The adhesive and immunomodulating properties of the multifunctional \textit{Staphylococcus aureus} protein Eap

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Adherence of \textit{Staphylococcus aureus} to the host tissue is an important step in the initiation of pathogenesis. At least 10 adhesins produced by \textit{S. aureus} have been described and it is becoming clear that the expression of these adhesins and their interactions with eukaryotic cells involve complex processes. Some of these, such as the fibronectin-binding proteins (FnBPs) and Clumping Factor A, are well characterized. However, in the last 10 years a number of novel \textit{S. aureus} adhesins have been described. Functional analyses of these proteins, one of which is Eap (extracellular adherence protein, also known as Map and p70), are revealing important information on the pathogenesis of staphylococcal disease. More than 10 years after the first report of Eap, we are beginning to understand that this protein, which has a broad spectrum of functions, may be a critical factor in the pathogenesis of \textit{S. aureus}. This review will focus on the interactions of Eap with eukaryotic cells, plasma proteins and the extracellular matrix as well as on the recently recognized role of Eap as an important mediator in the immune response to staphylococcal infection.

Background

Eap (extracellular adherence protein) was isolated independently by a number of groups in the 1990s. Youssif \textit{et al.} (1991) reported a highly cationic 70 kDa (p70) protein from the cell surface of \textit{Staphylococcus aureus} Wood 46 that had high affinity for the glomerular basement membrane of rats. A 60 kDa fibrinogen-binding protein was identified by Bodén & Flock (1992), and McGavin \textit{et al.} (1993) reported a staphylococcal surface protein of different molecular masses (either 72 or 60 kDa) in different strains (FDA574 and Newman, respectively) capable of binding to a number of different extracellular matrix (ECM) glycoproteins including bone sialoprotein, fibronectin and fibrinogen. This 72 kDa protein was later characterized by Jonsson \textit{et al.} (1995) and called Map. The 60 kDa protein reported by both Bodén & Flock (1992) and McGavin \textit{et al.} (1993) was characterized by Palma \textit{et al.} (1999) and termed Eap. The disparity in size was later explained by Hussain \textit{et al.} (2001) in their study on 140 clinical isolates of \textit{S. aureus}. In this study, it was found that Eap could be classified into three groups based on PCR analysis of the coding genes: Group I (a 1-8 kb PCR product), Group II (a 2 kb PCR product, including the laboratory strain Newman) and Group III (a 2-4 kb PCR product only found in strain Wood 46). Sequence analysis showed that p70, Map and Eap exhibited extensive homology and it was concluded that these are all analogues of the same protein (Hussain \textit{et al.}, 2001). Although Kuroda \textit{et al.} (2001) reported four Eap analogues in strain N315, this finding appears to be strain-specific, since in other strains examined to date, Eap is present in a single copy. Eap was found in all but 5 of 140 \textit{S. aureus} strains examined (Hussain \textit{et al.}, 2001) but it was not detected in reference strains or clinical isolates of \textit{Staphylococcus epidermidis}.

