Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 6172–6176, July 1992
Microbiology

Protein F, a fibronectin-binding protein, is an adhesin of the group A streptococcus *Streptococcus pyogenes*

(fibronectin receptor/microbial adherence/virulence)

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Communicated by Stanley Falkow, March 6, 1992

ABSTRACT **Binding to fibronectin has been suggested to play an important role in adherence of the group A streptococcus *Streptococcus pyogenes* to host epithelial cells; however, the identity of the streptococcal fibronectin receptor has been elusive. Here we demonstrate that the fibronectin-binding property of *S. pyogenes* is mediated by protein F, a bacterial surface protein that binds fibronectin at high affinity. The gene encoding protein F (*prtF*) produced a functional fibronectin-binding protein in *Escherichia coli*. Insertional mutagenesis of the cloned gene generated a mutation that resulted in the loss of fibronectin-binding activity. When this mutation was introduced into the *S. pyogenes* chromosome by homologous recombination with the wild-type allele, the resulting strains no longer produced protein F and lost their ability to bind fibronectin. The mutation could be complemented by *prtF* introduced on a plasmid. Mutants lacking protein F had a much lower capacity to adhere to respiratory epithelial cells. These results demonstrate that protein F is an important adhesin of *S. pyogenes*.**

The initial event of most infectious diseases involves adherence of the infecting microorganism to tissues of the host by a process that is mediated by specific interactions between molecules present on both host and microbial cells (1). Many pathogens, including both bacterial (2–5) and parasitic species (6), bind to the eukaryotic matrix protein fibronectin and there is evidence to suggest that this fibronectin binding promotes adherence of these microorganisms to their target epithelial cells (5, 7). However, a rigorous assessment of the role of fibronectin binding in microbial adherence has not been possible because of the lack of a microorganism with a defined mutation in the receptor mediating fibronectin binding.

The Gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus), the causative agent of a number of suppurative diseases that are sometimes followed by rheumatic fever or acute glomerulonephritis, is an example of an organism that binds to fibronectin (4, 8). *S. pyogenes* initially attaches to and colonizes epithelial cells of the skin and pharyngeal mucosa (9). The fact that exogenous fibronectin inhibits this adherence (10) and that the organism preferentially adheres to fibronectin-coated epithelial cells (11) has suggested that fibronectin plays a major role in the adherence of *S. pyogenes*.

Further studies on the role of fibronectin binding in the adherence of *S. pyogenes* have been complicated by the fact that the streptococcal surface structure that is responsible for binding to fibronectin has not been clearly identified. Lipoteichoic acid (LTA), a polymer of glycerophosphate linked with the lipid moiety glycerophosphoryldiglucosyl diglyceride (12) has been implicated in binding to fibronectin. LTA is a major constituent of the cell wall of *S. pyogenes* and can inhibit the binding of fibronectin to intact streptococcal cells (10). However, it is the lipid moiety of LTA that interacts with fibronectin (10), and this region of the molecule is normally intercalated into the streptococcal cell membrane (13). The observation that the antiphagocytic M protein, a major cell-surface protein of *S. pyogenes* [reviewed by Fischetti (14)], can form a complex with purified LTA *in vitro* (15) has suggested that M protein, or some other uncharacterized surface protein, can orient LTA to expose the lipid moiety on the streptococcal cell surface and, thus, facilitate binding of the bacterium to fibronectin (7).

Other investigations have suggested that a surface protein of *S. pyogenes* distinct from either M protein or LTA is responsible for binding of the organism to fibronectin (8, 16–18). Fibronectin-binding proteins have been cloned and characterized from several Gram-positive organisms including *Staphylococcus aureus* (19) and one species of group C streptococci (*Streptococcus dysgalactiae*) (20). A gene encoding a fibronectin-binding protein has also been cloned from *S. pyogenes* (18). The role any of these proteins may have in promoting microbial adherence and an analysis of their contribution to the fibronectin-binding phenotypes of the intact microorganisms awaits construction of strains with defined mutations in the genes encoding these proteins.

