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The Fibronectin-Binding Protein of *Streptococcus pyogenes*, SfbI, Is Involved in the Internalization of Group A Streptococci by Epithelial Cells

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Streptococcus pyogenes **organisms (group A streptococci) are considered to be highly adhesive extracellular pathogens. However, it has recently been reported that *S. pyogenes* has the capacity to efficiently invade eukaryotic cells. In this study, we demonstrate that the interaction of *S. pyogenes* fibronectin-binding protein (SfbI) with fibronectin on nonphagocytic HEp-2 cells triggers bacterial internalization. Blocking of the SfbI adhesin by either antibodies against the whole protein or antibodies against the fibronectin-binding domains of SfbI, as well as pretreatment of HEp-2 cells with purified SfbI protein, prevents both *S. pyogenes* attachment and internalization. Inert latex beads precoated with the purified SfbI protein are ingested by eukaryotic cells, demonstrating that SfbI is per se enough to trigger the internalization process. Experiments performed with a recombinant SfbI domain encompassing the two fibronectin-binding regions of the SfbI molecule demonstrated that these binding regions are essential and sufficient to activate uptake by HEp-2 cells. These results demonstrate that the fibronectin-binding protein SfbI is involved in both *S. pyogenes*' attachment to and ingestion by HEp-2 cells and contribute to elucidation of the underlying molecular events leading to eukaryotic cell invasion by *S. pyogenes*.**

Streptococcus pyogenes (group A streptococcus) is an old pathogen that has renewed its reputation as an aggressive infectious agent in the last few years (15). This versatile pathogen can colonize different tissues and cause a wide range of diseases, such as throat and skin infections, highly invasive infections that may result in sepsis and shock, and serious sequelae, including rheumatic fever and acute glomerulonephritis (2).

Bacterial attachment to epithelial cells and colonization of mucosal surfaces are prerequisites for streptococcal infections (1). *S. pyogenes* adherence to host cells is a very complex process which involves multiple adhesins (3, 4, 32). Previous studies showed that fibronectin-binding proteins are essential for attachment to respiratory epithelial cells (10, 26) and Langerhans' cells (19) and that M protein mediates adhesion to keratinocytes (19) as well as to respiratory epithelial cells (32). Different streptococcal fibronectin-binding proteins have been described, of which the closely related proteins SfbI and F have been studied most extensively (10, 26, 27). Previous studies have documented the key role played by these proteins in adherence and pathogenesis (10, 11, 26). SfbI mediates streptococcal adherence to human respiratory epithelial cells via its fibronectin-binding domain (26). Epidemiological studies revealed that the SfbI-encoding gene (*sfbI*) is present in more than 70% of *S. pyogenes* isolates (30).

Although streptococci have been considered a model for extracellular pathogens, it has recently been shown that *S. pyogenes* has the capacity to invade and persist within human epithelial cells (18). However, the role played by streptococcal invasion of host cells in the biology of this microorganism remains to be elucidated. Moreover, the components involved in this process have not yet been identified.

Bacterial binding to the eukaryotic glycoprotein fibronectin, which in turn is bound to cell surface integrins, has been demonstrated to be essential for efficient attachment for several pathogens (24, 31). However, it is not known whether this interaction is also relevant to the streptococcal internalization process. This prompted us to analyze the involvement of the fibronectin-binding protein SfbI in the invasion of epithelial cells by *S. pyogenes*. In this study we demonstrate that SfbI not only mediates bacterial attachment to the eukaryotic cell line HEp-2 but is also required and sufficient to achieve efficient invasion of these epithelial cells.

MATERIALS AND METHODS

Bacterial strains and media. *S. pyogenes* A40 (T type 12), which expresses SfbI protein, was from the strain collection of our institute and was originally isolated from a throat culture. *S. pyogenes* DSM2071 (German Culture Collection) was used as a template for the amplification of *sfbI* gene fragments by PCR. *Escherichia coli* JM101 (22) was used for cloning and expression of recombinant fusion proteins. *S. pyogenes* strains were grown in Todd-Hewitt broth (Oxoid, Basingstoke, England) supplemented with 1% yeast extract (THY) or on THY agar. *E. coli* strains were grown in Luria-Bertani broth or agar (22).

Cell culture. The human laryngeal epithelial cell line HEp-2 (ATCC CCL23) was cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal calf serum (FCS; GIBCO), 5 mM glutamine (Flow Laboratories, Inc., McLean, Va.), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in an atmosphere containing 8% CO_2. For adherence and invasion assays, cells were resuspended at a concentration of approximately 3×10^5 cells per ml in DMEM and seeded into 24-well tissue culture plates (Nunc), which were then incubated for 24 h. For microscopic assays, cells were seeded onto 12-mm-diameter glass coverslips (Nunc) placed on the bottom of the tissue culture plates.