Maximal production of Eap occurs during the late-exponential phase of growth and has been reported to be regulated by both \textit{agr} and \textit{sar} (Dunman \textit{et al.}, 2001). Analysis of Eap production at 4 and 19 h (corresponding to late-exponential phase and stationary phase) has shown that 70% of Eap is found in the culture supernatants.
An unusual property of Eap is its ability to bind back to the S. aureus cell surface (Fig. 1). Externally added Eap can re-bind to S. aureus, both to endogenous surface-located Eap by an Eap–Eap interaction, and to other components on the bacterial surface. This re-binding is independent of endogenous Eap production (Hussain et al., 2002). Therefore, since Eap-negative mutants are as capable as the wild-type strain of binding exogenous Eap and of adhering to Eap/Map-coated surfaces (Hussain et al., 2002; Kreikemeyer et al., 2002), this suggests that there are several Eap-binding structures on the cell surface capable of binding Eap. Flock & Flock (2001) identified a neutral phosphatase as a putative target and Kreikemeyer et al. (2002) identified at least two S. aureus cell surface proteins that were capable of binding Eap. These findings suggest that there may be several Eap structures on the S. aureus cell surface with different affinities for Eap. Further evidence that Eap binds back to the S. aureus cell surface comes from the work of Vuong et al. (2002), who showed that binding of Eap to the S. aureus cell wall is dependent on the d-alanylation of teichoic acids. Eap does not bind to the surface of an S. aureus dlt mutant. This may be due to an impaired binding of Eap to teichoic acids, possibly due to altered electrostatic interactions. A non-specific interaction between Eap and the bacterial surface is implied by the large number of Eap molecules (10^5) that can bind to the surface and that the binding is difficult to saturate (Palma et al., 1999). Thus a specific receptor function is less likely. The biological role of these various interactions of Eap with itself and with bacterial surface structures is presently unclear; however, it is tempting to speculate that these multiple interactions may play a role in the architecture of the complex populations, and may thus contribute to the stability of these bacterial clusters during colonization and infection. Kreikemeyer et al. (2002) propose that coating of surfaces with secreted Eap may allow S. aureus to bind to surfaces not already coated with ECM proteins, thereby providing an additional adhesion mechanism. The Eap may play an important role in S. aureus adhesion. In order to investigate this, isogenic disruption mutants of Eap in strains Newman and Phillips were independently constructed (Hussain et al., 2002; Kreikemeyer et al., 2002) and the ability of these mutants to bind to plasma proteins was compared. No significant difference in the binding to fibronectin, fibrinogen (Chavakis et al., 2002; Hussain et al., 2002; Kreikemeyer et al., 2002) or vitronectin (Kreikemeyer et al., 2002) was found. This is most likely due to the presence of other adhesins such as the fibronectin-binding proteins that may be more important than Eap for adhesion to plasma proteins. Deletion of the fibronectin-binding proteins only results in a 40% reduction in adherence (Dziewanowska et al., 1999), suggesting that S. aureus may have a number of adhesins with overlapping functions or that adherence is the result of the additive effects of a number of different adhesins. Analysis of double or triple mutants may help clarify the extent to which Eap is involved in binding of S. aureus to plasma proteins.

At least seven plasma proteins have been found to bind Eap (Palma et al., 1999). The binding of Eap to vitronectin was shown to involve a specific protein–protein interaction because a radiolabelled vitronectin peptide could bind to either native or recombinant Eap while peptides of a similar size or charge could not (Jonsson et al., 1995). Palma et al. (1999) demonstrated direct Eap–Eap interactions and that Eap is able to form oligomers. With this property it was postulated that Eap would cause bacterial aggregation, and such behaviour was subsequently demonstrated (Palma et al., 1999). However, analysis of strain Newman and its isogenic Eap-deficient mutant on glass slides in the presence of externally added Eap revealed enhanced agglutination of both the wild-type and mutant strain, thereby indicating that agglutination is independent of endogenous Eap production (Hussain et al., 2002).

Role of Eap in adherence

The early studies on Eap showed that it was a protein with broad binding specificity but with particular affinity for the ECM and plasma proteins (Fig. 1). This suggested that
secreted and re-bound to the *S. aureus* cell surface has also been reported to be important in ICAM-1 (intercellular adhesion molecule-1)-dependent adhesion of *S. aureus* to endothelial cells (Chavakis et al., 2002).

**Interaction of Eap with matrix suprastructure**

Using immunogold-labelled preparations of matrix derived from human cartilage and skin, we could demonstrate that recombinant His-tagged Eap, expressed in *Escherichia coli,*
bonds to native matrix suprastructure. The interaction is specific and restricted to perifibrillar structures, adjacent to the major collagen fibrils (U. Hansen, M. Hussain, P. Bruckner, M. Herrmann & B. Sinha, unpublished data). The exact mode of binding and possible host ligands await further analysis.

Adherence to eukaryotic cells

The importance of Eap in adherence of S. aureus to eukaryotic cells has been shown by Hussain et al. (2002), Kreikemeyer et al. (2002) and Haggar et al. (2003) and in Fig. 1. All three studies have reported decreased adherence of an eap mutant to both fibroblasts and epithelial cells, with an 80% reduction in adherence to epithelial cells reported by Kreikemeyer et al. (2002). Addition of exogenous Eap was shown to increase the adherence of both the wild-type and the eap mutant to fibroblasts (Hussain et al., 2002), again suggesting that re-binding of Eap to S. aureus appears to be independent of endogenous Eap and further supports the idea that there are several S. aureus surface proteins or other components capable of binding Eap. Anti-Eap antibodies were shown to significantly decrease adherence of Newman to epithelial cells and also to reduce the adhesion to fibroblasts (Hussain et al., 2002). Similarly, anti-Eap antibodies were shown to decrease the adherence of strain Phillips to a Map substrate (Kreikemeyer et al., 2002).