In this report, we demonstrate that the high-affinity fibronectin-binding property of *S. pyogenes* is mediated by a fibronectin-binding surface protein, which we have named protein F. Insertional inactivation of the gene encoding protein F generated mutants of *S. pyogenes* that no longer bound to either soluble or immobilized fibronectin. Furthermore, these mutants were defective in their ability to adhere to respiratory epithelial cells when tested *in vitro*.

MATERIALS AND METHODS

Bacterial Strains and Media. *Escherichia coli* strains DH5α (Bethesda Research Laboratories) and HB101 (21) were used for molecular cloning and fibronectin-binding experiments. *S. pyogenes* JRS4 (22) produces a type 6 M protein and is a spontaneous streptomycin-resistant derivative of strain D471 from the Rockefeller University collection. The construction of JRS75, an isogenic M^- derivative of JRS4, has been described (23). Luria broth (24) was used to culture *E. coli*, and *S. pyogenes* strains were grown in Todd–Hewitt medium (Difco) supplemented with 0.2% yeast extract (THY). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml for *E. coli*; kanamycin, 500 μg/ml for *S. pyogenes* and 25 μg/ml for *E. coli*; streptomycin, 1000 μg/ml for *S.*

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Abbreviation: LTA, lipoteichoic acid.

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pyogenes; spectinomycin, 50 μg/ml for *E. coli*; chloramphenicol, 20 μg/ml for *E. coli*.

Plasmids and DNA Manipulation. The plasmids pHSG575 (25) and pCL1921 (26) are cloning vectors based on the *E. coli* plasmid pSC101. The ΩKm-2 element is described elsewhere (27). The plasmid pLZ12 is an *E. coli*/streptococcal shuttle vector that has been used for complementation analyses in *S. pyogenes* (27). Chromosomal DNA was purified from *S. pyogenes* after growth in THY broth supplemented with 20 mM glycine (28, 29). Restriction endonucleases and ligases were used according to the recommendations of the manufacturers. When required, incompatible DNA restriction fragments were joined by ligation after treatment with T4 DNA polymerase to produce blunt fragment ends.

Transformation of Bacteria. Plasmid DNA was introduced into *E. coli* by the method of Kushner (30). *S. pyogenes* was transformed by electroporation (27, 29).

Analysis of Fibronectin Binding. A plasmid library of streptococcal chromosomal DNA prepared in *E. coli* DH5α (see *Results*) was screened to identify clones with fibronectin-binding activity as follows: Overnight cultures of individual *E. coli* clones (1 ml) were harvested by centrifugation (13,000 × *g*; 5 min) and resuspended in a solution containing 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, and soybean trypsin inhibitor (0.1 mg/ml). The suspensions were sonicated for 10 sec at the maximum setting (model 185, Branson) for a total of four times each. Debris was removed by centrifugation (13,000 × *g*; 10 min) and the supernatants (lysates) were tested for fibronectin-binding activity (see below). The periplasmic fraction of selected clones was also prepared (31). Lysates or periplasm (0.2 ml) were mixed with 0.7 ml of a solution of phosphate-buffered saline and 1% (vol/vol) Tween 20 (PBS/T) and supplemented with 1×10^5 cpm of ^{125}I-labeled fibronectin (^{125}I-fibronectin) (specific activity, 3.8×10^6 cpm per μg of protein) in a microcentrifuge tube. After 2 hr of incubation with end-over-end rotation at room temperature, 0.1 ml of a suspension of JRS75, prepared by suspending an overnight culture to an optical density of 1.0 at 600 nm (OD_{600} = 1.0) using PBS/T, was added and the mixture was incubated for an additional 2 hr. A suspension (0.1 ml) of *E. coli* HB101 in PBS/T (OD_{600} = 10.0) was then added to facilitate collection of the streptococci by centrifugation (13,000 × *g*; 10 min). The supernatant fluids were carefully removed and the radioactivity associated with the pellets was determined. When no *E. coli* lysate was added (control sample), the radioactivity associated with the cell pellet was 54,000 ± 3500 cpm. The background of ^{125}I-fibronectin binding to the tubes alone was 1500 ± 300 cpm and neither DH5α nor HB101 bound to fibronectin under the conditions of this assay. The analysis of binding of *S. pyogenes* to various concentrations of fibronectin was performed essentially as described (16) using *E. coli* HB101 to facilitate collection of the streptococci (see above). Bovine fibronectin was iodinated by the chloramine-T method (32).

