The extracellular fibrinogen-binding protein (Efb) from *S. aureus* binds divalently to fibrinogen and gives rise to a specific antibody response

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Abstract

Staphylococcus aureus is an important human and animal pathogen that causes a wide range of infections. These infections can be very serious and sometimes hard to get rid of, because of the many virulence factors the bacteria produce during infections.

This project was a research of the extracellular fibrinogen-binding protein, Efb, which is a 15.9 kDa protein that has been shown to be an important virulence factor during *S. aureus* infections.

The purpose with the project was to find out if the protein has more than one binding site to fibrinogen and if people produce antibodies against Efb.

This was performed with methods such as affinity chromatography, ELISA, coagulation test and western blot. It was shown that Efb has two binding sites to fibrinogen. One is placed on the C-terminal part of Efb and the other on the N-terminal. It was also shown that the production of antibodies against Efb rises significantly in people during an ongoing infection.

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1. Introduction

General background

Staphylococcus aureus is the leading cause of human and animal wound infections. There is a broad spectrum of diseases that can result from *S. aureus* infections. The most common ones are harmless like skin infections and abscesses. But in some cases the infections can develop into severe life-threatening diseases, such as endocarditis, osteomyelitis and septicaemia [1].

S. aureus is one of the most common causes of infections in the industrialized world and the development of antibiotic-resistant *S. aureus* strains poses severe health threats to hospitals and communities.

Before the penicillin was developed staphylococcal infections often had fatal outcomes. But in 1942 when penicillin became available, the staphylococcal infections were treated successfully. Ever since then the antibiotic-resistance has increased within a very short time. In the 1950's frequent outbreaks of staphylococcal infections in hospitals were found to be associated with penicillin-resistant strains of S*taphylococcus aureus*, caused by the bacteria's production of β-lactamase (also called penicillinase), which breaks down the penicillin wall. A new antibiotic, methicillin, was developed in the 1960's to treat the increasing penicillin-resistant strains of *S. aureus*. But one year later it seemed that the bacteria had developed a methicillin-resistance (MRSA) as well [2].

Today hospital strains of *S. aureus* are often resistant to a variety of different antibiotics, many of them are resistant to all antibiotics except vancomycin, the antibiotic used to treat MRSA strains [2].

Methicillin-resistance is widespread and most MRSA strains are also multiply resistant. Infections caused by MRSA strains are associated with high rates of morbidity and mortality. Lately clinical isolates of S. aureus with reduced susceptibility and resistance to vancomycin has been identified [2].

This increasing problem makes the development of alternative treatments of *S. aureus* necessary. One alternative for treating *S. aureus* infections is to develop a vaccine against the bacteria.

Staphylococcus

Staphylococcus belongs to the family of gram-positive cocci, Micrococcaceae. Micrococcaceae consists of four genera: Planococcus, Stomatococcus, Micrococcus and Staphylococcus, of which the latter is the only genus with a medical importance.

Staphylococcus is a gram-positive, non-motile, facultative anaerobic bacterium. They have a spherical shape (0.5-1.0 µm in diameter) and grow mostly in grape-like clusters, but also in pairs, short chains and singly. This configuration helps to distinguish them from streptococci, which are also spherical cells that usually grow in chains.

Staphylococcus bacteria were first observed by Robert Koch in 1878, when he described the bacteria in human pus. The name Staphylococcus was introduced by Alexander Ogston in 1883 and is originate from the Greek word staphyle, which means "bunch of grapes". In 1884 Rosenbach described and named two different types of staphylococcus according to the colours of their colonies. *S. aureus*, because of the golden yellow colonies they produce and *S. albus*, because of their white colonies. *S. albus* were later renamed to *S. epidermidis* [3].

S.aureus is the only human infective species in the Staphylococci family that is coagulase-positive. Other coagulase-positive staphylococci species have been recognized in different mammals, *S. intermedius* in dogs, *S. hyicus* in pigs and *S. delphini* in dolphins. So far 13 coagulase-negative staphylococci and 1 coagulase-positive have been discovered in humans. The 13 coagulase-negative staphylococci are grouped under that very name, coagulase-negatives [2].

Staphylococcus aureus

Staphylococcus aureus are a part of the human normal flora. Many people are colonized with *S. aureus*, about 20% of the population are carriers of the bacteria [1]. This means that serum from healthy people colonized with *S. aureus* is found to be consisting of antibodies against S*taphylococcus aureus*.

The bacteria are found and live harmlessly on human skin, commonly inside the nose, in the armpit, the groin and genital area. But if the skin is punctured or broken the bacteria can enter the wound and cause an infection.

S. aureus is a very adaptive bacterium and it can infect almost every tissue in the body. The infections caused by the bacteria are most commonly skin infections, like boils, impetigo, and cellulitis which are limited to a small area of the skin. But if the *S. aureus* enters the blood stream it can lead to severe infections in other parts of the body such as the lungs, bones, joints, heart, blood, and central nervous system [11].

S. aureus infections are passed on via contact of the skin. To be able to cause the infection the bacteria has to bind to components of the cell surface or to the extracellular matrixes. As long as the bacterium hasn't attached it is accessible for both antibodies and antibiotic.

Virulence factors associated with *S. aureus*.

S. aureus produces a wide range of virulence factors, which all have different functions. They help the bacteria to stay alive inside the host by enable the attachment of the bacteria to host tissue, others participate in the spread of the bacteria to distal or deeper tissue [3] and some help the bacteria with the evasion of the host defences.

The pathogenesis for the majority of diseases caused by *S. aureus* is multifactorial, which makes it difficult to determine the precise role of a specific virulence factor produced by the bacteria.

The virulence factors can be divided into two major groups: Cell wall-associated proteins and Extracellular proteins.

Cell wall-associated proteins

S. aureus produces many proteins on its surface. These are usually responsible for the attachment to host tissue. Some of these proteins are, clumping factor (Clf), fibronectin-binding protein (FnBP), protein A and collagen-binding proteins.

Cumping factor (Clf) is the major cause of *S. aureus* adhesion to fibrinogen. It binds to the γ-chain on fibrinogen. The name clumping factor has its origin from the reaction between bacteria and fibrinogen, when *S. aureus* cells are suspended in plasma. The cells form macroscopic clumps (clumping) when the dimeric plasma protein, fibrinogen, binds to clumping factor on the bacterial surface [4].

