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# SpeB modulates fibronectin-dependent internalization of *Streptococcus pyogenes* by efficient proteolysis of cell-wall-anchored protein F1

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SpeB is a cysteine proteinase and virulence determinant secreted by the important human pathogen *Streptococcus pyogenes*. Recent investigations have suggested a role for SpeB in streptococcal entry into human cells. However, conflicting data concerning the contribution of SpeB to internalization have been presented. Protein F1 is a cell-wall-attached fibronectin (Fn)-binding protein that is present in a majority of streptococcal isolates and is important for internalization. This study shows that protein F1 is efficiently degraded by SpeB, and that removal of protein F1 from the bacterial surface leads to reduced internalization. Whereas M1 protein and protein H, two additional surface proteins of *S. pyogenes* that bind human plasma proteins, are protected from proteolytic degradation by their ligands, protein F1 is readily cleaved by SpeB also when in complex with Fn. This finding, and the connection between the presence of Fn at the bacterial surface and entry into human cells, suggest that SpeB plays a role in the regulation of the internalization process.

## INTRODUCTION

*Streptococcus pyogenes* is one of the most common and significant human bacterial pathogens and is the causative agent of diseases such as pharyngitis, impetigo and erysipelas. On occasion, infection with *S. pyogenes* causes more serious conditions, such as sepsis, a toxic shock syndrome and necrotizing fasciitis. Non-suppurative and clinically important sequelae to streptococcal infections include glomerulonephritis and rheumatic fever. For a recent review of *S. pyogenes* pathogenicity, see Cunningham (2000).

Adhesion to tissues or cells is an initial step in microbial colonization of the host. For many bacteria, adhesion is mediated by binding to fibronectin (Fn), a human plasma protein and an important component of the extracellular matrix. Fn-binding has long been recognized as an important feature of *S. pyogenes* adhesion (Abraham *et al.*, 1983), and several streptococcal proteins have been shown to contribute to this binding. These include M- or M-like proteins (Cue *et al.*, 1998; Frick *et al.*, 1995; Schmidt *et al.*, 1993), FBP54 (Courtney *et al.*, 1994), PFBP (Rocha & Fischetti, 1999), Fba (Terao *et al.*, 2001), glyceraldehyde-3-phosphate

dehydrogenase (Pancholi & Fischetti, 1992), protein F2 (Jaffe *et al.*, 1996), streptococcal opacity factor (Courtney *et al.*, 1999) and protein F1/SfbI (Hanski & Caparon, 1992; Talay *et al.*, 1992). The relative importance of these proteins most likely varies between different streptococcal serotypes, but the gene encoding protein F1, or its allelic gene variant *sfbI*, has been identified in a majority of isolates (Goodfellow *et al.*, 2000; Natanson *et al.*, 1995). Protein F1 is a 70 kDa cell-wall-attached protein, with two binding sites for Fn: a COOH-terminal site with similarity to Fn-binding regions in Fn-binding proteins of *Staphylococcus aureus* and *Streptococcus dysgalactiae*, and a second more NH<sub>2</sub>-terminally located site that binds Fn with higher affinity (Sela *et al.*, 1993). These binding regions bind to different parts of Fn (Ozeri *et al.*, 1996; Talay *et al.*, 2000).

La Penta *et al.* (1994) demonstrated that *S. pyogenes*, previously considered an exclusively extracellular pathogen, can internalize into eukaryotic cells. This process generally requires Fn, although Fn-independent internalization has also been described (Molinari *et al.*, 2000). Fn acts as a bridging molecule and mediates internalization by interacting both with bacterial surface proteins and with  $\alpha_5\beta_1$  integrins on host cells (Cue *et al.*, 2000). Several reports have demonstrated that protein F1, or Sfb1, can mediate internalization through binding to Fn (Jadoun *et al.*, 1997, 1998; Molinari *et al.*, 1997; Okada *et al.*, 1998).

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Abbreviation: Fn, fibronectin.

Most *S. pyogenes* strains secrete the cysteine proteinase SpeB. The enzyme is secreted as a 40 kDa zymogen, and autocatalytic cleavage generates an active 28 kDa proteinase (Bustin *et al.*, 1970; Doran *et al.*, 1999). The mature SpeB has broad proteolytic activity and degrades or activates a number of different human proteins, including fibrinogen (Matsuka *et al.*, 1999), Fn (Kapur *et al.*, 1993b), immunoglobulins (Collin & Olsén, 2001a, b), interleukin-1 $\beta$  (Kapur *et al.*, 1993a) and a metalloprotease (Burns *et al.*, 1996). SpeB also releases pro-inflammatory kinins from kininogens (Herwald *et al.*, 1996). Besides its ability to cleave host proteins, SpeB exerts proteolytic activity on *S. pyogenes* proteins, including M and M-like proteins (Berge & Björck, 1995; Raeder *et al.*, 1998; Rasmussen & Björck, 2001).

Conflicting results concerning the contribution of SpeB to *S. pyogenes* internalization into human cells have been reported and the possible mechanisms whereby SpeB influences internalization are not well understood. Whereas SpeB has been shown to increase internalization (Tsai *et al.*, 1998), two other studies have demonstrated decreased internalization with SpeB-producing bacteria (Burns *et al.*, 1998; Chaussee *et al.*, 2000). Finally, one study indicates that the effect of SpeB on internalization is dependent upon the amount of hyaluronic acid capsule present in the strain studied and that inactivation of the proteolytic activity of SpeB has only a minor effect on internalization (Jadoun *et al.*, 2002). Here, we focus on the requirement of Fn for *S. pyogenes* internalization by studying the effect of SpeB on Fn-binding in a strain that binds Fn through protein F1. We find that the binding of Fn is highly sensitive to SpeB, thus providing a possible mechanism for proteolytic regulation of *S. pyogenes* entry into human cells.