Analysis of Binding of *S. pyogenes* to Fibronectin. *S. pyogenes* strains were cultured overnight at 37°C in THY, harvested by centrifugation, and resuspended in PBS/T (OD_{600} = 0.1). Aliquots of the bacterial suspensions (0.5 ml) were mixed with equal volumes of PBS/T and were supplemented with soluble ^{125}I-fibronectin at various concentrations. After a 2-hr incubation at room temperature, the amount of bound fibronectin was determined as explained above. Background values, obtained from tubes that contained no streptococci, were determined for each concentration of fibronectin added and were subtracted from the values obtained with streptococci.

Protein Transfer Assay for Identification of Fibronectin-Binding Proteins. Surface protein extracts of various *S. pyogenes* strains were prepared with phage lysin (33), separated by electrophoresis on a SDS/10% polyacrylamide gel and transferred to a poly(vinylidene difluoride) membrane (Immobilon-P; Millipore). Additional protein-binding sites on the membrane were blocked by a 5-hr incubation at room temperature in Tris-buffered saline (TBS; 10 mM Tris·HCl/145 mM NaCl, pH 7.4) containing 0.2% (vol/vol) Tween 20 and 0.2% (vol/vol) Tween 80. The membrane was subsequently placed in a solution of TBS containing ^{125}I-fibronectin (≈6×10^6 cpm) at room temperature. After overnight incubation, the membrane was washed extensively with eight changes of TBS, dried, and then subjected to autoradiography.

Adherence of *S. pyogenes* to Epithelial Cells. Proliferating epithelial cells isolated from hamster trachea (HTE cells) were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum as described (34). Confluent monolayers were dissociated and seeded onto glass coverslips at a density of 100,000 cells per coverslip, incubated for 18 hr, and then washed three times with MEM. *S. pyogenes* strains from overnight cultures in THY medium were harvested by centrifugation, washed twice in MEM, and resuspended in MEM to equivalent concentrations (OD_{600} = 2.0).

Viability determinations and analysis by light microscopy confirmed that the suspensions contained the same number of bacterial cells. Aliquots of the bacterial suspensions (0.05 ml) were added to HTE cell cultures containing a single coverslip in a final vol of 0.5 ml. The mixture was then gently rotated for 4 hr at 37°C in an atmosphere of 5% CO_2/95% air. Nonadherent streptococci were removed by immersing the coverslips in PBS with agitation. This procedure was repeated for a total of 12 consecutive immersions. The cells were fixed in methanol and stained with Gram stain. The degree of bacterial adherence to the HTE cells was then analyzed by light microscopy.