Construction and purification of SfbI fusion proteins. His-Tag fusion proteins were constructed for studying SfbI-cell interactions, whereas glutathione *S*-transferase (GST)-fusion proteins were constructed for the immunization of rabbits because of the adjuvant effect of the GST (29). A 1,581-bp *Bam*HI/*Eco*RI or

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*Bam*HI/*Sal*I fragment encompassing positions 274 to 1854 of the *sfbI* DNA sequence (27) was cloned into pGEX2T (Pharmacia) and pQE30 (Qiagen), thereby generating the recombinant plasmids pGST2 and pSTH2, respectively. These plasmids code for GST-SfbI and His-Tag-SfbI fusion derivatives (H2 fragment) lacking the N-terminal signal peptide and the C-terminal wall and membrane anchor regions. A 615-bp *Bam*HI/*Eco*RI or *Bam*HI/*Sal*I fragment encompassing positions 1240 to 1854 of the *sfbI* gene (27) was cloned into pGEX2T and pQE30, thereby generating the recombinant plasmids pGST12 and pSTH12, respectively. These plasmids encode GST-SfbI and His-Tag-SfbI fusion derivatives (H12 fragment) which lack the N-terminal half of the SfbI molecule and the C-terminal wall and membrane anchor regions but retain the two fibronectin-binding regions (i.e., the fibronectin-binding repeat region and the nonrepetitive fibronectin-binding spacer region). Then, a 984-bp *Bam*HI/*Sal*I fragment encompassing positions 274 to 1257 of the *sfbI* gene (27) was cloned into pGEX2T and pQE30, thereby generating the recombinant plasmids pGSTH10 and pSTH10, respectively. These plasmids encode GST-SfbI and His-Tag-SfbI fusion derivatives (H10 fragment) which contain the surface-exposed regions of SfbI lacking both the C-terminal and the fibronectin-binding regions.

Overexpression and purification of GST-fusion proteins from *E. coli* lysates were performed as previously described (28), whereas the His-Tag fusion proteins were purified under native conditions according to Qiagen protocols.

DNA manipulations were performed as described by Sambrook et al. (22), restriction and modification enzymes were obtained from New England Biolabs, Inc., and PCR amplifications were performed with a PCR reagent kit (Perkin-Elmer Cetus) in accordance with the manufacturer's instructions.

Antibody preparation and purification of anti-SfbI IgG. Rabbit polyclonal anti-*S. pyogenes* serum was prepared by immunizing a rabbit with heat-killed streptococci (70°C for 1 h). To obtain polyclonal antibodies against the SfbI fusion proteins H2, H12, and H10, rabbits were immunized twice subcutaneously with 500 μg of the purified recombinant proteins encoded by pGST2, pGST12, and pGST10. The serum immunoglobulin G (IgG) fraction containing the anti-SfbI antibodies was separated from sera by affinity chromatography on a protein A-agarose column as previously described (28). The antibodies specific for the GST domain and those cross-reacting with *E. coli* proteins were removed from the IgG fractions by repeating affinity chromatography on a column of agarose coupled with *E. coli* proteins and GST. The collected IgG fractions were shown to recognize SfbI protein but not GST in Western blots at dilutions ranging from 1:50 to 1:10,000. $F(ab')_2$ fragments were generated by pepsin digestion and separated from Fc fragments by standard procedures (12). The rabbit polyclonal antibody against the group C streptococcal fibrinogen-albumin-IgG-binding protein (anti-FAI antibody) used as a control in the inhibition experiments has been previously described (28).

Invasion assay. The invasion of bacteria was quantified by a standard antibiotic protection assay (6). Briefly, bacteria were inoculated in THY and grown at 37°C for 18 h. Then, they were collected by centrifugation (3,000 × *g*, 15 min), washed once with phosphate-buffered saline (PBS, pH 7.4), and resuspended in HEPES-DMEM (GIBCO) supplemented with 1% FCS. Approximately 3×10^7 bacteria were added per well (infection rate, 1:100), and plates were further incubated for 2 h. The wells were then washed twice with PBS, and extracellular bacteria were killed by adding fresh medium containing penicillin (5 μg/ml) and gentamicin (100 μg/ml). After a 2-h incubation, the monolayers were rinsed twice with PBS and then lysed with 500 μl of Triton X-100 (0.025% in H_2O). Appropriate bacterial dilutions were plated to determine the number of viable internalized bacteria. Results are expressed as averages of the numbers of bacteria recovered per well for three independent determinations ± standard errors of the means (SEM) in a single assay. The assays were repeated five times with similar results, and only the results from a single experiment are shown. Statistical analysis was performed by Student's paired *t* test. The A40 strain used throughout this study was considered highly invasive, since between 10^4 and 10^5 bacteria were recovered per well after 4 h of infection. The percent internalization of the A40 strain from results of a total of 11 tests was in the range of 0.1 to 1.9 (mean ± SEM, 0.68 ± 0.2). For inhibition assays, cell monolayers were preincubated with different concentrations of purified recombinant SfbI fusion protein for 60 min before the infection.