## METHODS

**Bacterial strains, growth conditions, and plasmids.** The *S. pyogenes* AP strains are from the *S. pyogenes* strain collection, Institute of Hygiene and Epidemiology, Prague, Czech Republic. JRS4 is a spontaneous streptomycin-resistant derivative of the M6 strain D471 from the Rockefeller University collection (Scott *et al.*, 1986). 86858 is of serotype M12 (Bohach *et al.*, 1988). The MC25 (Collin & Olsén, 2000) and BM27.6 (Kihlberg *et al.*, 1999) strains are derived from the AP1 strain. MC25 lacks M1 protein and BM27.6 lacks protein H at the bacterial surface. *Escherichia coli* DH5 $\alpha$  strains carrying plasmids pLZ12 and pPTF8 (Hanski & Caparon, 1992) were kindly provided by Dr Michael Caparon, Washington University School of Medicine, St Louis, MO, USA. Plasmid pLZ12 is an *E. coli*-*S. pyogenes* shuttle plasmid and plasmid pPTF8 carries the *prtF1* gene, encoding protein F1. The plasmids were purified and electroporated into the AP1 strain, thus generating chloramphenicol- and kanamycin-resistant transformed strains, AP1(pLZ12) and AP1(pPTF8). *S. pyogenes* strains were grown in Todd-Hewitt broth (Difco) with 0.2% yeast extract (Difco) (THY) or C-medium (CM) in 5% CO<sub>2</sub> at 37 °C. CM consists of 0.5% (w/v) Proteose Peptone no. 2 (Difco) and 1.5% (w/v) yeast extract dissolved in CM buffer (10 mM potassium phosphate, 0.4 mM MgSO<sub>4</sub>, 17 mM NaCl pH 7.5) (Gerlach *et al.*, 1983). For growth of AP1(pLZ12) and AP1(pPTF8), 500  $\mu$ g kanamycin ml<sup>-1</sup> and 3  $\mu$ g

chloramphenicol ml<sup>-1</sup> was added. For growth of MC25, 150  $\mu$ g kanamycin ml<sup>-1</sup> was added, and for BM27.6 1  $\mu$ g tetracycline ml<sup>-1</sup> was added.

**Azocasein assay.** To determine proteinase activity, bacteria were cultured in CM with or without the addition of 10  $\mu$ M trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) (Sigma), a cysteine proteinase inhibitor. The cultures were centrifuged and supernatants were incubated with an equal volume of activation buffer (1 mM EDTA and 10 mM DTT in 0.1 M NaAc/HAc, pH 5.0) for 30 min at 37 °C. An equal volume of 2% azocasein (Sigma), dissolved in activation buffer, was added and incubation proceeded for 30 min. Azocasein was precipitated by adding trichloroacetic acid (TCA) to a concentration of 5% (w/v) followed by centrifugation at 15 000 g for 5 min. A 300  $\mu$ l sample of the supernatant was mixed with 700  $\mu$ l H<sub>2</sub>O and the A<sub>366</sub> was determined in 1 ml cuvettes (Sarstedts, Germany) using a Novaspec II spectrophotometer.

**Purification and active-site titration of SpeB.** SpeB was purified from the medium of the AP1 strain as described previously (Berge & Björck, 1995). The purified enzyme was aliquoted and stored at -80 °C until further use. The amount of active SpeB was determined by performing an azocasein assay with the addition of various concentrations of the cysteine proteinase inhibitor E64. SpeB was preincubated for 15 min at 37 °C with different concentrations of E64 up to an equimolar ratio. Thus, the fraction of active SpeB was equal to the lowest amount of inhibitor required to totally abolish the proteinase activity. Preparations used in this study were 46% and 39% active. Throughout the paper the concentration of active SpeB is given.

**Analyses of the proteolytic effects of SpeB.** To determine the effects of SpeB on bacterial binding of intact Fn, the 30 kDa fragment of Fn, fibrinogen or IgG, bacteria were grown overnight, harvested, washed once in PBS and resuspended in PBS to an approximate concentration of 1 $\times$ 10<sup>10</sup> cells ml<sup>-1</sup>. SpeB was activated as above, and various amounts were added to the samples, together with DTT to a final concentration of 1 mM. The samples were incubated at 37 °C for 1 h and the bacteria were pelleted and resuspended in PBS supplemented with 0.05% Tween 20 and 0.02% sodium azide (PBSAT). Iodoacetamide (final concentration 6 mM) was added to inactivate SpeB. Bacteria were diluted to appropriate concentrations and binding assays with the respective radiolabelled ligands were performed (see below).

To determine the protective effect of bound plasma proteins against SpeB treatment, bacteria were cultured and harvested as above and incubated for 30 min in PBS supplemented with 10% plasma. Alternatively, the bacteria were incubated with ligands for proteins F1, M1 and H in concentrations corresponding to 10% plasma (Fn, 0.03 mg ml<sup>-1</sup>; fibrinogen, 0.3 mg ml<sup>-1</sup>; IgG, 1.2 mg ml<sup>-1</sup>; or albumin, 5 mg ml<sup>-1</sup>), or, for Fn, corresponding to undiluted plasma (0.3 mg ml<sup>-1</sup>). Activated SpeB and DTT (final concentration 1 mM) were added to the solution, or to washed bacteria. Alternatively, PBS and DTT were added. The bacteria were incubated for 1 h at 37 °C, and the reaction was terminated with iodoacetamide (final concentration 6 mM). To remove bound plasma proteins, bacteria were treated for 5 min with 0.1 M glycine-HCl, pH 2.0, and washed three times in PBS. Bacteria were diluted to appropriate concentrations and binding assays with the respective radiolabelled ligands were performed.

Binding assays were performed using intact human Fn (Sigma), a 30 kDa NH<sub>2</sub>-terminal fragment of Fn (Sigma), human fibrinogen (Sigma), or human IgG (Sigma), radiolabelled with Iodo-Beads (Pierce) according to the manufacturer's instructions. Free <sup>125</sup>I was separated from labelled protein on a PD10 column (Amersham

Pharmacia Biotech). The radiolabelled protein was incubated with bacteria for 30 min in PBSAT. After centrifugation (3000 *g* for 20 min), the radioactivity of the pellets was determined and expressed as a percentage of added radioactivity, deducting binding of the protein to the polypropylene tubes. Calculations for statistical significance were made using an unpaired Student's *t*-test, two-tailed. Comparisons were made between the binding of the different radiolabelled ligands to exponential-phase or stationary-phase bacteria, and no significant differences were seen. For the Scatchard plots, competitive binding assays were performed. In these assays, a fixed amount of radiolabelled protein was mixed with various amounts of unlabelled protein and added to AP1(pPTF8) bacteria. The amount of bound protein (*x*-axis) was plotted against the quotient of bound to free protein (*y*-axis) and a linear regression analysis was performed using the Instat (GraphPad) computer program.