RESULTS

Identification of Protein F. To analyze the role of fibronectin binding in adherence, we first identified the streptococcal fibronectin receptor. *S. pyogenes* JRS75 contains a defined deletion of the structural gene for the type 6 M protein (*emm6.1*) (23) and binds to fibronectin (16). Chromosomal DNA of JRS75 was partially digested with *Sau*3A, ligated into the *Bam*HI site of the plasmid vector pHSG575, and used to transform *E. coli* DH5α. The resulting plasmid library was then screened for fibronectin-binding activity. The genes for streptococcal surface proteins are usually transcribed and translated in *E. coli*; however, the gene products either appear in the periplasm (35) or are associated with the inner membrane (36) and are not expressed on the surface of the outer membrane. Therefore, whole-cell lysates of individual clones were prepared by sonication and tested for a binding activity that could inhibit the binding of ^{125}I-fibronectin to intact cells of *S. pyogenes*. Of 300 plasmid clones tested, 1 was identified that expressed a binding activity (Fig. 1). Binding activity was also detected in preparations of periplasm from the clone (Fig. 1). From these data, along with the observation that the binding activity was sensitive to trypsin (data not shown), we concluded that we had identified a streptococcal fibronectin-binding protein, which we named protein F.

Characterization of the Gene Encoding Protein F and Construction of a Mutant Allele. Analysis of the chimeric plasmid encoding the gene for protein F (*prtF*) demonstrated that it contained an 8-kilobase (kb) fragment of the JRS75 chromosome (pPTF1; Fig. 2). A series of chimeric plasmids were then constructed to further localize *prtF* (Fig. 2). Analysis of the fibronectin-binding activity of strains harboring these plasmids suggested that *prtF* was located within the 2.9-kb *Eco*RV fragment present in pPTF5 (Fig. 2). Analysis of protein F expressed by *S. pyogenes* demonstrated that it is a

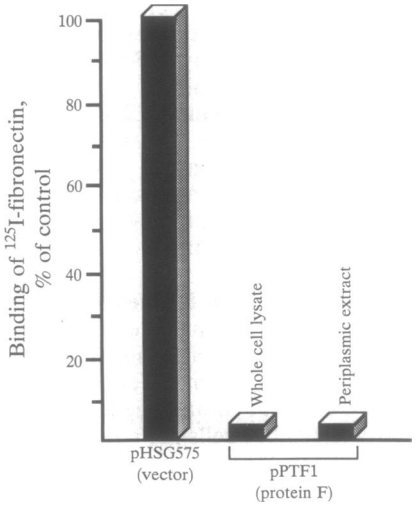

FIG. 1. Protein F inhibits binding of fibronectin to *S. pyogenes*. Extracts of *E. coli* strains harboring the indicated plasmids were examined for their ability to inhibit binding of fibronectin to *S. pyogenes* JRS75.

120,000-kDa surface protein (see below). Because a protein of this size would require a coding region of at least 2.7 kb, it is unlikely that this *Eco*RV fragment can contain more than one gene. The location of the entire *prtF* coding region in this *Eco*RV fragment was supported by the observation that protein F produced by *E. coli* strains harboring pPTF5 was the same size as the streptococcal protein (120,000 kDa; data not shown) and was confirmed by complementation analysis (see below). Insertional mutagenesis of *prtF* using an Ω element, which contains a kanamycin-resistance determinant flanked by strong transcription and translation termination signals (ΩKm-2) (27), demonstrated that insertion into the *Pst* I site, but not the *Sca* I site, abolished expression of fibronectin-binding activity (pPTF6.1 and pPTF7.1; Fig. 2). The mutant allele of *prtF* generated by insertion of ΩKm-2 into the *Pst* I site was designated *prtF*::Ω-1.