Assessment of *S. pyogenes* attachment and invasion by double immunofluorescence analysis. Immunofluorescence techniques were used to determine the numbers of extracellular and intracellular bacteria. Samples were processed 45 min after infection (see above), as longer incubation times resulted in large numbers of attached and/or ingested bacteria, which made quantification impossible. After infection, cells were washed and fixed with 3.7% formaldehyde in PBS. To block nonspecific antibody binding, 1% FCS in PBS was added for 30 min and then cells were incubated with a 1:100 dilution of a rabbit *S. pyogenes* antiserum for 1 h, washed three times with PBS, and further incubated with fluorescein isothiocyanate-labelled goat anti-rabbit IgG antibodies (Sigma) diluted 1:50. After 45 min the monolayers were washed three times, permeabilized with 0.1% Triton X-100 in PBS, washed four times, and incubated with the first antibody for another hour. The monolayers were then washed and further incubated for 45 min with a 1:50 dilution of tetramethylrhodamine isothiocyanate-labelled goat anti-rabbit IgG (Sigma) to stain both extracellular and intracellular bacteria. After being washed, samples were mounted and analyzed with a fluorescence microscope (Orthoplan 2; Leitz, Wetzlar, Germany). In each experiment, 50 cells were analyzed and the attachment and ingestion rates were determined by averaging the numbers of extracellular and intracellular bacteria per cell. The results were expressed as means ± SEM of three determinations performed on separate days. Statistical analysis was performed by Student's paired *t* test, and the differences were considered significant at a P of ≤ 0.05. As negative controls, nonpermeabilized monolayers were exposed to both fluorescent conjugants.

For inhibition assays, cell monolayers were preincubated with purified recombinant SfbI fusion protein (1 μg per well) for 60 min before the infection or bacteria were preincubated for 45 min with the crude decomplemented serum (1:10 dilution) or with the purified IgG fraction (25 μg of protein per test).

Interaction of SfbI-coated latex beads with HEp-2 cells. Approximately 10^8 latex beads (3-μm diameter; Sigma) were washed three times in PBS and then coated with 5 μg of the SfbI polypeptides encoded on pSTH2 (H2), pSTH12 (H12), and pSTH10 (H10) in 50 μl of PBS at 4°C overnight. Coated beads were washed once in PBS and then blocked with 200 μl of 10-mg/ml bovine serum albumin in PBS for 1 h at room temperature. Beads were washed twice in PBS and once in HEPES-DMEM (1% FCS) and then resuspended in 2 ml of HEPES-DMEM (1% FCS). The coupling efficiency was assessed as approximately 100% by flow cytometry with a FACScan (Becton Dickinson) and polyclonal antibodies against SfbI. HEp-2 cells were grown on 12-mm-diameter glass coverslips for scanning electron microscopy or on 6-cm-diameter tissue culture plates (Nunc) for transmission electron microscopy, and 300 μl or 1 ml of beads was added per well or plate, respectively. The cells were incubated for 1 h at 37°C (5% CO_2), washed five times with PBS, and fixed.

Electron microscopy. Cells were fixed in a solution containing 3% glutaraldehyde and 5% formaldehyde in cacodylate buffer for 45 min on ice and then washed with cacodylate buffer. For scanning electron microscopy, samples were dehydrated in a graded series of acetone and subjected to critical-point drying with CO_2. Samples were then covered with a 10-nm-thick gold film and examined with a Zeiss 982 Gemini digital scanning microscope. For transmission electron microscopy, samples were processed, sectioned, and examined as previously described (9).

RESULTS

The *S. pyogenes* SfbI protein is required for efficient attachment to and internalization by HEp-2 cells. The SfbI protein has been demonstrated to be a key factor for streptococcal attachment (26, 27). The SfbI domains involved in the interaction with human fibronectin and the regions encompassed by the recombinant proteins used throughout this work are shown in Fig. 1.