**Adhesion and internalization assays.** AP1(pLZ12) or AP1(pPTF8) was grown overnight, washed once in PBS, treated with SpeB (10 µg active enzyme ml<sup>-1</sup> for 1 h at 37 °C) and diluted in minimal essential medium containing Earle's salts (MEM) (Life Technologies) and 10% fetal bovine serum. Human pharyngeal epithelial Detroit 562 cells (ATCC CCL-138) were grown in MEM supplemented with antibiotics, glutamine and 10% fetal bovine serum. Near-confluent monolayers of cells were washed three times in PBS and three times in non-supplemented MEM to remove antibiotics and serum. Subsequently, 1 ml bacterial suspension (5 × 10<sup>5</sup> c.f.u. ml<sup>-1</sup>) was added to each well and the plate was incubated for 2 h with 5% CO<sub>2</sub> at 37 °C, followed by five washes with PBS and release of adherent bacteria by trypsin treatment and cell lysis as described by Berry & Paton (1996). Alternatively, to determine the number of internalized bacteria, the wells were washed and incubated for another 2 h in 1 ml MEM supplemented with 100 µg gentamicin and 5 µg penicillin, followed by washing with PBS three times to remove antibiotics. Internalized bacteria were released as above. Viable counts were performed and the number of adherent and internalized bacteria was expressed as percentage of added bacteria. Calculations for statistical significance were made using an unpaired Student's *t*-test, two-tailed. The viability of the cell monolayers was examined by light microscopy and by the Suicide-Track test kit for apoptosis (Oncogene). No signs of necrosis or apoptosis were seen and there were no differences between the experimental setups.

**Western blot experiments.** Proteins were separated by SDS-PAGE and blotted onto Immobilon-P PVDF filters. The filters were subjected to immunodetection with polyclonal rabbit anti-Fn antibodies (Sigma) diluted 1:5000. Horseradish-peroxidase-conjugated goat anti-rabbit secondary antibodies were used and immunodetection was performed by chemiluminescence (Nesbitt & Horton, 1992).

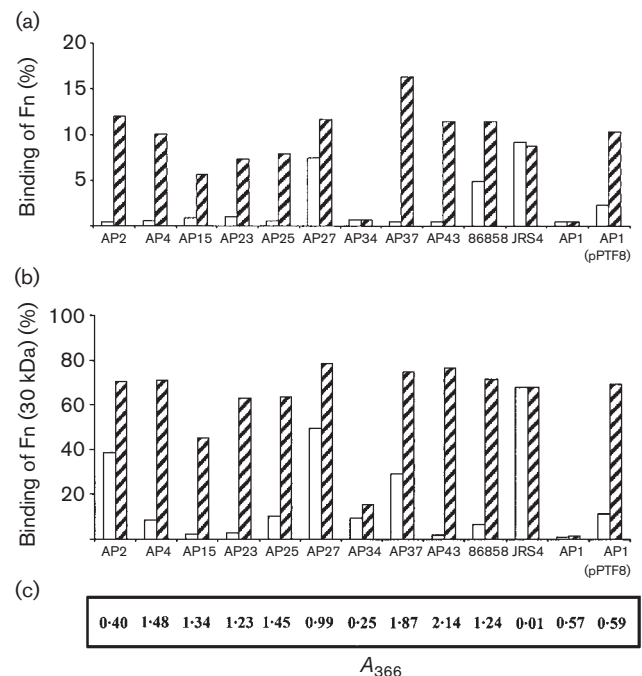
## RESULTS

### Growth of *S. pyogenes* in medium favouring SpeB expression reduces Fn-binding

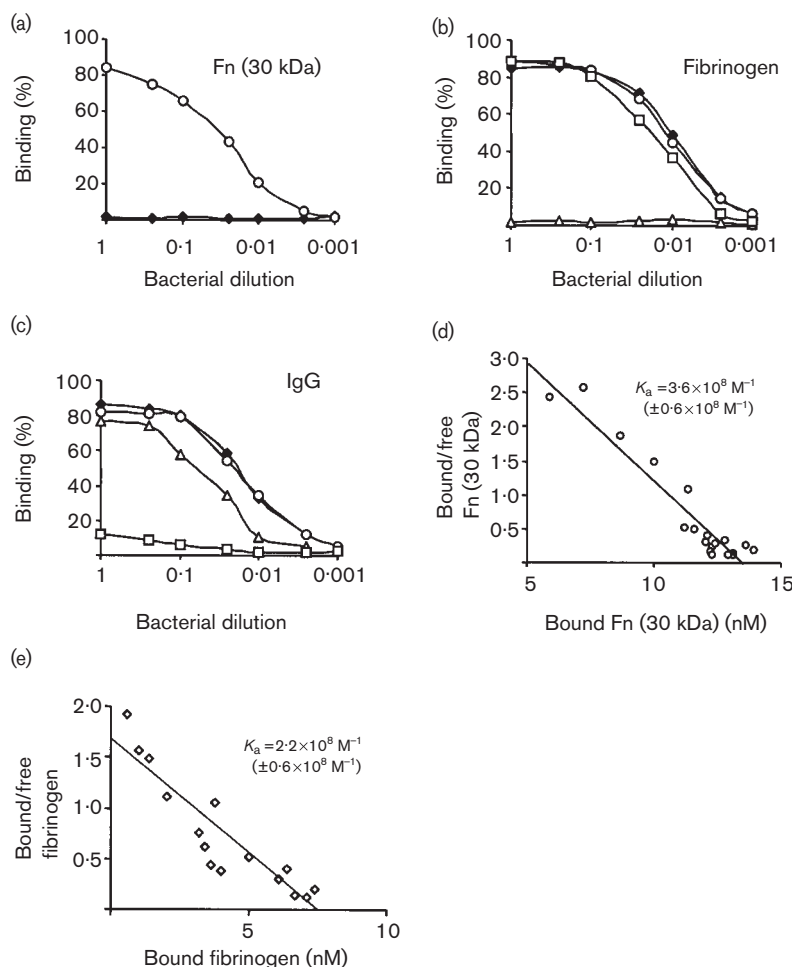
Proteolytic activity in the growth medium of several *S. pyogenes* strains was determined after overnight growth in CM, using azocasein as a substrate (data not shown). Twelve strains with proteolytic activity in the supernatant and one strain (JRS4) with no detectable activity were selected for further experiments. Growth in CM favours the expression of the streptococcal cysteine proteinase SpeB and the presence of the cysteine proteinase inhibitor E64 abolished enzymic activity in all examined strains.

The strains were examined for binding of radiolabelled Fn. All strains examined, except strain AP1, bound Fn when grown in the presence of E64, while all strains with proteolytic activity in the supernatant bound significantly less radioactivity when grown without E64 (Fig. 1a). Fn-binding proteins in Gram-positive bacteria bind the NH<sub>2</sub>-terminal part of Fn (Schwarz-Linek *et al.*, 2003). Therefore, the binding of a radiolabelled NH<sub>2</sub>-terminal fragment of Fn (30 kDa fragment) to bacteria grown in C-medium with or without E64 was determined. As with full-length Fn, growth without E64 markedly reduced binding of the 30 kDa fragment to strains with proteolytic activity in the supernatant (Fig. 1b). Thus, the presence of active SpeB appears to reduce Fn-binding to the bacteria. The binding of the 30 kDa fragment to the strains was higher, and showed less variation between experiments, than that of full-length Fn, making the 30 kDa fragment a more accurate tool for further studies.