Protein A is a surface protein that binds IgG molecules by their Fc region, instead of the Fab region which is most common. This wrong orientation of IgG molecules on the bacterium surface results in a prevention of opsonization and phagocytosis [3].

Fibronectin-binding proteins bind to fibronectin and enable the bacteria to enter the eucaryotic cells, which help the bacteria to survive longer in the host. This is why infections that seem to be gone suddenly arise again [9].

Collagen-binding proteins promote the attachment to collagen and they can be very useful for the bacterial attachment to disrupted endothelium where collagen has been exposed [3].

Extracellular proteins

S. aureus produce and secrete a large number of extracellular proteins. These have many different functions, for instance there are toxins like α-toxins, which are membrane-damaging toxins that are secreted during infection. If the bacteria secrete α-toxins in food they can give rise to food poisoning While the bacteria dies during heating, the toxin doesn't which results in food poisoning [10].

Other extracellular proteins are coagulase, Eap and the extracellular fibrinogen-binding protein (Efb).

Coagulase is a traditional marker for identifying *S. aureus* in the clinical microbiology laboratory. *S. aureus* is the only one in the human staphylococci family that produces the protein. Coagulase binds to prothrombin which causes a change in the prothrombin configuration and converts it into thrombin. Thrombin binds to fibrinogen and fibrinogen polymerization takes place. The fibrin threads that are produced form a protective covering around the bacteria and form an abscess, which makes it inaccessible for antibodies and antibiotic [11].

Extracellular adherence protein, Eap, has a very broad spectrum of binding interactions to host components. *S. aureus* strains that fail to produce Eap exhibit significantly reduced affinity for eucaryotic tissue and lack the ability to colonize and invade host tissue [12].

The extracellular fibrinogen-binding protein, Efb, has been studied in this project. It has several functions during infections, so far it's been established that Efb binds to fibrinogen and that it inhibit the complement cascade by binding to the important protein, complement

C3b. It has also been proofed to be a contributing factor of the delayed wound healing during *S. aureus* infections, which is a hallmark for *S. aureus* [12].

Fibrinogen

Fibrinogen is a 340kDa plasma glycoprotein that is synthesised in the liver. It is found in high concentrations (3mg/ml) in human blood plasma and plays a major role in the process of clotting blood [2].

Fibrinogen consists of six polypeptide chains which are three pairs of non-identical polypeptide chains: Aα, Bβ and γ, linked together by disulfide bridges. The molecule is arranged symmetrically into three globular domains with the E domain in the middle and two distal domains [2].

Fibrinogen is soluble in plasma, but during the process of blood clotting (see blood clotting) fibrinogen transforms into its insoluble form, fibrin [2].

Blood clotting

The ability of the body to control the flow of blood following vascular injury is important for continued survival. The process by which the body prevents blood loss is called coagulation.

Coagulation is a complex process during which a cellular system comprised of cells called platelets, which circulate in the blood, form a platelet plug over damaged blood vessels. This is followed by blood clotting, where the transformation of fibrinogen to fibrin takes place.

When a blood vessel is wounded and the endothelial cells are damaged, coagulation is initiated within 20 seconds. Platelets are activated and adhere to the collagen in the endothelium that becomes exposed following rapture of endothelial lining of vessels and forms a plug at the site of injury. When the platelets are adhered they release the contents of their granules, which activate other platelets and white blood cells. Fibrinogen works as a link between the platelets.

The next step in blood cougulation is the transformation of fibrinogen to fibrin. This is initiated by prothrombin, which transforms to its active form, thrombin, with the help of factor X. Thrombin causes a break in fibrinogen, a peptide is released and starts to polymerize by binding to other peptides released from fibrinogen, and the insoluble fibrin threads are produced. The fibrin threads form a web over the aggregated platelets, which make the plug more stable [6-7].

Disorders in coagulation can cause too much or too little clotting. This can lead to increased bleeding or thrombosis (the formation of a clot inside a vessel) or embolism (the migration of a clot or another object from one part of the body and causes a blockage in the blood vessel).

Extracellular fibrinogen-binding protein (Efb)

Efb is a 15.9 kDa fibrinogen-binding protein that is produced and secreted by *S. aureus* during infections. In previous studies the *efb* gene was found in all *S. aureus* strains tested, but was lacking in coagulase-negative species [3].

Efb consists of 136 amino acids (AA's) and contains two almost identical repeats of a 22 AA-segment in the amino (N-) terminal region. Similar repeating is found in the *S. aureus*-produced protein, coagulase, in its carboxyl (C-) terminal region. These two regions in Efb and coagulase both bind to fibrinogen [3].

It has been shown that Efb is an important virulence factor in *S. aureus* infections. It does not only bind to fibrinogen but can also, according to earlier studies, interfere with platelet aggregation and the complement cascade in the host.

Platelets are, as described on the previous page, responsible for the coagulation of blood. By binding to each other and to fibrinogen the platelets form a plug at the site of injury, platelet aggregation. Efb can inhibit this platelet aggregation via its interaction with a receptor on the platelet surface followed by a novel type of binding to fibrinogen. Efb binds fibrinogen in a way that makes it inaccessible for the platelets and the platelet aggregation process. This inhibits the formation of the platelet plug. Therefore Efb causes prolonged bleeding and delayed wound healing [13], which is a hallmark for *S. aureus* infections.

The complement system represents the most "primitive" line of defence against infectious agents. The activation of the complement cascade leads to opsonophagocytic killing of the bacteria, by the binding of the important complement component, C3b, on the bacterial surface [1].
S. aureus has the ability to produce a polysaccharide-consisting capsule around itself. This capsule appears to play a key role in the protection against complement-mediated phagocytosis. However, capsule production occurs mostly during the later stages of bacterial growth. It is suggested that the complement-inhibitory properties of Efb may serve to neutralize complement activation initiated by *S. aureus* during the early stages of infection, when capsule production has not been initiated. Efb binds to C3b with its complement-binding region, the C-terminal. This prevents the C3 activation by directly preventing C3b's binding to activator surfaces and inhibits the complement-mediated lysis and opsonophagocytosis [1].

Vaccine

There is research going on to develop a vaccine against *S. aureus*, but so far the successes have been limited.

The problem is that several vaccines have been based on whole bacteria. But since *S. aureus* pathogenesis involves a complex combination of bacterial virulence factors, a multi-component vaccine might be more effective.