To assess the role of SfbI in *S. pyogenes* internalization, the effect of bacterial pretreatment with polyclonal antibodies directed against the GST-SfbI fusion proteins H2 and H12 (fibronectin-binding regions) was evaluated (Fig. 2). As expected, the attachment of the *S. pyogenes* A40 strain was inhibited by pretreatment with both anti-H2 and anti-H12 crude sera as well as by purified IgG anti-H2 and its $F(ab')_2$ fragments (Fig. 2). An even greater effect was observed on bacterial internalization with inhibition rates of between 96 and 80% compared to that of the untreated control. Similar results were obtained with either the crude serum specific for the fusion protein without the anchor (H2) or against the fibronectin-binding domain (H12) (Fig. 2). This suggests that binding to fibronectin is required to achieve both bacterial attachment and subsequent internalization. The observed effect was dose dependent, with maximum inhibition occurring at a dilution of 1:10 (data not shown). The inhibition of attachment observed after bacterial pretreatment with preimmune serum or IgG may be due to the ability of group A streptococci to bind IgG via its Fc domain, which in turn may lead to adhesin masking. However, the poor inhibition observed with antibodies specific for the SfbI H10 fragment (no fibronectin-binding domains) or the FAI protein (group C streptococcal protein that cross-reacts with the A40 M protein) ruled out a major contribution of nonspecific coating to the observed effect. The results obtained with the anti-FAI antibody suggest that although protein M also mediates adherence to HEp-2 cells (32), in the A40 strain SfbI is the major adhesin.

SfbI-mediated interaction of *S. pyogenes* with HEp-2 cells appears to be required not only for bacterial adherence but also for bacterial internalization. To confirm this hypothesis, HEp-2 cells were pretreated with the His-Tag-SfbI derivative

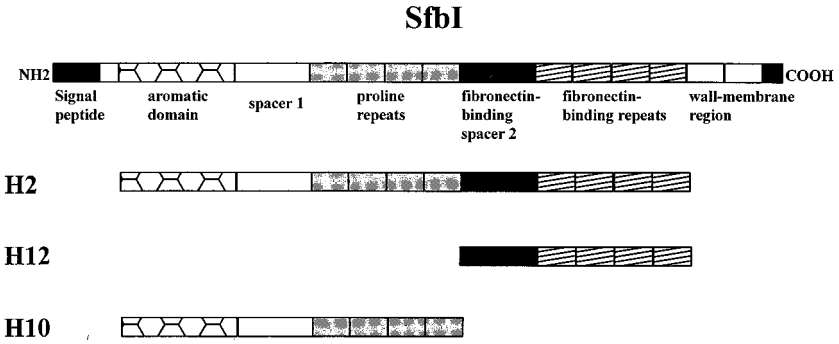


FIG. 1. Schematic representation of the fibronectin-binding protein SfbI. The SfbI regions encompassed by the fusion proteins that were used throughout this work (H2, H12, and H10) are also indicated.

H2 and the number of viable intracellular bacteria recovered 4 h after infection was recorded. Cellular pretreatment with the H2 polypeptides resulted in a concentration-dependent inhibition of bacterial invasion (Fig. 3A). Complete blocking of A40 invasion was observed with 1 μg of the protein; hence, this concentration was used for subsequent studies.

A reduction in the number of viable bacteria recovered from infected cells may be due either to a global reduction of internalized bacteria in every cell or to a reduction in the number of infected cells (i.e., bacteria may infect efficiently only a proportion of the cellular population). To discriminate between these two hypotheses and to evaluate the effect of the SfbI protein in both attachment and internalization, double immunofluorescence studies after a 45-min infection were performed. When cells were pretreated with the H2 polypeptide, both the attachment and the internalization of the *S. pyogenes* strain were almost completely abolished (means ± SEM, 1.5 ±

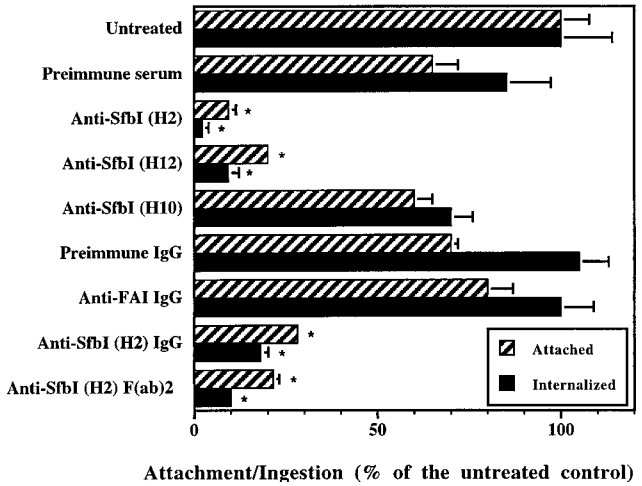
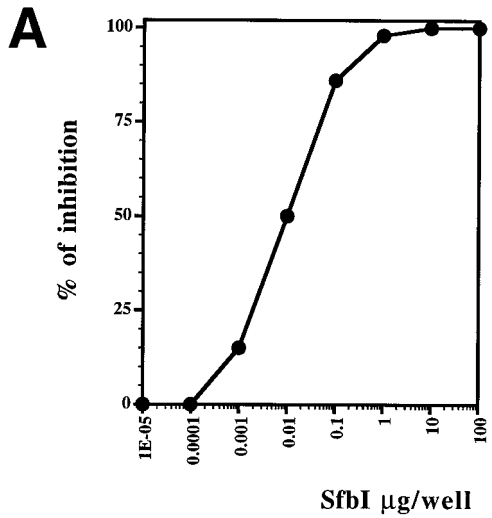