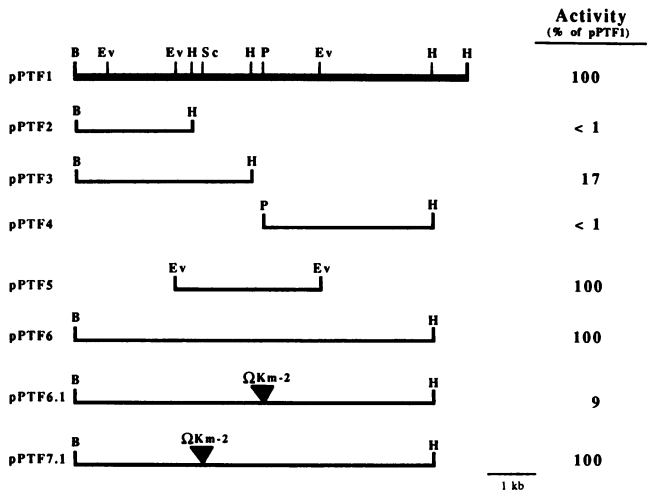

FIG. 2. Restriction maps and mutagenesis of plasmids that contain the gene encoding protein F (*prtF*). Chimeric plasmid pPTF1 contains an 8-kb fragment of the JRS75 chromosome that includes *prtF*. As indicated, fragments of pPTF1 were subcloned to further localize *prtF*. A *Bam*HI/*Sal* I fragment containing the entire insert of pPTF1 was introduced into *Bam*HI/*Sal* I-digested pCL1921 to construct pPTF7 (data not shown). Insertion of ΩKm-2 into the *Pst* I site of pPTF6 and the *Sca* I site of pPTF7 generated pPTF6.1 and pPTF7.1. Fibronectin-binding activity was determined as described for Fig. 1; however, 100% represents inhibition of binding of ^{125}I-fibronectin to JRS75 produced by a lysate of the *E. coli* clone harboring pPTF1. For clarity, the cloned streptococcal sequences are shown as linear molecules and the plasmid vectors are not shown. B, *Bam*HI; Ev, *Eco*RV; H, *Hin*dIII; Sc, *Sca* I; P, *Pst* I; S, *Sal* I.

Protein F Is the Major Fibronectin Receptor of *S. pyogenes*. To provide direct evidence that protein F is the fibronectin receptor of *S. pyogenes*, the mutant allele *prtF*::Ω-1 was used to replace the resident *prtF* allele of a wild-type *S. pyogenes*. The plasmid containing *prtF*::Ω-1 (pPTF6.1; Fig. 2) was converted to a linear molecule by digestion with *Eco*RI and was used to transform *S. pyogenes* JRS4 with selection for the kanamycin-resistance determinant of ΩKm-2. Southern blot analysis of several kanamycin-resistant transformants confirmed allelic replacement (data not shown). One transformant (SAM1) was chosen for further analysis and its ability to bind to fibronectin was compared to JRS4.

Surface proteins were isolated from JRS4 and SAM1 after digestion of the streptococcal cell wall with phage lysin (33), separated by SDS/PAGE, transferred to a poly(vinylidene difluoride) membrane, and reacted with ^{125}I-fibronectin. Autoradiography of the treated membrane revealed that JRS4 possessed a fibronectin-binding surface protein of ≈120,000 kDa (Fig. 3*A*, lane 3). An additional larger binding species