The vaccine should be based antigens derived from important virulence factors produced by the bacteria during different stages of the infection.

Aim of the study

This project has been focused on the extracellular fibrinogen-binding protein, Efb. The qualities and characterisitcs of the protein have been studied.
Two questions were asked in the beginning of the project:
Do people produce antibodies against Efb during *S. aureus* infections?
Have Efb more than one binding site to fibrinogen?

2. Methods

Cultivation of *S. aureus*

S. aureus strain Newman was grown on a blood agar plate and incubated at 37 ºC over night. Bacteria colonies of *S. aureus* from the incubated blood agar plate were inoculated into four flasks of Brain Heart Infusion and put on a shaker (HT Infors AG) at 121 rpm over night at 37 ºC.

Purification of Efb

The bacterial culture, was centrifuged at 7500 rpm for 25 minutes at 4 ºC. The pellet was discarded while the supernatant was filtrated by suction. To be able to control the purification steps all the way during the process a 1 ml-sample was taken after each step.

Affinity chromatography

Affinity chromatography was performed at 4 ºC. Fibrinogen was coupled to a CNBr-activated Sepharose (GE Health care) in a column according to the manufacturer's manual.

The column was washed at 4 ml/min with both PBS and 0.7 % acetic acid with the pH 3.5. These fluids were alternating for a few cycles and finished with a PBS-wash. The Efb-containing supernatant was then circulated through the column at low speed (3 ml/min) over night.

The column was washed once with PBS. A detector, coupled to the column, registered the proteins being washed out from. When no more proteins were registered, it was assumed that only the fibrinogen binding proteins were left in the column. To eluate these proteins a 0.7 % acetic acid with the pH 3.5 was run through the column. The detector registered the proteins with a large peak, which could be assembled in a separate tube.

In the final step the column was washed with PBS + 20 % ethanol and 0.7 % acetic acid with the pH 3.5. These two fluids were alternated in the column and the cycle was ended with PBS + 20 % ethanol.

Fast protein liquid chromatography (FPLC)

Fast protein liquid chromatography (FPLC) is an ion exchange chromatography, where the proteins in the supernatant are separated depending on their charge distribution.

The supernatant was dialysed in a 0,04M phosphate buffer with pH 6.5 (A-buffer) over night. The FPLC-system was washed for 6 minutes. During which A-buffer and B-buffer, a 0.04M phosphate buffer with 1M NaCl, were alternating.

The dialysed sample was filtrated and loaded into the FPLC-system where the proteins were attached to a Mono-S-gel (Pharmacia, Uppsala) in the column. A-buffer was pumped into the system and gradually B-buffer was too, finishing with 100 % B-buffer.

The more B-buffer pumped into the system the weaker the binding of the proteins to the Mono-S-gel got, which led to a separation of the proteins. They were assembled in fractions in small tubes. Three peaks were registered by the detector and each one of them represented a different protein. By earlier experience the peaks were known to be 1. coagulase 2. Efb and 3. Eap.

Identification of Efb

Protein determination

A standard curve was prepared by mixing 200 µl dye-reagent (Bio-Rad Protein Assay) with 1 to 6 µl standard (Albumin, Bovine serum, BSA), 1 mg/ml, in six wells on a round bottomed microtiter plate (greiner bio-one).

Purified fractions from the purification process were also mixed with dye-reagent, 5 µl sample to 200 µl reagent.
At other times the samples were mixed with the dye-reagent in the same way as the standard, 1 to 6 µl sample to 200 µl dye-reagent in six wells. PBS was used as a blank, where 5 µl PBS was mixed with 200 µl reagent.

The results were analysed in a microtiter plate reader (Labsystems integrated EIA Management System) at 620 nm.

Antibody recognition of Efb and Eap

A binding of Efb and Eap to immobilised fibrinogen was performed using the methods for binding ELISA, with some modifications. All the binding ELISA's in this project were performed in 96-well microtiter plates (Costar).

Fibrinogen (Sigma Chemical Co.) at 1µg/ml was applied to the wells on a microtiter plate and incubated over night at room temperature. The plate was washed 4 times in PBS with 0.05 % Tween 20 (MERCK eurolab), PBST. This PBST-wash was repeated after every incubation time during this experiment.

Selected fractions from the purification, diluted 1/4, were applied to the wells, 100 µl/well. As positive controls, Efb-proteins at 1 µg/ml and Eap-proteins diluted 1/100 were used. There were two negative controls, one with PBS and conjugate and one with antibodies and conjugate. The plate was incubated for 1 hour at 37 ºC and then washed with PBST.

The bound samples were detected with sheep Efb-antibodies and sheep Eap-antibodies 100 µl/well, diluted 1/3000 for 1 hour at 37 ºC. This was followed by an incubation of HRP-conjugated sheep-antibodies produced in rabbit (DAKO) with 100 µl/well diluted 1/1000 for 1 hour at 37 ºC. The plate was washed with PBST after each step.

The colour was developed by applying TMB-chromogen substrate (DAKO Cytomation), 100 µl/well and the reaction was stopped with 1M HCl (+ 3M H_2SO_4), 100 µl/well. The absorbance was read at 450 nm in a microtiter plate reader (Labsystems integrated EIA Management System).

Coagulase test of purified proteins

Horse plasma, 100 µl, was incubated with 25 µl of selected fractions from the purification process, 5, 6, 11, 12, 17 and 18. Incubation with coagulase was used as a positive control and PBS as a negative control. The samples containing coagulase were coagulated after the incubation, which gave a positive result in those cases (see table 1).

SDS-PAGE and Western blot on FPLC-purified proteins

Chosen samples from the purification process were concentrated by centrifugation and evaporation followed by resolving the pellet in loading buffer, in a ratio of 1:4. A polyacrylamide gel and a stacking gel were prepared, according to manufacturer's manual. The samples were heated and loaded onto the gel with a low molecular marker and run for 45 minutes. The gel was washed in blotting buffer, a solution of tris base, glycine and methanol with the ph 8.3, and put in the transfer-cassette. The samples were transferred from the gel to a nitrocellulose membrane (BioBlot-NT Blotting Membrane) via an electroblot at 100 V with cooling for 1 hour. The membrane was washed for 3x5 minutes in washing buffer (0,1 % PBST) and blocked in blocking buffer, a 2 % BSA-solution with 0.1 % Tween 20, for 1 hour at room temperature on a shaking table. The membrane was once again washed for 3x5 minutes in washing buffer and probed with sheep Efb-antibodies, diluted 1/1000, on a wiggling table over night at room temperature. The membrane was washed 3x5 minutes and the bound antibodies were detected with HRP-conjugated sheep-antibodies produced in rabbit diluted 1/1000 for 2 hours at room temperature on a shaking table. The membrane was washed a final time for 3x5 minutes in washing buffer. The bands on the membrane were developed using 4-chloro-1-naphtol tablets (Sigma) prepared according to the manufacturer's manual.