FIG. 2. Inhibition of *S. pyogenes* attachment and ingestion by pretreatment with anti-SfbI antibodies. *S. pyogenes* organisms were pretreated with antibodies, and after infection of HEp-2 cells, the numbers of extracellular and intracellular bacteria were quantified by double immunofluorescence analysis. The numbers of attached (mean ± SEM, 27 ± 2.5) and ingested (mean ± SEM, 16 ± 1.8) bacteria per cell in the absence of inhibitors (untreated) were recorded as 100%. Results are expressed as percentages of the untreated bacteria and are mean values of three independent experiments; SEM are represented by lines. The results are statistically significant compared with values for the untreated control, with a P of ≤0.05 (*). As controls, bacteria were pretreated with the preimmune serum, the purified IgG fraction corresponding to the preimmunized rabbit, and anti-FAI IgG.



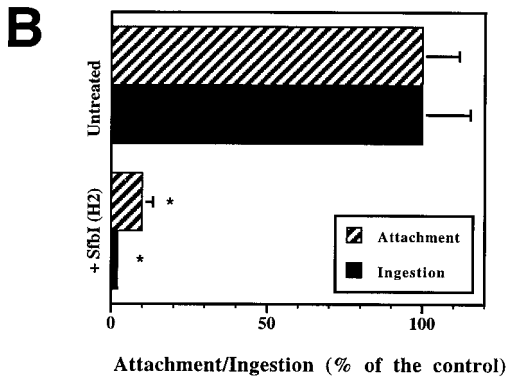


FIG. 3. Effect of pretreatment of HEp-2 cells with SfbI on *S. pyogenes* invasiveness. (A) The H2 polypeptide was added at different concentrations to the wells containing cells and further incubated for 1 h before infection with the SfbI-positive strain A40. Results are expressed as percentages of inhibition compared to values for the untreated control and are means of results of three independent tests; the SEM were lower than 10%. 1E-05, 1×10^{-5}. (B) The effect of HEp-2 cell pretreatment with the H2 peptide (1 μg per well) on bacterial attachment and invasion was evaluated as described in Materials and Methods. Results are expressed as percentages of the values for the untreated control and are means of results of three independent experiments; SEM are represented by lines. The results are statistically significant compared with results with the untreated control, with a P of ≤0.05 (*). The untreated control attachment and invasion averages ± SEM were 17 ± 2 and 13 ± 2 bacteria/cell, respectively.