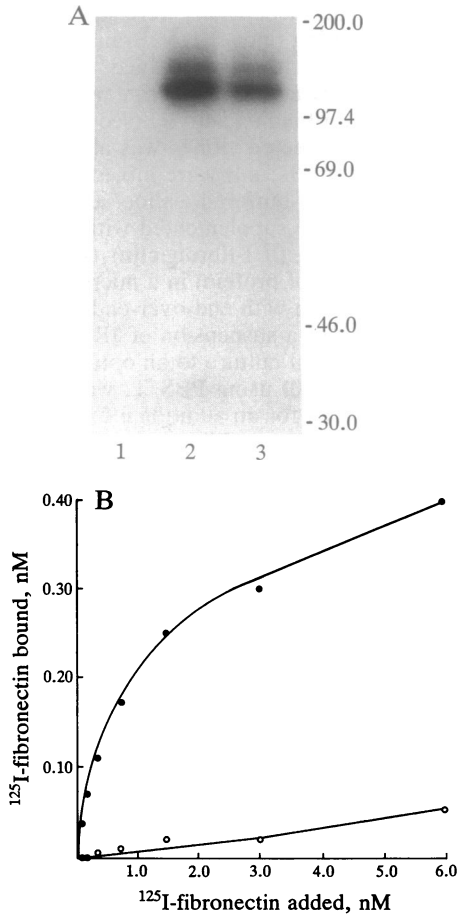

FIG. 3. Protein F is the major fibronectin receptor of *S. pyogenes*. *S. pyogenes* SAM1 is derived from JRS4 by allelic replacement of *prtF* with *prtF*::Ω-1. Transformation of SAM1 with pPTF8 produced SAM1 (pPTF8). (*A*) Autoradiograph of proteins from phage lysin extracts of SAM1 (lane 1), JRS4 (lane 3), and SAM1 (pPTF8) (lane 2) that had been separated by SDS/PAGE, transferred to a poly(vinylidene difluoride) membrane, and incubated with ^{125}I-fibronectin. Numbers on right represent migration of protein standards (kDa). (*B*) Binding of soluble ^{125}I-fibronectin to JRS4 (●) and SAM1 (○). Data represent means of duplicate determinations, which differed by <5%. Concentrations indicate final concentration of fibronectin in the incubation mixture.

was also observed (Fig. 3A), which, as has been described for other streptococcal surface proteins (35), likely results from cell wall components that have remained associated with the protein. None of the fibronectin-binding proteins was present in surface protein extracts of SAM1 (lane 1), even after the membrane was subjected to autoradiography for extended periods of time. The 2.9-kb *Eco*RV fragment containing *prtF* (Fig. 2) was then inserted into the shuttle vector pLZ12, which can replicate autonomously in *S. pyogenes* (28), and the chimeric plasmid (pPTF8) used to transform SAM1. The resulting strain produced a pattern of fibronectin-binding surface proteins identical to JRS4 (lane 2), which indicates that the plasmid copy of *prtF* can complement the mutation in SAM1. Analysis of the binding of intact cells to soluble fibronectin revealed that SAM1 had lost the capacity to bind fibronectin at high affinity when compared with JRS4 (Fig. 3B). While the binding of fibronectin to JRS4 exhibited the characteristics expected of a ligand–receptor interaction, the residual low level of binding to SAM1 was characterized by a low affinity and it showed no tendency to reach saturation (Fig. 3B). These data establish that the fibronectin-binding proteins observed in extracts of JRS4 represent protein F and that protein F is expressed on the surface of *S. pyogenes*.

Protein F Is an Adhesin for Respiratory Epithelial Cells. To assess the role of protein F in the adherence of *S. pyogenes* to host epithelial cells, we compared the abilities of SAM1 and JRS4 to adhere to a proliferating, nontransformed hamster respiratory epithelial cell culture (HTE cells) (34). While JRS4 was found to adhere to HTE cells in large numbers (Fig. 4A), few adherent bacteria were observed when SAM1 was incubated with HTE cells (Fig. 4B). We estimate that at least 100-fold less bacteria of SAM1 than JRS4 adhered to HTE cells when assayed under the same conditions. Aggregation of streptococcal cells mediated by the M protein has been shown to contribute to the adherence of *S. pyogenes* to some epithelial cell types (16). To confirm that bacterial aggregation was not contributing to the large difference in adherence observed between SAM1 and JRS4, we tested the adherence of a strain isogenic to JRS4 that contains a defined deletion of the M protein structural gene (JRS145; M.C., R. T. Geist, J. Perez-Casal, and J. R. Scott, unpublished data). This strain does not aggregate and we observed that it adhered to HTE cells in a manner identical to that of JRS4 (Fig. 4C). Allelic replacement of *prtF* with *prtF*::Ω-1 in JRS145 produced a strain (SAM2) that was as defective as SAM1 in adherence to HTE cells (Fig. 4D). These data show that it is solely the presence or absence of a functional protein F that determines whether *S. pyogenes* will adhere to these epithelial cells.