Antibody recognition of Efb

Antibody recognition of Efb was investigated using the methods for binding ELISA (see Antibody recognition of Efb and Eap), with some modifications.

Two ELISA-plates were coated with the purified fractions 3-26. They were diluted in a threefold serie from top to bottom and incubated at room temperature over night. The plates were aftercoated, without washing them first, with 2 % BSA for 1 hour at 37 ºC. This was followed by an incubation of sheep Efb-antibodies diluted 1/3000 for 1 hour at 37 ºC. The bound antibodies were detected with HRP-conjugated anti-sheep antibodies produced in rabbit. The colour was developed by applying TMB-chromogen substrate and 1M HCl (+ 3M H_2SO_4) was added to stop the reaction. The results were read in a microtiter plate reader at 450 nm (see diagram 1).

The characteristics of Efb

A recombinant Efb (rEfb) molecule and both the amino (N-) terminal (Efb-N) and the carboxyl (C-) terminal (Efb-C) part of the Efb molecule was used in the following experiments.

The incubations that weren't incubated in room temperature were incubated at 37 ºC in the experiments.

Binding of fibrinogen to recombinant Efb

This method involves a crosstitration of immobilised rEfb and free fibrinogen. It was performed according to the methods for binding ELISA (see Antibody recognition of Efb and Eap), with some modifications.

A microtiter plate was coated with rEfb in a twofold serial dilution from 20-0.1 µg/ml, left to right and incubated over night at room temperature. The plate was blocked with 2 % BSA, without washing it first, for 1 hour at 37 ºC. This was followed by a 1 hour incubation of fibrinogen diluted in a twofold serial dilution from 10-0.08 µg/ml, top to bottom. Rabbit fibrinogen-antibodies (DAKO) diluted 1/500 were applied and the plate was incubated for 1 hour. To detect the bound fibrinogen, HRP-conjugated rabbit-antibodies produced in swine (DAKO) diluted 1/2000 were applied and incubated for 1hour. The colour was developed with TMB-chromogen substrate and the reaction was stopped with 1M HCl (+ 3M H_2SO_4). Finally the results were read in a microtiter plate reader at 490 nm.

This experiment was also performed with the coating of Efb-C and Efb-N. The molecules were diluted in a twofold serial dilution from 10-0.156 µg/ml, left to right, four rows per protein. The plate was blocked with 2 % BSA followed by incubation with four different concentrations of fibrinogen: 4, 1, 0.2 and 0 µg/ml, one concentration per row and Efb-molecule for 1 hour. Detection of the bound fibrinogen was done as described above.

Competitive inhibition

A microtiter plate coated with 1µg/ml rEfb was blocked with 2% BSA for 1 hour.

A constant concentration of fibrinogen, 2 µg/ml, was mixed with different proteins in a round-bottomed plate. The proteins were diluted in a threefold serial dilution, from 200-0.003 µg/ml, left to right, one protein per each row. The plate was incubated for 20 minutes at room temperature and 80 µl of each concentration from each protein was transferred from the round-bottomed plate to the rEfb coated microtiter plate and incubated at 37 ºC for 1 hour. Detection of fibrinogen binding was done as described above.

Coagulation test on recombinant Efb

A base solution was prepared with coagulase (from the purification process) diluted 1/10 and horse plasma, the two ingredients were mixed in a ratio of 1/10. This ratio was found during pilot experiments. Nine tubes were prepared with the base solution and rEfb (905 µg/ml), Efb-N (300 µg/ml) and Efb-C (400 µg/ml) was distributed to three tubes each as inhibitors. They were added in different volumes: 40, 10 and 0 µl (positive control), except rEfb, which was added in the volumes 20, 5 and 0 µl (positive control). The tubes were incubated at 37 ºC until the positive controls were coagulated. The results could be read by the eye (see table 2).

Binding of Efb to fibrinogen

This experiment was performed by using the methods for binding ELISA (see Antibody recognition of Efb and Eap), with some modifications.

A microtiter plate coated with a twofold serial dilution of fibrinogen from 40-0.02 μg/ml, left to right, was blocked with 2 % BSA for 1 hour. Three different proteins, rEfb, Efb-C and –N, were applied in three different concentrations, 4, 1 and 0.2 μg/ml, one concentration per row, and incubated for 1 hour. The bound Efb-molecules were detected with sheep Efb-antibodies diluted 1/2000 for 1 hour. This was followed by an incubation of HRP-conjugated sheep-antibodies produced in rabbit diluted 1/1000 for 1 hour.
The colour was developed by adding TMB-chromogen substrate and the reaction was stopped with 1M HCl (+ 3M H_2SO_4). The results were analysed in a microtiter plate reader at 450 nm.

The experiment was also performed with immobilised Efb-forms and free fibrinogen: Two microtiter plates were coated with rEfb, Efb-C and Efb-N in a twofold serial dilution from 40-0.02 μg/ml, left to right, four rows per protein, and incubated over night at room temperature. The plates were blocked with 2 % BSA followed by an incubation of four different concentrations of fibrinogen: 2, 0.5, 0.1 and 0 μg/ml, one concentration per row and Efb-molecule, for 1 hour. Rabbit fibrinogen-antibodies diluted 1/500 were added and the plates were incubated for 1 hour. To detect the bound fibrinogen HRP-conjugated rabbit antibodies produced in swine diluted 1/2000 were applied. Detection of the bound fibrinogen was done as described above.