No binding of Fn or the 30 kDa fragment to the AP1 strain was detected. However, AP1 bacteria expressing protein F1 [AP1(pPTF8)] bound high levels of the radiolabelled 30 kDa fragment (Fig. 1b). The binding to 400-fold-diluted



**Fig. 1.** Growth of *S. pyogenes* in conditions favouring proteolysis affects bacterial Fn-binding. Different *S. pyogenes* strains were grown overnight (20 h) in CM without (open bars) or with (hatched bars) the cysteine protease inhibitor E64. (a) Binding of radiolabelled Fn. (b) Binding of radiolabelled 30 kDa Fn fragment. Binding is shown as the percentage of added radioactivity measured in the bacterial pellets. (c) The supernatants from bacteria grown without E64 were analysed for proteolytic activity by using an azocasein assay. The cleavage of azocasein was measured as A<sub>366</sub>.



**Fig. 2.** Protein-binding properties of various *S. pyogenes* strains. (a) Binding of the radio-labelled 30 kDa Fn fragment to different dilutions of AP1 (◆) or AP1(pPTF8) (○) bacteria. (b) Binding of radiolabelled fibrinogen to different dilutions of AP1 (◆), AP1(pPTF8) (○), MC25 (△) or BM27.6 (□) bacteria. (c) Binding of radiolabelled IgG to different dilutions of AP1 (◆), AP1(pPTF8) (○), MC25 (△) or BM27.6 (□) bacteria. (d) Scatchard plot for the interaction between the 30 kDa Fn fragment and  $2 \times 10^7$  AP1(pPTF8) bacteria. The affinity constant  $\pm$  SD is indicated. (e) Scatchard plot for the interaction between fibrinogen and  $4 \times 10^6$  AP1(pPTF8) bacteria. The affinity constant  $\pm$  SD is indicated.

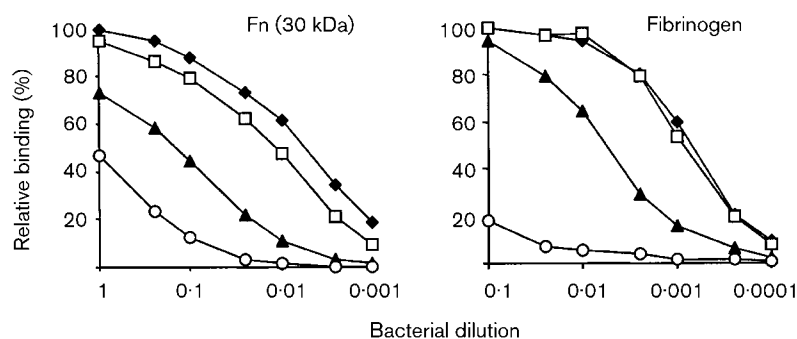
AP1(pPTF8) bacteria was clearly above background, whereas undiluted AP1 bacteria (Fig. 2a) showed no binding, demonstrating that the interaction between the 30 kDa fragment and AP1(pPTF8) bacteria is mediated only by protein F1. Furthermore, neither AP1 nor AP1(pPTF8) expressed SpeB when grown in THY medium (data not shown), which made this strain an accurate tool for studying the effects of SpeB on the binding of Fn and other plasma protein ligands to *S. pyogenes*, without interference of endogenous SpeB. To further characterize the binding properties of this strain, binding of radiolabelled fibrinogen and IgG to AP1, AP1(pLZ12), AP1(pPTF8), MC25 (a mutant of AP1 lacking surface-associated M1 protein: Collin & Olsén, 2000), or BM27.6 (a mutant of AP1 lacking surface-associated protein H: Kihlberg *et al.*, 1999) bacteria was determined. The results, shown in Fig. 2(b, c), demonstrate that binding of fibrinogen to the AP1 strain is mediated exclusively by the M1 protein. Binding of IgG to MC25 bacteria was somewhat reduced, whereas the BM27.6 strain showed very low IgG-binding, confirming earlier results that protein H is the main contributor to IgG-binding in AP1 bacteria (Kihlberg *et al.*, 1999). The AP1(pLZ12) strain behaved like the AP1 strain in all

binding assays and the binding data for this strain are not shown.

Scatchard plots were performed to determine the affinity constants for the interactions between AP1(pPTF8) bacteria and the 30 kDa fragment (Fig. 2d) or fibrinogen (Fig. 2e). The  $K_a$  values for the two interactions were very similar:  $3.6 \times 10^8 \text{ M}^{-1}$  and  $2.2 \times 10^8 \text{ M}^{-1}$ , respectively, and comparable to previously published affinity constants for the interactions between protein F and fibronectin ( $1.8 \times 10^8 \text{ M}^{-1}$ ; Katerov *et al.*, 1998) and M1 protein and fibrinogen ( $2.5 \times 10^8 \text{ M}^{-1}$ ; Ringdahl *et al.*, 2000). The calculated number of binding sites at the bacterial surface was  $8.1 \times 10^4$  for the 30 kDa fragment, and  $2.2 \times 10^5$  for fibrinogen.

### Protein F1 is sensitive to proteolysis by SpeB

Growth in CM significantly reduced the binding of labelled 30 kDa fragment to bacteria expressing protein F1 [Figs 1 and 2a, AP1(pPTF8)]. SpeB cleaves and releases fragments of M1 protein, protein H, SclB and the C5a peptidase from the bacterial surface (Berge & Björck, 1995; Rasmussen & Björck, 2001). To evaluate whether protein F1 also is a



**Fig. 3.** Sensitivity of bacterial surface proteins to SpeB treatment. AP1(pPTF8) bacteria were grown in THY, harvested, and treated with different concentrations of SpeB (□, 0.1 µg ml<sup>-1</sup>; ▲, 2 µg ml<sup>-1</sup>; ○, 10 µg ml<sup>-1</sup>; ◆, untreated). Binding of the radiolabelled 30 kDa Fn fragment and fibrinogen was determined for different bacterial dilutions. The relative binding compared to the binding to undiluted and untreated bacteria ( $2 \times 10^9$  c.f.u. ml<sup>-1</sup>) is shown. Data are from one representative experiment.