Role of Eap in invasion of host cells by S. aureus

Although S. aureus was considered for many years to be an extracellular organism, it is now accepted that it possesses the ability to adhere to and invade non-professional phagocytic cells (Dziewanowska et al., 1999; Peacock et al., 1999; Sinha et al., 1999, 2000). Haggar et al. (2003) found that wild-type strain Newman was internalized more efficiently compared to an eap-negative mutant. Addition of exogenous Eap was shown to increase the internalization of both the wild-type strain Newman and the Eap mutant strain by fibroblasts as well as enhancing internalization of clinical isolates and TM300 (Staphylococcus carnosus). Furthermore, pretreatment of staphylococcal cells with anti-Eap antibodies reduced internalization (Haggar et al., 2003), suggesting that Eap plays an important role in the internalization of S. aureus strain Newman by MRC-5 and HACAT cells. However, these findings are in contrast to those of Kreikemeyer et al. (2002), who found that all eap-negative mutant bacteria were internalized by HEp-2 cells, whereas most but not all of the wild-type bacteria were internalized. These differences may be due to the different strains used (Phillips vs Newman) because they may express Eap and fibronectin-binding proteins to a different extent. Moreover, the different host cells (MRC-5 and HACAT vs HEp-2), which vary in their expression of fibronectin, may also have affected the outcome of the experiments. Thus the exact contribution of Eap to the internalization process remains to be clarified.

Is there a eukaryotic receptor for Eap?

The ability of Eap to bind back to S. aureus and the importance of Eap for adherence of S. aureus to eukaryotic cells suggest that Eap may act as a bridge between the eukaryotic cell and the bacterium (Table 1). ICAM-1 on endothelial cells has been shown to bind Eap and it was also shown that this interaction blocked the adhesion of monocytes to an endothelial cell layer (Chavakis et al., 2002). ICAM-1 is also present on fibroblasts and epithelial cells. However, it has not yet been determined if ICAM-1 is the receptor on these cells that is responsible for binding Eap or whether other receptors are involved. It is also plausible that plasma proteins attached to the eukaryotic cell surface, such as fibronectin, serve as substrates for Eap.

Eap as a virulence factor

In vivo studies have recently commenced to determine the importance and role of Eap in S. aureus pathogenesis. The existence of Eap analogues raises the question of whether some of the analogues are more potent virulence factors than others. A difference in expression levels of Eap and the efficacy of internalization has been noted (Haggar et al., 2003). However, whether there is a correlation between an Eap analogue and the severity or type of infection remains to be investigated.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Receptor</th>
<th>Consequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>APCs</td>
<td>ICAM-1?</td>
<td>Possible interference with interactions between APCs and T cells by blocking the interaction of ICAM-1 (APCs) and LFA-1 (T cells), resulting in inhibition of T-cell response</td>
<td>Lee et al. (2002)</td>
</tr>
<tr>
<td>T cells</td>
<td>Unknown</td>
<td>Apoptosis of T cells</td>
<td>Chavakis et al. (2002)</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>ICAM-1</td>
<td>Inhibition of extravasation of leukocytes</td>
<td>Hussain et al. (2002);</td>
</tr>
<tr>
<td>Epithelial cells and fibroblasts</td>
<td>Unknown (fibronectin?)</td>
<td>Adherence and internalization of S. aureus</td>
<td>Kreikemeyer et al. (2002); Haggar et al. (2003)</td>
</tr>
</tbody>
</table>
The interactions of Eap with eukaryotic cells (Table 1), particularly its binding to ICAM-1, point to Eap as an important virulence factor of \textit{S. aureus}. ICAM-1 is an important factor in the pathogenesis of \textit{S. aureus}, affecting both mortality and severity of disease (Verdrengh et al., 1996). Chavakis et al. (2002) showed that Eap could bind to ICAM-1 on endothelial cells and block the interactions of ICAM-1 with Mac-1 and LFA-1 (lymphocyte function-associated molecule-1). Binding of Eap to ICAM-1 inhibited leukocyte adhesion to endothelial cells and as a result prevented leukocyte extravasation. Eap was also shown to inhibit neutrophil recruitment during peritonitis, suggesting that Eap may be functioning as an anti-inflammatory agent (Chavakis et al., 2002; reviewed by Rhee et al., 2003). Therefore, the effects of blocking ICAM-1 interactions with leukocytes and the inhibition of neutrophil recruitment (Chavakis et al., 2002) and possibly the resulting immunosuppression may be important factors that determine the outcome of \textit{S. aureus} infection.

