmon resonance was determined as described in the text. Mean values of the rate constants and of the equilibrium dissociation constant are given below the respective sensorgrams. The following conditions and concentrations were used (bottom to top sensorgrams of each panel, respectively): Fn at 1476, 1362, 1135, 1022, 908, 815, 558, 341, 227, 114, nM; Fg at 590, 1,180, 1,770, 2,360, 2,950, 3,540, 4,130, 5,020 nM; Vn at 47.7, 49.5, 57.5, 66, 91.6, 100, 125 and 150 nM; and Tsp-1 at 17.78, 22.22, 26.67, 35.5, 31.11, 44.44, 53.33, 71.11, 88.89 nM.

Fig. 2. Analysis of rEap tandem repeat domains. (A) Schematic representation of the domain constructs used in this study. (B) Constructs were expressed using vector pQE30UA and purification was achieved in a single step on Ni-NTA resin. Shown are Coomassie Blue-stained SDS-PAGE (upper left) and ligand overlay assay membranes, as indicated. For ligand overlay assays, nitrocellulose membranes with blotted proteins were probed with biotinylated Fn, Fg, Vn, Tsp-1, and Cn I, respectively, and protein-protein interaction was detected with avidin in an enzymatic color reaction as detailed in Methods and Materials.

Fig. 3. Adherence of strain Newman to human fibroblasts and endothelial cells. (A) Confluent layers of fibroblasts were inoculated with strain Newman without addition of protein (control wells) or strain Newman supplemented with either Eap protein or rEap tandem repeat domains and incubated for 2 h. Bacteria were detached, and the viable count was determined. Results are expressed as means, error bars show standard deviations (n=3; *, p <0.05 versus control). (B) Microtiter plate wells were coated with endothelial cells and subsequently blocked with 1% BSA. Bacteria were grown in BHI broth overnight, washed with PBS and added to microtiter wells in the
presence of Eap constructs. After 1 h, wells were washed, anti-staphylococcal antibody added, followed by AP-conjugated goat anti-rabbit antibody was added. AP color substrate was used, and optical density was determined at 405 nm.

![Fig. 4. Invasion of human fibroblasts and human endothelial cells by strain Newman.](http://iai.asm.org/)

Confluent layers of fibroblasts (Fig. 4A) and endothelial cells (Fig. 4B) were inoculated with strain Newman without addition of protein (control well) or strain Newman supplemented with either Eap protein or Eap tandem repeat domains and incubated for 2 h. Wells were further incubated with lysostaphin to kill extracellular bacteria. Host cells were lysed, and the viable count was determined. Data are presented as mean CFU of three and two experiments in Fig. 4A and Fig. 4B, respectively. Error bars show standard deviations (*: p<0.05; **: p <0.01 versus control).

![Fig. 5. Effect of Eap full length protein and Eap domains on peripheral blood mononuclear cells (PBMCs).](http://iai.asm.org/)

PBMCs were stimulated with indicated concentrations of Eap or Eap tandem repeat domains for 72h (A) and Eap or D1-3 in cis (B). PBMCs without addition of any exogenous protein but culture medium served as negative controls. Data are presented as mean counts per minute of triplicate determinations. One representative experiment out of two is presented in Fig. 5A and B, respectively.
Table 1. Primers used in this study to generate rEap tandem repeat domains.

<table>
<thead>
<tr>
<th>Tandem repeat domain</th>
<th>Primers: position in \textit{eap7}*</th>
<th>Sequence</th>
<th>No. of aa / molecular mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>F1: 133-159, R1: 460-480</td>
<td>GGA TAT TCT AAA ATA CAG ATT CCA TAT TTG AAC ATT TGC TTT TGC CTC</td>
<td>116 / 13017</td>
</tr>
<tr>
<td>D2</td>
<td>F2: 469-494, R2: 774-798</td>
<td>GCA AAT GTT CAA GTG CCG TAT ACA AT TTT TGC TTC TTT ATC TTT CGC TTG C</td>
<td>110 / 12188</td>
</tr>
<tr>
<td>D3</td>
<td>F3: 798-822, R3: 1087-1114</td>
<td>GTA AATAAT CAA GTG CCA TAT TCA TTT TAC TTT CGA AAC TGT TTT TAC AGT</td>
<td>105 / 11912</td>
</tr>
<tr>
<td>D4</td>
<td>F4: 1114-1137, R4: 1411-1437</td>
<td>GCG GAG CGT TAT GTA CCA TAT ACA A AAG CGC TTT ATT AGT TTT AGT GTG TTG</td>
<td>108 / 12243</td>
</tr>
<tr>
<td>D5</td>
<td>F5: 1458-1482, R5: 1732-1755</td>
<td>ACT AAA GTG AAG TTT CCA GTA ACG TTT AAA TTT AAT TTC AAT GTC TAC</td>
<td>98 / 11031</td>
</tr>
</tbody>
</table>

\* = EMBL accession number AJ243790
Table 2. Summary of binding characteristics of fibronectin (Fn) and fibrinogen (Fg) to the recombinant tandem repeat domains D1 to D5

<table>
<thead>
<tr>
<th>Binding partner/Net Charge</th>
<th>D1 (immobilized amount: 40 pg)</th>
<th>D2 (immobilized amount: 75 pg)</th>
<th>D3 (immobilized amount: 219 pg)</th>
<th>D4 (immobilized amount: 93 pg)</th>
<th>D5 (immobilized amount: 91 pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fn*</td>
<td>Yes*</td>
<td>Yes*</td>
<td>Yes*</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Fg</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>K&lt;sub&gt;D&lt;/sub&gt; = 61.9 nM</td>
<td>K&lt;sub&gt;D&lt;/sub&gt; = 27 nM</td>
<td>K&lt;sub&gt;D&lt;/sub&gt; = 6.78 nM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Net charge KR-ED§ (%)</td>
<td>8 (7.4)</td>
<td>12 (11.1)</td>
<td>14 (13.6)</td>
<td>11 (10)</td>
<td>5 (5.2)</td>
</tr>
</tbody>
</table>

*No model available to fit the data: Fn shows the strongest binding to peptide D3. Fn with a concentration of 454 nM gives a signal of approximately 22 RU during binding to D1 (immobilized amount: 40 pg), and approximately 37 RU during binding to D2 (immobilized amount: 75 pg), however, Fn with a concentration of 477 nM gives a signal of approximately 280 RU during binding to D3 (immobilized amount: 219 pg).

§KR: lysine and arginine; ED: glutamic acid and aspartic acid.
Fig. 1
**Fig. 4A**

![Bar graph for CFU (x10^4)](image)

- Control
- Eap
- D1
- D2
- D3
- D4
- D5

**Fig. 4B**

![Bar graph for CFU (x10^5)](image)

- Control
- Eap
- D1
- D2
- D3
- D4
- D5
- D1-3

**Note:**

- CFU (x10^4)
- CFU (x10^5)
- Data points represent mean ± standard error of the mean (SEM)