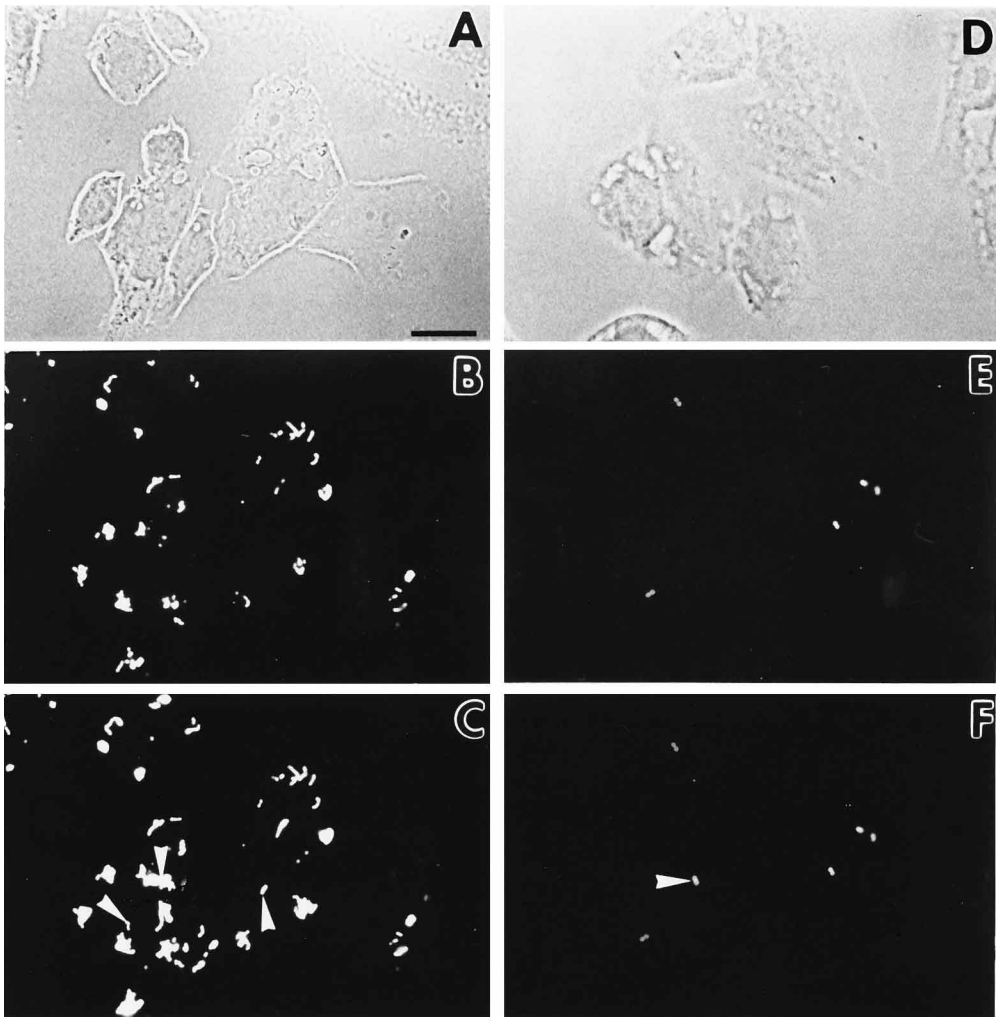


FIG. 4. Microscopic analysis of *S. pyogenes* interactions with untreated and SfbI-treated HEp-2 cells. Untreated (A to C) or H2-treated (D to F) HEp-2 cells were infected with the *S. pyogenes* A40 strain, and after 45 min of infection, samples were subjected to a double immunofluorescence assay. (B and E) Attached bacteria were labelled with fluorescein isothiocyanate. (C and F) Attached plus internalized bacteria were labelled with tetramethylrhodamine isothiocyanate. (A and D) Phase-contrast micrographs of the corresponding microscopic fields are shown. The H2 polypeptide was used at 1 μg/assay. Ingested bacteria are indicated by the arrowheads. Bars, 15 μm.

0.4 and 0.25 ± 0.1 bacteria/cell, respectively, versus 17 ± 2 and 13 ± 2 bacteria/cell in the untreated sample) (Fig. 3B and 4). We observed uniform inhibition of bacterial association with the eukaryotic cells, which was characterized by a global reduction in the number of attached bacteria and an almost complete abolishment of internalization. Pretreatment of HEp-2 cells with antibodies specific for fibronectin also resulted in 50% inhibition of bacterial uptake (data not shown). These results confirmed that SfbI-mediated binding to fibronectin is required for bacterial internalization.

Uptake of SfbI-coated latex beads by HEp-2 cells. The previous inhibition experiments demonstrated that SfbI protein is essential for the uptake of the A40 strain by HEp-2 cells. However, bacterial cells display multiple structures on their surfaces, and thus, we cannot exclude the possibility that structures other than SfbI are involved in bacterial uptake by nonphagocytic cells. To assess whether SfbI protein per se is able to mediate invasion, inert latex beads were coated with the purified His-Tag-SfbI derivatives H2 and H12 (fibronectin-binding domains). Latex beads were allowed to interact with HEp-2 cells in a fashion similar to that of intact bacteria, and then cells were monitored by scanning electron microscopy (Fig. 5). Uncoated and latex beads coated with H10, a SfbI derivative lacking the fibronectin-binding domains, hardly associated with HEp-2 cells (not shown). In contrast, the H2-coated particles not only bound to but were also efficiently taken up by HEp-2 cells (Fig. 5A). Latex beads coated with the H12 polypeptide, which encompasses the fibronectin-binding domains (fibronectin-binding repeats and spacer), attached to and were efficiently ingested by eukaryotic cells (Fig. 5B to F), exhibiting a morphological uptake pattern identical to that seen with beads coated with the H2 polypeptide. Transmission electron microscopy was performed with the HEp-2 cells infected with H2-coated latex beads (Fig. 6) to confirm the intracellular localization of the latex beads (Fig. 6C). Therefore, the role of SfbI in the streptococcal invasion process is confirmed, since the whole protein or even polypeptides encompassing only its fibronectin-binding domain are sufficient to trigger the uptake process of inert particles without the contribution of other bacterial factors.