substrate for SpeB, AP1(pPTF8) bacteria were treated with different concentrations of SpeB, and the binding of fibrinogen (ligand for the M1 protein; Kantor, 1965) and the 30 kDa fragment was analysed. In these experiments, any reduction of the binding of the ligands would be due to cleavage of these bacterial surface proteins by SpeB. The binding of the 30 kDa fragment and fibrinogen to serial dilutions of bacteria shows that protein F1 is more susceptible to SpeB cleavage than the M1 protein (Fig. 3). Even at a low concentration (0.1 µg ml<sup>-1</sup>), SpeB treatment reduced the binding of the 30 kDa fragment to  $2 \times 10^7$  bacteria ml<sup>-1</sup> by 31% ( $\pm 11\%$ ; 95% confidence interval). With 2 µg ml<sup>-1</sup> of SpeB, the binding was reduced by 81% ( $\pm 8\%$ ; 95% confidence interval). In contrast, treatment with 0.1 µg ml<sup>-1</sup> of SpeB reduced the fibrinogen binding by only 0.3% ( $\pm 5.3$ ; 95% confidence interval), whereas at 2 µg ml<sup>-1</sup> of SpeB, the reduction was 20% ( $\pm 13\%$ ; 95% confidence interval). The results demonstrate that cleavage of protein F1, compared to the M1 protein, occurs at much lower SpeB concentrations. At a higher concentration of SpeB (10 µg ml<sup>-1</sup>), the binding of both ligands is markedly affected. Thus, proteinase-treated bacteria bound less radiolabelled 30 kDa fragment and fibrinogen than a 100-fold smaller number of untreated bacteria (Fig. 3). To determine the SpeB cleavage site(s) of protein F1, AP1(pPTF8) and AP1(pLZ12) bacteria were treated with various concentrations of SpeB and the supernatants were subjected to Western blotting with radiolabelled Fn or the 30 kDa fragment. No bands reacting with the radiolabelled probes were detected. Alternatively, the supernatants were separated by SDS-PAGE and silver stained (data not shown). No additional bands were seen when comparing supernatants from AP1(pPTF8) and AP1(pLZ12). These results suggest that protein F1 is completely degraded when exposed to SpeB on the bacterial surface.

### SpeB treatment reduces the internalization of bacteria expressing protein F1

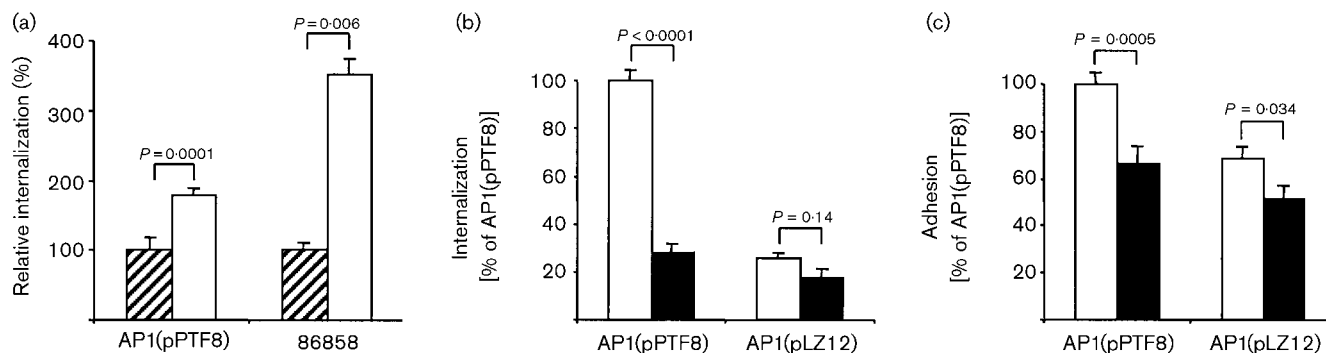
Since SpeB has been reported both to decrease (Chaussee *et al.*, 2000) and to increase (Tsai *et al.*, 1998) internalization of *S. pyogenes*, the effect of SpeB on internalization into epithelial cells was determined. AP1(pPTF8) expressing protein F1 and the high SpeB-expressing 86858 strain were grown in C-medium, or in C-medium complemented with

E64, respectively. The addition of E64 to the growth medium increased the internalization of AP1(pPTF8) about twofold and the internalization of 86858 more than threefold (Fig. 4a). To study the effect of SpeB on internalization without interference from endogenous SpeB, AP1(pPTF8) and AP1(pLZ12), lacking protein F1, were grown in THY medium, washed and treated with 10 µg ml<sup>-1</sup> of SpeB, which removes >99% of proteins F1 and M1 from the bacterial surface (Fig. 3). On average, the internalization of untreated AP1(pPTF8) bacteria was 3.9%, which was significantly decreased by SpeB digestion (Fig. 4b). The internalization of digested AP1(pPTF8) was 27% that of untreated bacteria ( $\pm 8\%$ , 95% confidence interval). The small difference seen in internalization between untreated and treated AP1(pLZ12) bacteria was not significant, indicating that the M1 protein is of minor importance for internalization in the experimental system used.

To investigate whether the reduced internalization was accompanied by reduced adhesion, bacterial adhesion to epithelial cells was determined (Fig. 4c). The mean adhesion of untreated AP1(pPTF8) bacteria was 86%. SpeB treatment (see above) resulted in a modest, but significant ( $P < 0.001$ ), decrease in adhesion. The mean adhesion of the treated AP1(pPTF8) bacteria was 66% ( $\pm 13\%$ , 95% confidence interval) of untreated AP1(pPTF8) bacteria. SpeB-treated AP1(pLZ12) bacteria adhered slightly less than untreated AP1(pLZ12) bacteria ( $P < 0.05$ ). These results suggest that both proteins F1 and M1, and probably additional factors, promote adhesion to epithelial cells in the system used.