DISCUSSION

Because microorganisms have evolved multiple mechanisms to ensure their successful attachment to host cells, a critical evaluation of the role of fibronectin binding in adherence requires the construction and analysis of microorganisms with defined mutations in the genes encoding their fibronectin-binding proteins. By constructing a mutation in the gene encoding protein F, a fibronectin-binding protein that we have identified on the surface of the group A streptococcus, we have demonstrated that protein F is the principle fibronectin receptor of this organism and that it can serve as an adhesin for respiratory epithelial cells.

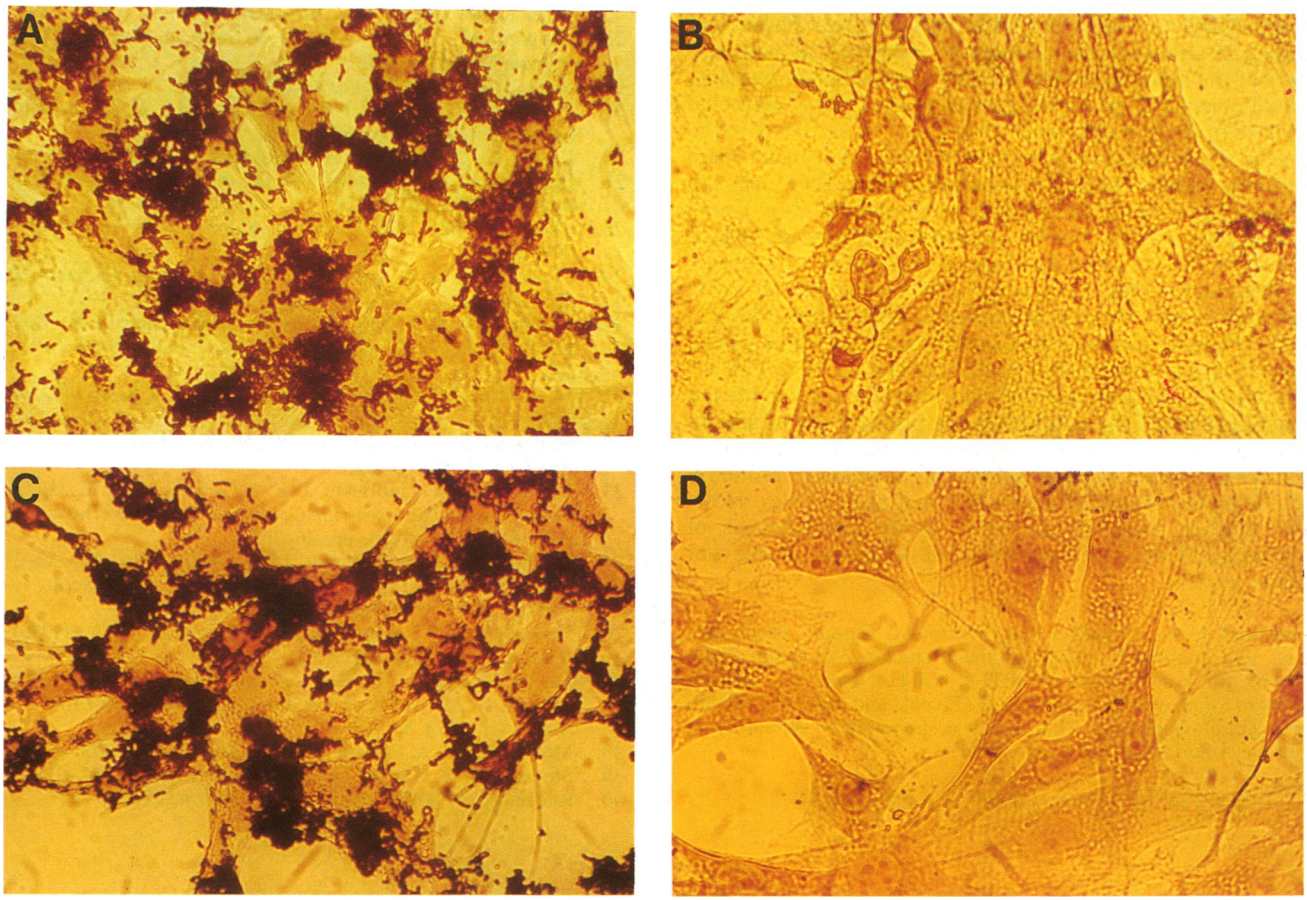

FIG. 4. Protein F is essential for adherence of *S. pyogenes* to respiratory epithelial cells. Adherence of group A streptococcal strains to HTE cells is shown. (*A*) JRS4 (protein F^+, M^+). (*B*) SAM1 (protein F^-, M^+). (*C*) JRS145 (protein F^+, M^-). (*D*) SAM2 (protein F^-, M^-). Streptococci are the small darkly staining cocci on the surface of the epithelial cells. (×1000.)

Other fibronectin-binding proteins have been purified (17) or cloned from *S. pyogenes* (18). However, it has not been shown that these proteins are expressed on the surface of *S. pyogenes* or whether they represent the fibronectin receptor of the intact bacterium. Additional experimentation will be required to determine the relationship of these proteins to protein F. A fibronectin-binding surface protein has been extensively characterized and cloned from the Gram-positive organism *S. aureus* (19). The nucleotide sequence of this gene predicts a protein product of 108,000 kDa (37), which is similar to the apparent size of protein F. The fibronectin-binding domain of this protein has been localized to a 38-amino acid motif that is repeated three times (37) and synthetic peptides that mimic the repeats can inhibit the binding of fibronectin to intact staphylococcal cells (37). Both the staphylococcal protein and intact cells of *S. pyogenes* bind to a site in the 29-kDa N-terminal domain of fibronectin that does not contain an RGD sequence (8, 38). In preliminary experiments, we have found that the product of the cloned staphylococcal gene can compete for fibronectin binding with protein F, suggesting that the two proteins may share some similarity. However, peptides