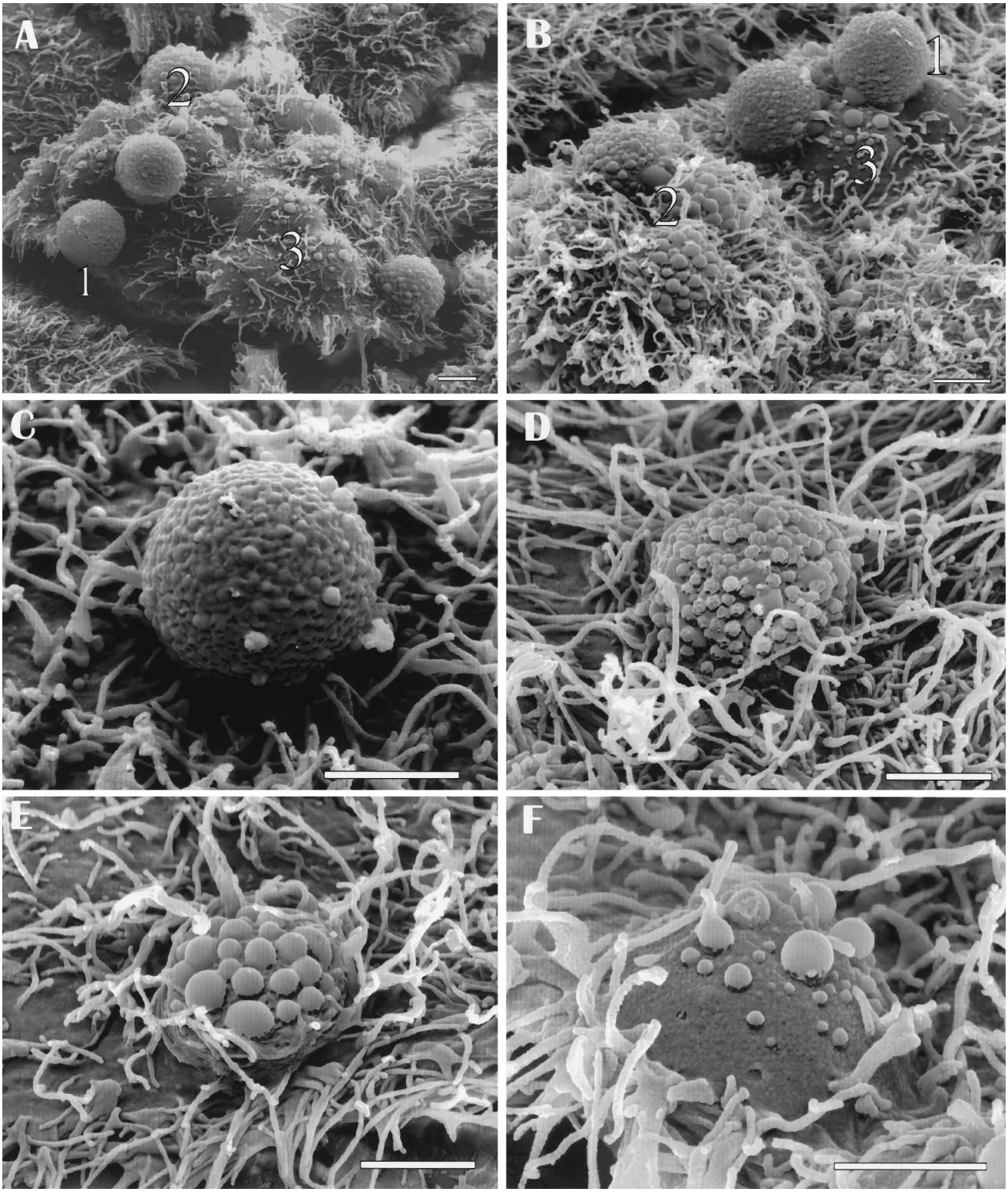


FIG. 5. Scanning electron microscopic analysis of the interaction of SfbI-coated latex beads with HEp-2 cells. Coated beads were allowed to interact with HEp-2 cells as described in Materials and Methods. Latex beads coated with H2 (A) and H12 (B) are attached (1) or in the process of internalization (2 and 3) by HEp-2 cells. (C to F) Sequence of the process of internalization of H12-coated latex beads by HEp-2 cells. Bars, 2 μm.