Using a wound infection model in mice, Chavakis et al. (2002) showed that there was no difference in the size of the abscess formed when mice were infected with wild-type strain Newman \textit{eap}-positive and an \textit{eap}-negative mutant. In a second model, where the bacteria were administered intravenously, no significant difference in the numbers of bacteria recovered from kidneys was seen. This finding is in contrast to that of Lee et al. (2002), who, using the same strategy, found that mice infected with a \textit{map}-negative strain (mutation in strain Newman) had a 25-fold reduction in kidney abscess formation. The reason for this discrepancy may be due to the time-scale of the experiments. Analysis by the Chavakis group was carried out after 5 days whereas the Lee group carried out an extended study over several weeks, suggesting an effect of Eap for chronic rather than short-term infections.

Lee et al. (2002) found a number of differences in mice infected with \textit{map}-positive and \textit{map}-negative strains over time. Although the initial stages of infection were similar in both sets of mice (in terms of weight loss and bacterial densities), 21 days post-infection the mice infected with the \textit{map}-negative mutant had returned to their normal weight whereas the mice infected with \textit{map}-positive strain remained below their weight prior to infection. Eight weeks post-infection, 57% of the \textit{map}-positive-infected mice had heart abscesses whereas none in the \textit{map}-negative-infected group exhibited this pathology.

Initial findings from Lee et al. (2002) suggested that modulation of the immune response by Eap (possibly by immunosuppression) was important and so they continued their study with \textit{nu/nu} mice, which are T-cell deficient, and control genotype \textit{nu/+} mice. It was reasoned that if the T-cell response was being modulated by Eap, then \textit{nu/nu} mice infected with a \textit{map}(−) strain should present with similar pathologies to \textit{nu/+} mice infected with a \textit{map}(+) strain. The study found that the severity of arthritis in the two groups was similar although \textit{nu/nu} mice infected with the \textit{map}(−) strain had higher arthritis and osteomyelitis scores compared with \textit{nu/+} mice infected with the \textit{map}(−) strain, indicating that Eap is also capable of modulating the T-cell response and affecting the severity of disease. The major difference between the groups was that no abscess formation was seen in the mice infected with the \textit{map}(−) strain whereas abscess formation was seen in all mice infected with the \textit{map}(+) strain, suggesting a T-cell-independent mechanism for abscess formation. The findings from Lee et al. (2002) taken together with the work of Verdrengh et al. (1996) and Chavakis et al. (2002) point to the possibility that the interactions of Eap with ICAM-1 are critical in determining the severity and outcome of disease. Binding of Eap to ICAM-1 on endothelial cells blocks the anti-inflammatory response. If Eap also binds to ICAM-1 on antigen presenting cells (APCs) then this could inhibit interactions of T cells and APCs (Fig. 1) resulting in immunosuppression. However, the effect of interactions between Eap and ICAM-1 on the outcome of disease over time, the interaction of Eap with T cells and whether other mechanisms are involved remain to be determined.

**Eap as an immunomodulator?**

The \textit{in vivo} studies to date indicate the importance of the immune response and the interaction of the immune response with Eap in the outcome of infection. Studies have now begun to elucidate how Eap modulates the host immune response. The interactions of Eap and ICAM-1 are likely to have profound effects on the immune response to \textit{S. aureus} infection and could be instrumental in deciding the outcome of infection. However, the findings from a number of studies also suggest that Eap could be involved in the development of immunotolerance or may facilitate the survival of intracellular \textit{S. aureus} by directing the immune response towards the humoral rather than cell-mediated response.

Eap (p70) is capable of inducing a time and dose-dependent increase in IgM and IgG synthesis in PBMC (peripheral blood mononuclear cell) cultures (Jahreis et al., 1995). However, enriched B-cell populations could not be activated to secrete immunoglobulins following stimulation with Eap, suggesting that Eap does not activate B cells directly (Jahreis et al., 1995). Jahreis et al. (1995) showed that Eap activation of T cells did not occur via lectin- or superantigen-like activity. Since publication of this study, work has focused on how Eap interacts with the T-cell response. Naturally, interest in the interactions of Eap with the T-cell response is also due to its partial homology to MHC class II molecules.