that mimic the binding domains of the fibronectin-binding proteins of *S. dysgalactiae* also compete for binding with the staphylococcal protein, even though these proteins show little amino acid homology (20). Thus, it appears that several different mechanisms for the interaction of bacterial receptors with fibronectin may exist. Further elucidation of these mechanisms will require direct probing of the interaction between the purified receptors and fibronectin.

Our mutagenesis studies, showing that strains of *S. pyogenes* that can no longer bind to fibronectin are also defective in their ability to adhere to respiratory epithelial cells, have provided direct evidence for the important role of fibronectin binding in streptococcal adherence. However, as with any study of the adherence of an exclusive pathogen of humans, careful consideration must be given to the type of epithelial cell used to analyze adherence (39). The HTE cells used in the present study have several characteristics that make them useful for studies of streptococcal adherence. HTE cells are a primary cell culture and are not transformed (34). Transformed cells usually exhibit altered expression of surface structures and are often aberrant in expression of receptors for fibronectin (40). Unlike isolated human buccal cells, which are commonly used in streptococcal adherence assays, HTE cells represent a uniform population of cells that can be cultured and assayed under defined conditions. Finally, HTE cells are derived from the respiratory tract and resemble the pharyngeal epithelial cells that are a primary site of attachment and colonization of *S. pyogenes*. Our *in vitro* studies with HTE cells have suggested that, as an adhesin, protein F may be an important virulence factor of *S. pyogenes*. However, further study of protein F in the pathogenesis of streptococcal infections awaits the development of relevant animal models.

We wish to thank Staffan Normark for his support, encouragement, and continued interest in these studies. We thank Philip Horwitz for construction of pPTF8, Linda Nixon and William Goldman for providing us with the HTE cells, and Vincent Fischetti for his gift of phage lysin. This work was supported by a grant-in-aid from the American Heart Association.

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