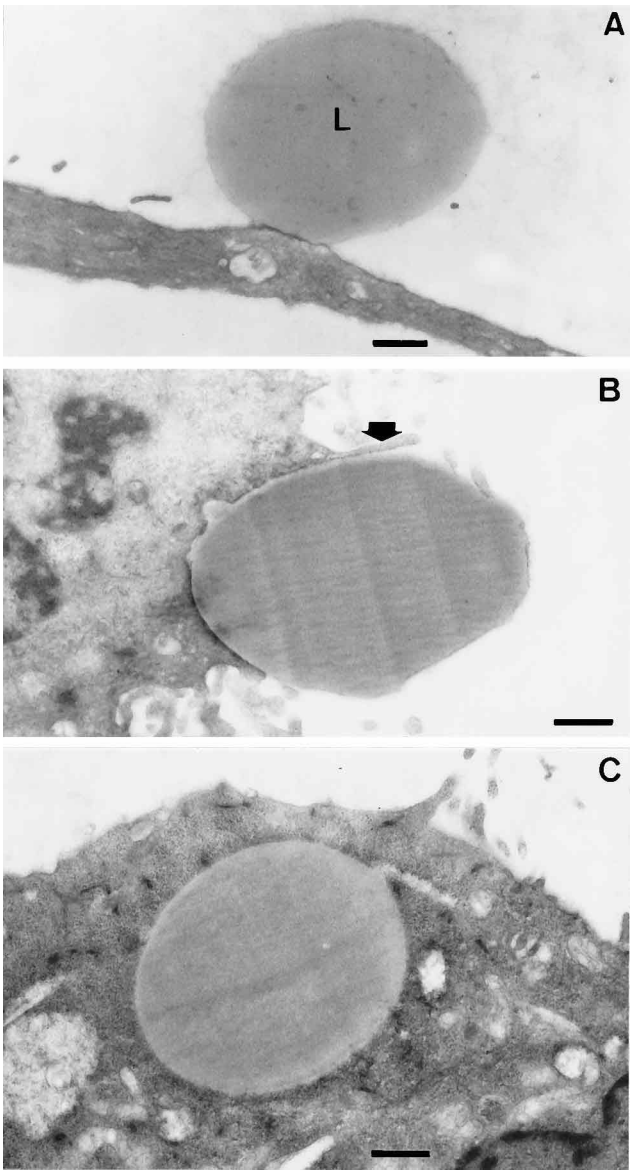


FIG. 6. Transmission electron microscopic analysis of the SfbI (H2)-coated latex bead uptake process by HEp-2 cells. (A) H2-coated latex bead (labelled L) attached to a cellular surface; (B) early stage of the uptake process, with the arrow indicating the membrane starting to flow around the bead; (C) final intracellular location of internalized H2-coated latex beads. Bars, 0.5 μm.

DISCUSSION

S. pyogenes was, until recently, a classic example of a highly adhesive extracellular pathogen. Therefore, research has been concentrated on the characterization of mechanisms leading to bacterial attachment to and colonization of mucosal surfaces. The binding of group A streptococci to the extracellular matrix protein fibronectin is considered an important virulence trait, since it allows bacterial colonization of host cells (25). In recent years, convincing evidence which supports the invasive potential of streptococci in general and *S. pyogenes* in particular has been produced (8, 18, 21). However, neither the invasin(s) nor the underlying mechanisms leading to bacterial internalization have been elucidated.

Our results demonstrate that the binding of SfbI protein to eukaryotic cells via fibronectin triggers bacterial internalization by nonphagocytic cells. The blocking of the SfbI adhesin by specific antibodies, either against the whole protein or against the fibronectin-binding domains of SfbI, prevented not only bacterial attachment but also internalization. Purified SfbI protein at a concentration of 1 μg per assay blocked streptococcal attachment and ingestion. This demonstrates that the interaction between SfbI, via its fibronectin-binding domain, and eukaryotic cells is essential for the entry process. The coating of inert latex beads with a purified polypeptide encompassing the fibronectin-binding domains of the SfbI protein was sufficient per se to activate the uptake process in HEp-2 cells. However, we cannot rule out the possibility that other structures may also be involved in the internalization processes of other *S. pyogenes* strains or in uptake by other eukaryotic cells.

The coating of bacteria with fibronectin normally leads to attachment to the surfaces of mammalian cells without subsequent internalization (7, 20, 31). In fact, it was believed that the only role of streptococcal fibronectin-binding proteins was to mediate attachment to host cells (5, 10, 11, 16, 26). However, other pathogens can use a similar strategy to invade eukaryotic cells. *Mycobacterium leprae*, an obligately intracellular pathogen, uses a fibronectin-binding protein to invade epithelial cells (23). The internalization of the bacillus Calmette-Guérin (*Mycobacterium bovis*) by the human bladder carcinoma cell line T-24 is mediated, at least in part, by the recognition of bacterium-bound fibronectin by an integrin (17).

The cellular receptors for fibronectin are members of the integrin superfamily of cell adhesion proteins. Integrins are large, αβ heterodimeric cell surface molecules that are involved in a variety of adhesive interactions (13). Integrin $\alpha_5\beta_1$ encompasses the classical fibronectin receptor, and fibronectin attachment to HEp-2 cells has been demonstrated to be mediated by this receptor (14). The direct binding of SfbI-positive bacteria to fibronectin, which in turn is attached via the RGD sequence to the cellular β_1 integrins, might allow *S. pyogenes* to use the eukaryotic receptor for entry into host cells. This hypothesis is also supported by the latex bead uptake and inhibition experiments. Other bacterial invasins may also bind directly to the integrins. In fact, the use of antibodies directed against $\alpha_5\beta_1$ resulted in blocking of invasin-mediated binding and internalization of *Yersinia pseudotuberculosis* (14, 33).

In conclusion, the results reported here highlight the role played by SfbI in streptococcal infections and allow us to understand the underlying molecular events of eukaryotic cell invasion by *S. pyogenes*. This may contribute to elucidation of the function of invasiveness during natural infections and to our knowledge of the strategies employed by facultative intracellular pathogens to enter host cells.

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