PBMCs stimulated with Eap display increased IL-4 (interleukin 4) synthesis (Jahreis et al., 2000). IL-4 is a cytokine involved in the differentiation of T cells into Th2 cells and the development of the humoral immune response. IL-4 can also regulate the expression of various surface molecules on macrophages and dendritic cells (e.g. Santin et al., 1999; Fernandez et al., 2001). 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 between activated T cells and MHC class II-bearing APCs. Only activated T cells appear to be affected by Map (Lee et al., 2002) and it is noteworthy that only activated T cells are affected by IL-4 (Yoshimoto & Paul, 1994). However, the effect of IL-4 on the interactions between activated T cells and APCs is most likely in addition to the effect of blockage of the ICAM-1 interactions by Eap (Chavakis et al., 2002), since ICAM-1 has also been shown to be involved in interactions with T cells and APCs (Dang et al., 1990; Hogg et al., 1991).

Eap has also been reported to inhibit the delayed type hypersensitivity response, which is a Th1-initiated response, and to induce T-cell death (Lee et al., 2002). Therefore, Eap has now been shown to be capable of modulating both the Th1 and Th2 responses. Skewing the immune response towards a Th2 response, rather than Th1 response, could affect the outcome of infection, because an insufficient Th1 response (i.e. decreased IFN-γ secretion) would result in decreased phagocytic activity leading to incomplete elimination of *S. aureus* and the development of chronic infection. In addition, directing the immune response away from a cell-mediated response may actually enhance the intracellular survival of *S. aureus*. Disrupting the balance between the antibody (Th2) and cell-mediated (Th1) responses may result in the development of immunotolerance, another means of causing chronic infection. In fact, the work of Lee et al. (2002), which showed continued disease in *map* (+)-infected mice 8 weeks post-infection, points to Eap as an important virulence factor in chronic *S. aureus* infections.

**Conclusions**

Over the last years, our understanding of Eap and its analogues has greatly evolved. Since its primarily ascribed function as a staphylococcal adhesin, Eap has unveiled surprising and fascinating aspects of its multifunctionality and has thus exemplified the biological importance of the increasingly understood family of secreted *S. aureus* proteins. Eap has been shown to be important in the adherence to and internalization of *S. aureus* by eukaryotic cells as well as being capable of modulating the inflammatory response through its interactions with ICAM-1. Although much progress has been achieved on pinpointing the spectrum of putative biological functions of Eap, important questions (Table 2) such as the molecular characterization of Eap interactions with its putative prokaryotic and eukaryotic receptor structures, mechanisms involved in the internalization of *S. aureus* by Eap, or the effect of Eap on intracellular events are currently not clear. These and other questions addressing the biological role of Eap, with particular emphasis on its role in the *in vivo* situation, are all presently under investigation. It is hoped that the advanced understanding of this exciting group of molecules may provide new insight into the pathogenesis of *S. aureus* infections, and their prevention and treatment, and may also indicate novel avenues in the use of bacterial products to treat non-bacterial disease.

**Table 2. Eap – unanswered questions**

<table>
<thead>
<tr>
<th>Question</th>
<th>Indication</th>
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<tbody>
<tr>
<td>Does Eap bind to eukaryotic receptors other than ICAM-1?</td>
<td>How do the <em>in vitro</em> findings relate to the <em>in vivo</em> situation, e.g. would the concentrations of Eap in the blood be sufficient to induce the cellular events seen in vitro?</td>
</tr>
<tr>
<td>How does Eap bind to matrix suprastructure? Which host components are involved?</td>
<td></td>
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<tr>
<td>How does Eap enhance internalization of <em>S. aureus</em>?</td>
<td></td>
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<tr>
<td>What happens following binding of Eap to eukaryotic cells?</td>
<td></td>
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<tr>
<td>Does Eap contribute to the intracellular survival of <em>S. aureus</em>?</td>
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<tr>
<td>How is Eap modulating the immune response?</td>
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<tr>
<td>Does Eap have targets other than ICAM-1?</td>
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**References**