### Plasma proteins protect proteins H and M1, but not protein F1, from cleavage by SpeB

Proteins F1, H and M1 bind various plasma proteins, which raised the question whether these surface proteins are also cleaved by SpeB in a plasma environment. In a first series of experiments, we investigated the binding of radiolabelled fibrinogen and the 30 kDa fragment to AP1(pPTF8) bacteria (Fig. 5a). The bacteria were preincubated with PBS or 10% plasma, washed, and treated with SpeB. Following digestion and washing, plasma proteins still bound to the bacterial surface were eluted with low



**Fig. 4.** Effect of SpeB on bacterial internalization and adhesion. (a) Internalization of strains AP1(pPTF8) or 86858, grown without (striped bars) or with (open bars) E64 in the growth medium, into a pharyngeal cell line. The values are from one representative experiment with triplicate samples,  $\pm$  SD. (b) Internalization of AP1(pPTF8) or AP1(pLZ12), untreated (open bars) or treated (black bars) with  $10 \mu\text{g ml}^{-1}$  active SpeB, into a pharyngeal cell line. The values are the means from four independent experiments, with triplicate samples in each,  $\pm$  SEM. (c) Adherence of AP1(pPTF8) or AP1(pLZ12), untreated (open bars) or treated (black bars) with  $10 \mu\text{g ml}^{-1}$  active SpeB, to a pharyngeal cell line. The values are the means from three independent experiments, with triplicate samples in each,  $\pm$  SEM.

pH. Bacteria were also incubated simultaneously with 10% plasma and SpeB, followed by washing and acid treatment. As a positive control, bacteria were preincubated with 10% plasma and treated with acid without the addition of SpeB. Following these pretreatments, bacteria were subjected to binding experiments with radiolabelled ligands. The results show that protein F1 is still highly susceptible to SpeB cleavage following preincubation with plasma (Fig. 5a). Moreover, when SpeB and plasma are added simultaneously to the bacteria, the binding of the 30 kDa fragment is still significantly reduced, despite the fact that plasma contains several additional substrates for SpeB and  $\alpha_2$ -macroglobulin, which is an inhibitor of SpeB (Rasmussen *et al.*, 1999). In contrast, plasma proteins bound to M1 protein efficiently protected this surface protein against degradation by SpeB.

The difference in susceptibility to SpeB was further emphasized when the binding of radiolabelled proteins to the bacteria, pretreated as above, was tested at a single bacterial concentration ( $5 \times 10^7$  c.f.u.  $\text{ml}^{-1}$ ) (Fig. 5b). In these experiments radiolabelled IgG was also included as a ligand to analyse the effect of SpeB on protein H, the dominating IgG-binding protein of AP1 bacteria (Åkesson *et al.*, 1990) (the schematic structure and protein-binding sites of proteins H and M1 are shown in Fig. 5c). The data demonstrate that binding of plasma proteins to proteins H and M1 prevents degradation by SpeB, whereas protein F1 is not protected. Also purified fibrinogen and IgG at concentrations corresponding to 10% plasma partially inhibited SpeB degradation of proteins H and M1, while Fn and albumin did not affect the removal of fibrinogen- and IgG-binding activity by SpeB. Moreover, none of these plasma proteins could protect protein F1 against SpeB cleavage (Fig. 5b). To further verify that bound Fn could not protect protein F1, AP1(pPTF8) bacteria were preincubated with purified Fn at a concentration

corresponding to undiluted plasma ( $0.3 \text{ mg ml}^{-1}$ ) and binding of radiolabelled Fn and the 30 kDa fragment to the bacteria was then determined. The reduction in binding after SpeB treatment was 78% ( $\pm 15\%$ , 95% confidence interval) for radiolabelled Fn and 82% ( $\pm 4.6\%$ , 95% confidence interval) for the 30 kDa fragment.

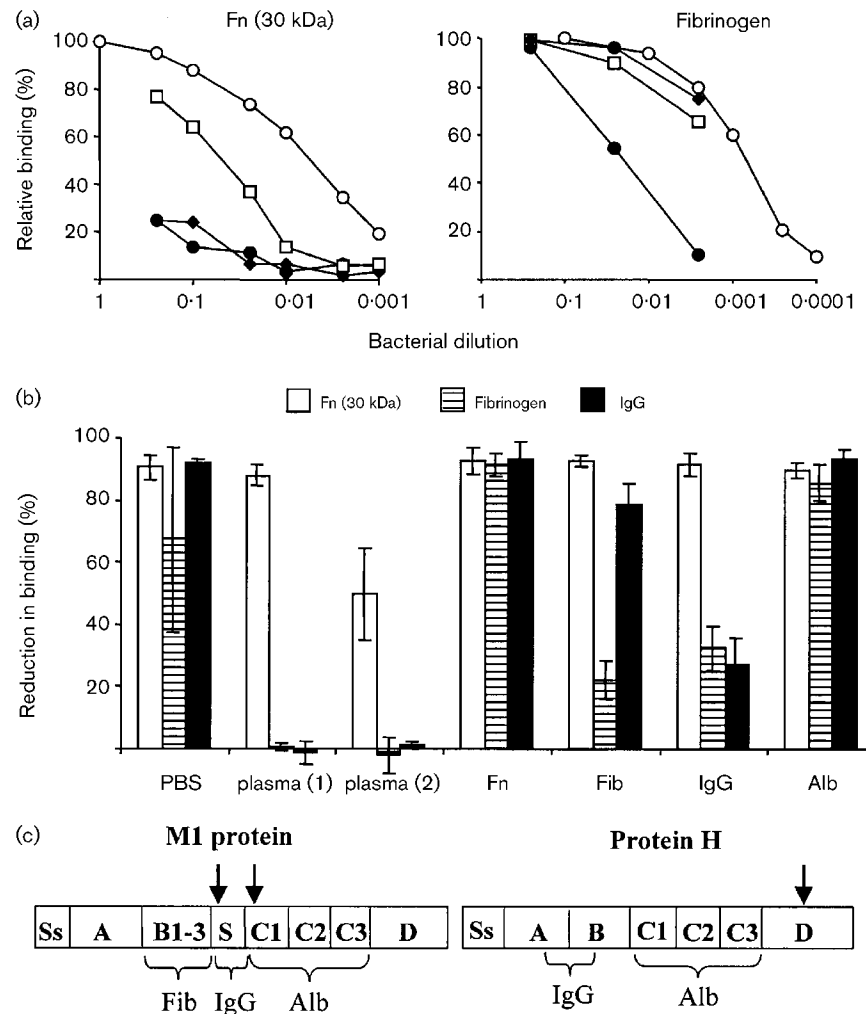
#### SpeB reduces the binding of Fn to different strains of *S. pyogenes* also after plasma preincubation

Different *S. pyogenes* strains that did not produce SpeB in THY medium (data not shown) were preincubated with PBS or 10% plasma, washed, treated with SpeB, and tested for binding of the 30 kDa fragment and fibrinogen (Fig. 6). In all strains tested, SpeB totally abolished the binding of the 30 kDa fragment after preincubation with PBS, and despite preincubation with plasma, the binding of the 30 kDa fragment was efficiently reduced in all strains. Strains of the M49 serotype lack the *prtF1* gene (Jaffe *et al.*, 1996), but Fn-binding in the AP49 strain is still sensitive to SpeB treatment, indicating that other Fn-binding proteins are also susceptible to proteolysis by SpeB. In contrast, after preincubation with PBS the fibrinogen-binding of some strains was not reduced by SpeB treatment, and after preincubation with plasma, the fibrinogen-binding in all examined strains was barely affected by SpeB. Our data demonstrate that SpeB can effectively regulate *S. pyogenes* Fn-binding, whereas fibrinogen-binding is more resistant to SpeB treatment, particularly in a plasma environment.