Phast-electrophoresis and Western blot

Western blotting was performed in a phast-electrophoresis system. Fibrinogen at 10 mg/ml was mixed with 1x loading buffer, boiled for a few minutes and loaded on an 8–25 % gradient phastgel with a low molecular weight marker. The electrophoresis was run for 130 Vh. The fibrinogen was transferred to a nitrocellulose membrane over night at room temperature by compression. The membrane was washed in 0.1 % washing buffer and blocked with blocking buffer for 30 minutes at room temperature. The nitrocellulose membrane was cut in four strips, which each one of them was separately incubated with one different protein with the concentration 2 μg/ml: rEfb, Efb-C, Efb-N respectively PBS (as a negative control) for 1 hour on a shaker at room temperature. The strips were washed together 3x5 minutes in washing buffer followed by incubation in a common box of sheep Efb-antibodies diluted 1/3000 for 1 hour on a shaker at room temperature. The bound proteins on the nitrocellulose membrane were detected with HRP-conjugated sheep-antibodies produced in rabbit diluted 1/3000 and incubated for 1 hour on a shaking table at room temperature. A solution of 4-chloro-1-naphtol was used to develop the potential bands on the strips.

Precipitation of Efb-fibrinogen complex

Fibrinogen was applied to the wells on a round-bottomed plate in three different concentrations in three different rows: 10, 5 and 2.5 mg/ml. Recombinant Efb was added to the plate in a threefold serial dilution from 1.0 mg/ml-0.5 µg/ml, left to right in each row. The precipitate formed was detected in a microtiter plate reader at 405 nm.

This experiment was also performed with the N- and C-terminal of Efb as inhibitors of the precipitation. A base solution of 50 µl fibrinogen with the concentration 2.5 mg/ml and 1 µl of rEfb with the concentration 50 µg/ml was added to 8 wells in two columns. To the first column, Efb-C was applied in the concentrations 200, 100, 40 and 0 µg/ml. Efb-N was added to the second column in the concentrations 150, 75, 30 and 0 µg/ml. The results were read in a microtiter plate reader at 405 nm.

Enzyme-linked immunosorbent assay (ELISA)

The antibody response against rEfb, Efb-C and Efb-N was studied in both sick (Patient serum from septicaemic patients from Örebro 2000) and healthy (blood donors from Örebro 2001) people in this experiment.

To find the right controls and concentrations to the following experiments, some pilot runs were performed (data not shown).

Microtiter plates were coated and incubated over night at room temperature, each with a specific protein: rEfb, Efb-C respectively Efb-N with the concentration 1 µg/ml. Eighteen patient sera diluted in four steps in a twofold serial dilution from 1/500 to 1/4000, from the top and four wells down or from the middle of the plate and four wells down, were added to the plate. Two doublets of a negative control diluted 1/1000 and five positive controls diluted 1/1000 of which four was applied as doublets and one was diluted in 8 steps in a twofold serial dilution from 1/250 to 1/32 000. The plate was incubated for 1 hour at 37 ºC and this was followed by washing the plates four times in 0.05 % PBST. The bound antibodies were detected with ALP-conjugated anti-human IgG produced in goat (DAKO) diluted 1/3000 for 2 hours at 37 ºC. The colour was developed by adding a phosphatase solution (Sigma), which was prepared according to manufacturer's manual. The results were finally analysed in a microtiter plate reader at 405 nm.

3. Results

Identification of native Efb:

The purification of fibrinogen binding proteins led to three purified proteins. The proteins were identified by electrophoresis followed by western blot and also by binding ELISA experiments. In the experiment "Antibody recognition of Efb" three peaks were detected by Efb-antibodies (See diagram 1). The peaks were already known to be coagulase, Efb and Eap from earlier studies, which was strengthened in this experiment. Although it is a bit unclear, three peaks can be distinguished in the diagram. The first one, fractions 1-8, is coagulase. The high absorbance values can be explained by the fact that Efb-antibodies are known to react against coagulase, because the C-terminal part of coagulase has a similar structure to the N-terminal part of Efb. The second peak, fractions 11-19, is Efb and the last one, fractions 22-24, is Eap. The Eap peak had already been recognize with Eap-antibodies in the experiment "Antibody recognition of Efb and Eap". The reason why Eap is at all registered by the Efb-antibodies is because the Eap-molecule is suspected to have an IgG-binding function, which means that the molecule binds unspecific to Efb-antibodies.

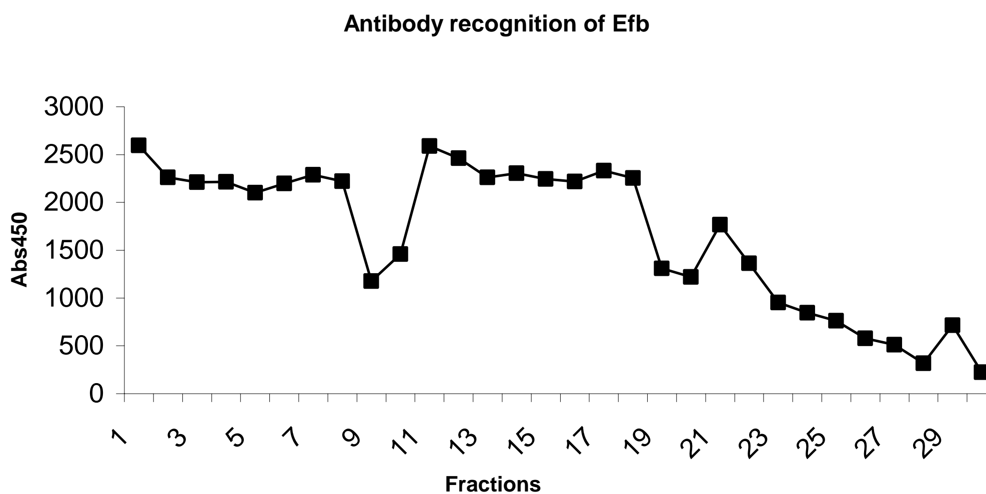


Diagram 1, The fractions from the purification step were detected by Efb-antibodies.

During the electrophoresis followed by western blot fraction 12 was found to have the same size as the Efb protein (data not shown). This makes the theory that the peak in the middle of diagram 1 is containing Efb more convincing.

To confirm that the first peak really was coagulase there was a coagulase test performed on selected fractions (5, 6, 11, 12, 17, 18). The result shows clearly that fractions 5 and 6 contain coagulase. Fraction 12 looks like it has some coagulative activity, while fractions 17 and 18 seem to lack this function entirely.

Fractions	Result
5	+++
6	++
11	+
12	++
17	0
18	0

Table 1, Results from a coagulase test, performed
on fractions from the purification process shown in the table.
0 equals no coagulation and +++ means that nothing inhibited the coagulation.

When recombinant Efb was provided by Brian Geisbrecht, a professor assistant at the University of Missouri-Kansas city, the project was focused on the characteristics of Efb:

Binding of fibrinogen to rEfb:

During an investigation of the binding of fibrinogen to Efb the optimal binding concentrations were established. In the experiment fibrinogen was bound to immobilised rEfb (see diagram 2) and the optimal concentrations were found to be 1µg/ml Efb and 1µg/ml fibrinogen.

The same results were found when the C-and N-terminal of Efb were coated and crosstitred with free fibrinogen (data not shown).

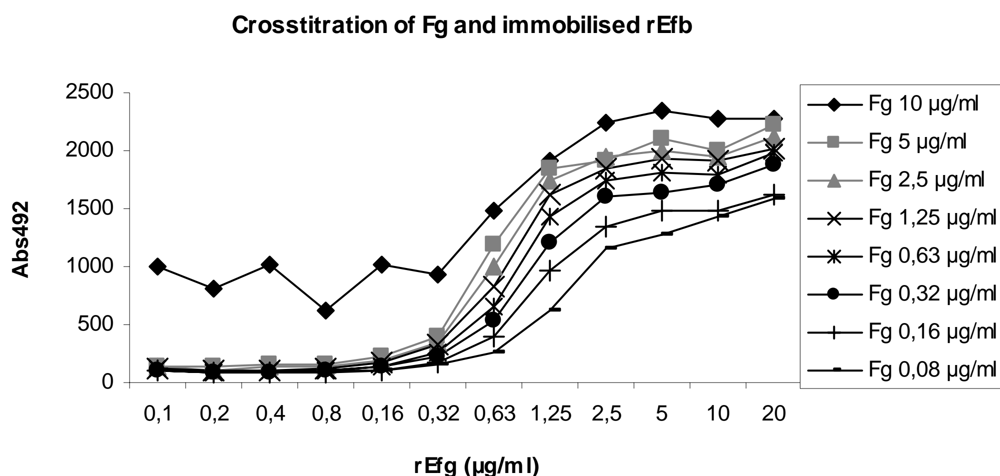


Diagram 2, Crosstitration of immobilised rEfb and free fibrinogen in a binding ELISA.

Competitive inhibition ELISA:

In a competitive inhibition, a mix of free fibrinogen and different Efb-molecules (rEfb, Efb-C, Efb-N, native Efb) and Eap (negative control) were first incubated together before addition to immobilised rEfb. The question was if the free Efb-molecules could inhibit the binding of fibrinogen to the immobilised rEfb. All the molecules did inhibit the binding, except the negative control, Eap (see diagram 3). Native Efb and rEfb reacted in similar way while the C- and N-terminal reacted in the same way. This shows that both the N- and C-terminal part of Efb bind to fibrinogen.

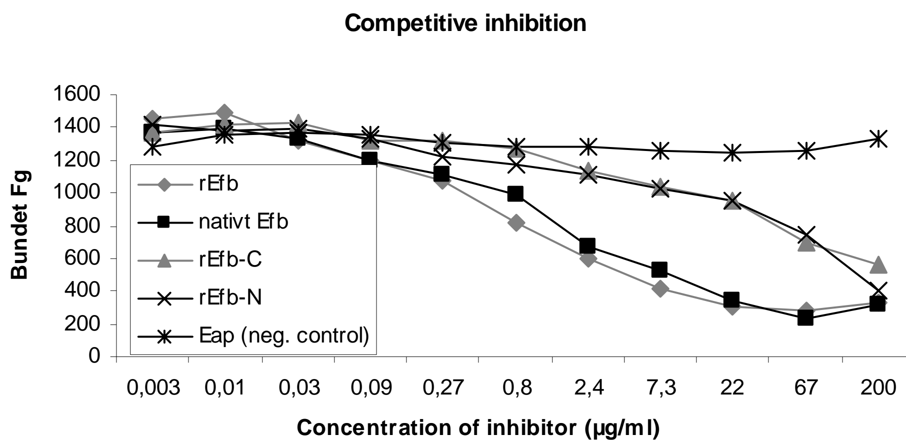


Diagram 3, Competitive inhibition, a rEfb-coated microtiter plate was incubated with a mix of fibrinogen and indicated molecules.

Binding of Efb-forms to fibrinogen:

To establish the previous results that both Efb-C and -N bind to fibrinogen, a direct binding was performed. The full-length rEfb, the C- and N-terminals of Efb were incubated with immobilised fibrinogen. The diagram below clearly shows that all the proteins bind to fibrinogen in the same way.

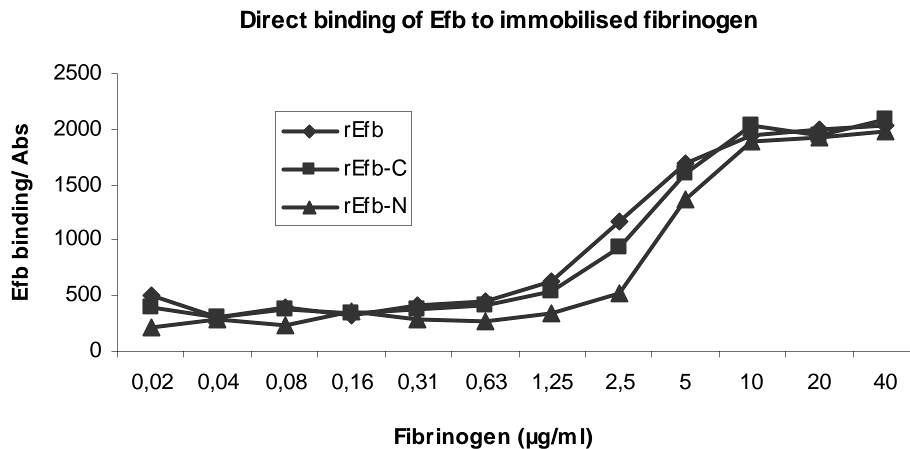


Diagram 4, The binding of free Efb molecules (0,2µg/ml) to immobilised fibrinogen.

Binding of fibrinogen to different forms of immobilised Efb:

To confirm that no unspecific bindings had taken place in the last experiment the method was run once more, but this time with immobilised Efb-molecules and free fibrinogen (diagram 5). The curve is similar to the previous experiment, confirming a binding between all forms of Efb and fibrinogen. (The graph shows one of several performed).