#### Fn bound to the bacterial surface can be released and processed by SpeB

Fn is cleaved by SpeB (Kapur *et al.*, 1993b). To investigate whether Fn is degraded also when bound to the bacterial

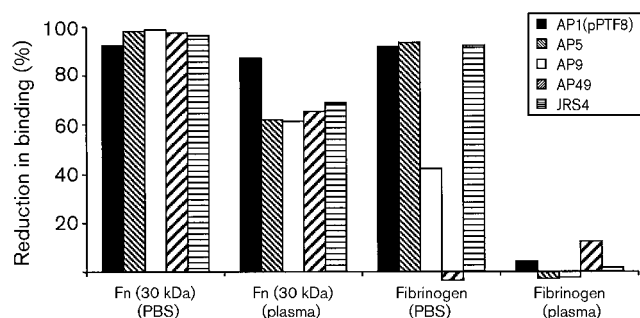




**Fig. 5.** Plasma proteins protect M1 protein and protein H, but not protein F1, against degradation by SpeB. (a) AP1(pPTF8) bacteria were pretreated as follows: (1) pre-incubation with plasma, washing with PBS, treatment with 0.1 M glycine, pH 2.0 (○); (2) preincubation with PBS, washing with PBS, digestion with SpeB, treatment with 0.1 M glycine, pH 2.0 (●); (3) preincubation with 10% human plasma, washing with PBS, digestion with SpeB, treatment with 0.1 M glycine, pH 2.0 (◆); (4) simultaneous preincubation with 10% human plasma and SpeB, washing with PBS, treatment with 0.1 M glycine, pH 2.0 (□). Following pretreatment, different dilutions of the bacteria (starting at  $2 \times 10^9$  cells  $\text{ml}^{-1}$ ) were tested for the binding of radiolabelled 30 kDa Fn fragment or fibrinogen. The relative binding compared to the binding of undiluted bacteria, pretreated with 10% plasma but not treated with SpeB, is shown. Data are from one representative experiment. (b) AP1(pPTF8) bacteria were preincubated, washed, digested with SpeB and treated with 0.1 M glycine, pH 2.0. Preincubations before SpeB digestion were as follows: PBS, PBS; plasma (1), 10% plasma; plasma (2), 10% plasma and SpeB simultaneously; Fn, purified Fn; Fib, purified fibrinogen; IgG, purified IgG; Alb, purified albumin. Subsequently, the binding of radiolabelled 30 kDa Fn fragment, fibrinogen or IgG to  $5 \times 10^7$  AP1(pPTF8) bacteria  $\text{ml}^{-1}$  was determined. The reduction in binding relative to bacteria preincubated with the respective ligand but not digested with SpeB is shown. Values are the means of three independent experiments  $\pm$  SEM. (c) Proteins M1 and H are schematically depicted. The  $\text{NH}_2$ -terminal signal sequences (Ss) and the binding sites for fibrinogen (Fib), IgG and albumin (Alb) are indicated. Arrows mark the sites preferentially cleaved by SpeB (Berge & Björck, 1995).

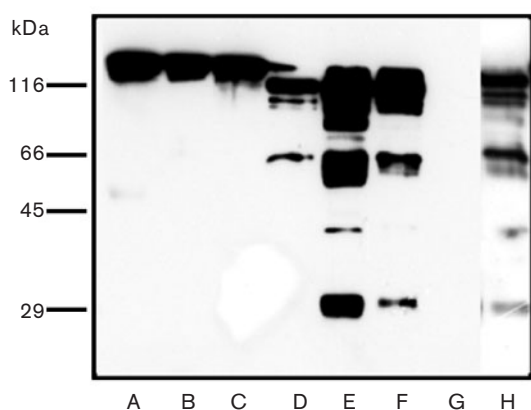
surface, AP1(pPTF8) bacteria were incubated with purified Fn or a 10% plasma solution for 30 min. The bacteria were washed and SpeB was added (final concentration  $10 \mu\text{g ml}^{-1}$ ). As a control, SpeB ( $10 \mu\text{g ml}^{-1}$ ) was added to purified Fn or 10% plasma. Bacteria were pelleted by centrifugation and supernatants were subjected to Western

blotting with anti-Fn antibodies (Fig. 7). Whereas purified Fn was completely degraded to distinct fragments (Fig. 7, lane D), Fn in the plasma sample was not cleaved (Fig. 7, lane C). In contrast, after binding to the bacterial surface, both purified Fn and Fn absorbed from plasma were cleaved by SpeB (Fig. 7, lanes E and F). To ensure that released



**Fig. 6.** Effect of SpeB digestion on the binding of the 30 kDa Fn fragment and fibrinogen to different *S. pyogenes* strains. Bacteria were incubated in PBS or plasma (10%), washed, and treated with SpeB ( $10 \mu\text{g ml}^{-1}$ ). Reduction in binding of the radiolabelled 30 kDa Fn fragment or fibrinogen to SpeB-treated bacteria ( $5 \times 10^7$  cells  $\text{ml}^{-1}$ ) is shown.

surface components of *S. pyogenes* did not react with the anti-Fn antibodies, bacteria preincubated with buffer were treated with  $10 \mu\text{g ml}^{-1}$  of SpeB as above; no material reacting with the antibody was released (Fig. 7, lane G). Limited cleavage of bound Fn was seen also when AP1(pPTF8) bacteria were incubated with PBS (Fig. 7, lane H), suggesting that some endogenous SpeB production occurred during the incubation period.



**Fig. 7.** Cleavage of fibronectin by SpeB. Proteins were separated by SDS-PAGE (10% acrylamide; reducing conditions) and the gel was subjected to Western blotting using an anti-Fn antibody. A, Untreated human plasma. B, Untreated purified Fn. C, Human plasma digested with SpeB. D, Purified Fn digested with SpeB. E, Supernatant from bacteria preincubated with human plasma, washed, and digested with active SpeB. F, Supernatant from bacteria preincubated with Fn, washed and digested with SpeB. G, Supernatant from bacteria preincubated with PBS, then digested with SpeB. H, Supernatant from bacteria preincubated with Fn, washed and incubated with PBS. All incubations were for 1 h and SpeB digestions were done with  $10 \mu\text{g}$  active enzyme  $\text{ml}^{-1}$ .