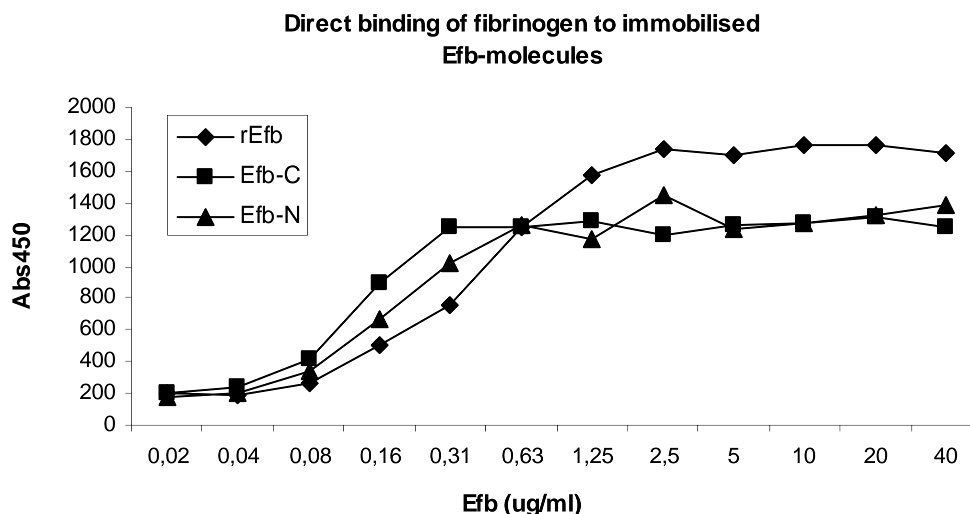
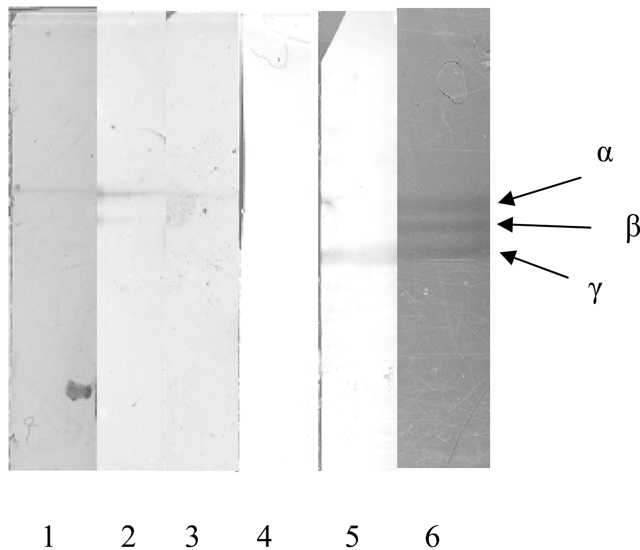


Diagram 5, Binding of free fibrinogen (2µg/ml) to immobilised Efb-molecules.

Western affinity blot:

In the experiment "Phast-electrophoresis and western blot" it was found that the full-length Efb and the N- and C-terminal of Efb all bind to the α-chain on fibrinogen (picture 1). The binding however between Efb-C and fibrinogen seem to be weaker than the binding between fibrinogen and the other molecules, based on the observed bond on the nitrocellulose membrane, which is hardly visible in the picture. That clumping factor binds to the γ-chain on fibrinogen was also confirmed in the same experiment.



1 Native Efb
2 N-terminal of Efb
3 C-terminal of Efb
4 Neg. control
5 Clumping factor (Clf)
6 Coomassie stained fibrinogen with its three chains α, β and γ

Picture 1, Fibrinogen transferred to a nitrocellulose membrane, probed with indicated proteins. Strip 6 is coomassie stained fibrinogen.

Precipitation of fibrinogen/Efb complex:

When it was established that Efb bind divalently to fibrinogen, another question rose, if Efb might be able to form a complex together with fibrinogen? This was investigated in a precipitation experiment (diagram 6). Fibrinogen was mixed with full-length Efb and a distinct precipitation was formed. It seems that an increasing concentration of fibrinogen requires an increasing concentration of Efb for the precipitation to take place, since the peaks in the diagram moves more to the right the higher the fibrinogen concentration is. The binding of fibrinogen in both ends of Efb probably results in a complex of Efb and fibrinogen, which precipitates if the molar ratio between them is right.

Efb-C and –N were applied to the precipitation mix in the following experiments and they were supposed to work as inhibitors of the precipitation. But it was not successful, since they did not inhibit the precipitation process (data not shown).

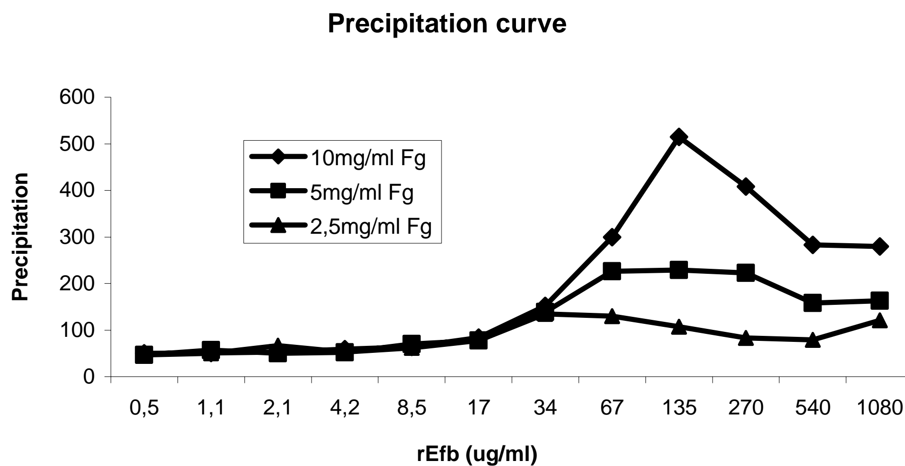


Diagram 6, The precipitation curve from a mix of free fibrinogen and rEfb.