## DISCUSSION

The ability of *S. pyogenes*, previously considered an exclusively extracellular pathogen, to enter eukaryotic cells has been demonstrated by several studies (see Courtney *et al.*, 2002, for a recent review). Bacterial Fn-binding seems to be crucial in this context, and the requirement of Fn for bacterial internalization has been firmly established (Cue *et al.*, 1998; Jadoun *et al.*, 1997; Ozeri *et al.*, 1998; Talay *et al.*, 2000). SpeB, secreted in substantial amounts by most *S. pyogenes* strains, has also been examined for a possible contribution to bacterial internalization. As the results of these previous investigations have been contradictory, the aim of the present study was to determine whether protein F1, the most thoroughly studied streptococcal protein responsible for Fn-binding and internalization, is a substrate for SpeB. Such an assumption was supported by previous findings showing that SpeB cleaves and releases other cell-wall-attached proteins of *S. pyogenes*. (Berge & Björck, 1995; Raeder *et al.*, 1998; Rasmussen & Björck, 2001).

The finding that protein F1 is cleaved from the streptococcal surface at low concentrations of SpeB suggests that loss of Fn-binding occurs soon after SpeB secretion starts. As expected, the removal of protein F1 from the bacterial surface results in a significant decrease in adhesion and internalization, emphasizing the importance of Fn for these processes. Interestingly, the cleavage of the M1 protein did not significantly affect internalization, suggesting a minor role for this protein in the internalization of the AP1 strain. The production and secretion of SpeB depends upon the nutritional state of the bacterium (Chaussee *et al.*, 1997). In most *S. pyogenes* strains, SpeB secretion *in vitro* will not take place until the bacteria start to reach the stationary growth phase, suggesting that SpeB production *in vivo* is favoured by the lack of nutrients. Cleavage of protein F1 by SpeB when the bacteria are short of nutrients will interfere with adhesion and internalization, and should facilitate bacterial spread and thus allow relocation to new and more fertile sites.

The susceptibility of streptococcal surface proteins to proteolytic cleavage presents a potential problem for the bacterium, as, in addition to proteinases produced by the bacterium, many host proteinases will be present at the site of infection (complement, contact phase, coagulation factors, and intracellular proteinases released from neutrophils and dying cells) (for a review see Rasmussen & Björck, 2002). One strategy by which *S. pyogenes* evades proteolysis and protects its surface proteins is by recruiting the broad-specificity proteinase inhibitor  $\alpha_2$ -macroglobulin through the surface protein GRAB. Thus,  $\alpha_2$ -macroglobulin bound to GRAB protects surface proteins from degradation by trypsin and SpeB (Rasmussen *et al.*, 1999). Some *S. pyogenes* strains, including AP1, do not bind  $\alpha_2$ -macroglobulin (Rasmussen *et al.*, 1999), but the present work identifies another mechanism whereby plasma proteins can protect proteins H and M1 from cleavage by SpeB. Bacterial

fibrinogen- and IgG-binding are sensitive to SpeB when AP1 bacteria are preincubated with PBS, but protected following preincubation with plasma. As fibrinogen-binding to AP1 bacteria is exclusively mediated by M1 protein, and IgG-binding mainly by protein H (Collin & Olsén, 2000; Kihlberg *et al.*, 1999; Fig. 2b, c), this suggests that proteins M1 and H are protected by the plasma proteins they bind. When examining which plasma proteins protect against SpeB cleavage, fibrinogen and IgG were found to protect M1-protein-mediated fibrinogen-binding. IgG binds with low affinity to the S domain of the M1 protein (Åkesson *et al.*, 1994), which is also the target for SpeB (Berge & Björck, 1995), suggesting that IgG bound to the S domain could sterically block SpeB. Alternatively, since IgG and fibrinogen are both substrates for SpeB (Collin & Olsén, 2001a, b; Matsuka *et al.*, 1999), M1-protein-bound IgG and fibrinogen could act as competing substrates. Protein H-mediated IgG-binding is protected by IgG, which indicates that IgG bound to protein H protects this protein from degradation by SpeB. This suggests substrate competition, as the binding site for IgG is located in the A and B domains (Åkesson *et al.*, 1994), whereas SpeB processes protein H in the D domain (Berge & Björck, 1995). Albumin binds to the C1–C3 domains of both the M1 protein and protein H (Åkesson *et al.*, 1994). Although SpeB cleaves the M1 protein in the C1 domain, albumin protects neither the M1 protein, nor protein H, from degradation by SpeB.

At the site of infection, plasma proteins of the inflammatory exudate are likely to cover the bacterial surface. As a result, proteins H and M1 will be at least partly protected from cleavage by SpeB. In contrast, plasma proteins offer little protection for protein F1, which is readily cleaved from the bacterial surface despite preincubation with plasma, even though Fn is a competing substrate for SpeB at the bacterial surface (Fig. 7). This striking difference implicates that the cleavage of Fn-binding proteins by SpeB could play an important biological role. Interestingly, and further supporting this notion, the difference was found in all strains examined (Fig. 6). The AP49 strain is of the M49 serotype, which does not carry the *prtF1* gene (Jaffe *et al.*, 1996). However, the binding of Fn to this strain is also susceptible to SpeB treatment. FbaB, a recently identified Fn-binding protein of *S. pyogenes* that is susceptible to SpeB cleavage (Terao *et al.*, 2002), could be responsible for the Fn-binding to this strain. In contrast, the fibrinogen-binding of some strains is highly resistant to SpeB also when plasma proteins are not present, and sensitive strains are protected by preincubation with plasma. The results suggest that Fn-binding surface proteins have evolved to be sensitive to SpeB, whereas some fibrinogen-binding proteins are inaccessible to SpeB per se, and some are protected by bound plasma proteins.

The significance of Fn-binding for streptococcal virulence is not clear. Fn-mediated entry of *S. pyogenes* into human cells has been thought to reflect an initial step in invasive

streptococcal infection. Recently, Fn-binding through the protein F1 homologue SfbI was shown to allow recruitment of collagens to the bacterial surface, thereby protecting the bacteria from phagocytosis (Dinkla *et al.*, 2003). On the other hand, isolates from cases of invasive disease often have low Fn-binding capacity (Natanson *et al.*, 1995) and internalize poorly (Molinari & Chhatwal, 1998). Furthermore, it was recently reported that a *Staphylococcus aureus* mutant deficient in a Fn-binding protein showed increased virulence in a rat model of pneumonia (McElroy *et al.*, 2002). These data, and those described here, underline the complexity of the molecular interplay between the different microbial and host factors that ultimately determine the outcome of an infection.

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