Coagulation test:

A coagulation test was performed, where the question was if Efb and the C- and N-terminals of Efb are able to prevent coagulation of blood plasma in the presence of coagulase (table 2)? During the experiment an inhibition of the coagulation could be observed with Efb-N as inhibitor and also with full-length Efb, although the N-terminal was more efficient. Meanwhile Efb-C did not inhibit the coagulation at all.

rEfb (905µg/ml)		Efb-C (400µg/ml)		Efb-N (300µg/ml)	
20 µl	+	40 µl	+++	40 µl	+/0
5 µl	+++	10 µl	+++	10 µl	++
0 µl	+++	0 µl	+++	0 µl	+++

Table 2, Coagulation of horse plasma with indicated Efb-molecules as inhibitors. + equals almost no coagulation and +++ means that nothing inhibited the coagulation.

Reactivity of human antibodies against Efb:

The antibody-response against full-length Efb and the N-and C-terminal of Efb was compared in septicaemic patients at acute and convalescent occasions (diagram 7). The results were analysed in a programme, where every titre from each patient was compared with a titred positive control. A significant increase of antibody concentration was seen against the full-length Efb, during ongoing infection. The antibodies did recognise Efb-C and Efb-N, but not in the same degree as the full-length molecule.

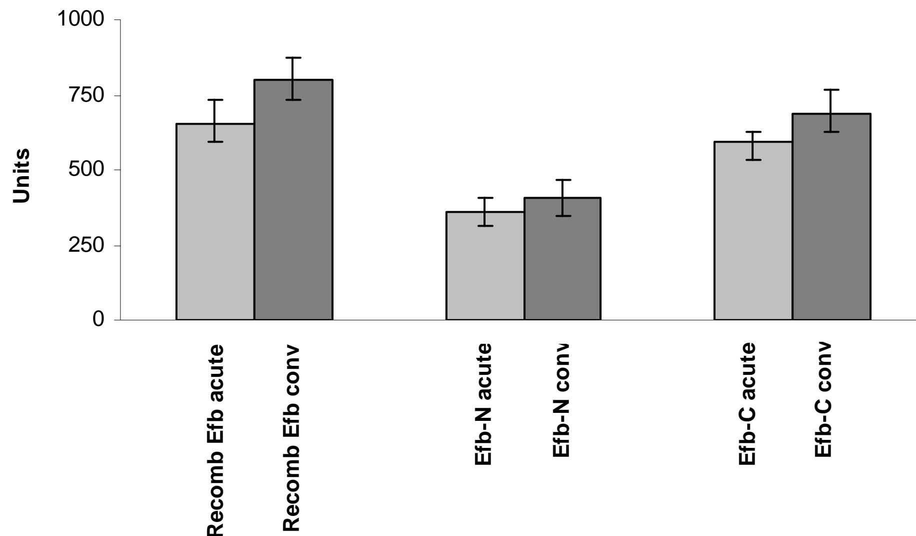


Diagram 7, The antibody-response against indicated Efb-molecules in septicaemic patients during an ongoing infection. The sera are taken from each person in two different phases during the infection. Acute phase, means that they've just become ill and the convalescent phase is during recovery.

Serum from healthy people was also studied in ELISA experiments. The results showed that many people have antibodies against Efb even if they aren't infected with the bacteria (data not shown).

4. Discussion

The functions and characteristics of the extracellular fibrinogen-binding protein, Efb, have been studied during this project. Since recombinant Efb (rEfb) and native Efb were shown to act in similar ways, rEfb was chosen for experiments in the study.

In this project it has been established that the Efb binding to fibrinogen is divalent. Also it seems likely that Efb and fibrinogen can form a complex together, which precipitates if the molar-ratio is right between them. If this process actually occurs in the body is not known, but if it would, fibrinogen would be consumed around the bacteria and be prevented to participate in the healing process, which would lead to a delayed wound healing.

This discovery including the results from earlier studies, where Efb has been found to prevent coagulation by binding to platelets, give the picture of Efb as a major reason for the delayed wound healing, which is a hallmark for *S. aureus* infections.

In an earlier study, wounds on rats were infected with mutated bacteria which lack the ability to produce Efb, while other rats were infected with the parental *S. aureus* strain. The results showed that the wounds infected with the mutated bacteria were healing faster than the wounds with the Efb-producing bacteria. This strengthens the suggestion that Efb is a contributing factor to delayed wound healing [14].

It has also been shown that both the N- and C-terminal of Efb bind to the α-chain on fibrinogen. If both the C- and N-terminal bind to the same part on the α-chain and if they compete for the same binding site is still unclear. However the complex formation between Efb and fibrinogen should not be possible if the two terminals were competing for the same binding site on fibrinogen. But there are more research left to be done before an answer can be found.

The question, if the C-terminal of Efb possibly could have a weaker binding to fibrinogen than the amino terminal, rose during the western blot experiment. This could also be an explanation why the molecule did not inhibit the coagulation of the horse plasma and the coagulase in the coagulation test, while Efb-N could. It is reasonable to believe that the homology between the two repeat domains in the N-terminal part of Efb and similar repeats in coagulase is the reason for the inhibitory effect that Efb-N has on coagulase.

People that have *S. aureus* infections have been shown to produce antibodies against Efb. Which confirm that the efb-gene is not a pseudogene, but is expressed in vivo during an infection.

Some people already have high levels of antibodies against Efb before they have been infected with *S. aureus*. This has been shown in experiments where healthy people have been tested for the presence of Efb-antibodies. This can be explained by the fact that a large part of the human population always is colonized by *S. aureus*. Others are colonized from time to time and some are never colonized by the bacteria. This forms very different starting points for every person. If people with lower levels of antibodies are more susceptible to *S. aureus* infections is under investigation.

Hypothetically the antibodies we produce should be protective, but the low level that is produced during an infection does not seem to be high enough. Since the infections often come back shortly after recovery. Could a higher concentration of antibodies be protective?

The antibodies in this project did recognise and bind to both the N- and C-terminal of Efb. However, the binding was not as specific as to full-length Efb. An explanation could be because of the epitopes on the Efb-molecule. When the molecule is cut in half some epitopes might disappear, so the individual terminals do not display the same amount of epitopes together as the full-length molecule.

MRSA is developing fast and new methods to prevent the dangerous *S. aureus* infections are needed. Vaccination against the bacteria could be successful and has been the goal for a long time. The vaccine has to be based on important virulence factors at different stages of the *S. aureus* infections.

The results that were found during the project and the results from earlier studies, give the picture of Efb as an important virulence factor during *S. aureus* infections. It can therefore be considered a likely candidate to be included in a vaccine against *S. aureus*.

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