***Streptococcus pyogenes* protein F promotes invasion of HeLa cells**

Nobuhiko Okada,¹† Ichiro Tatsuno,¹ Emanuel Hanski,² Michael Caparon³ and Chihiro Sasakawa¹

Author for correspondence: Nobuhiko Okada. Tel: +81 3 5791 6256. Fax: +81 3 3444 4831.
e-mail: okadan@platinum.pharm.kitasato-u.ac.jp

¹ Department of Bacteriology, Institute of Medical Science, University of Tokyo, Tokyo 108, Japan

² Department of Clinical Microbiology, The Hebrew University Hadassah Medical School, Jerusalem 91010, Israel

³ Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110, USA

Although the Gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus) has been considered an extracellular pathogen which adheres to human mucosal epithelium, the streptococcus possesses invasive capacity for cultured human epithelial cells. This study provides genetic and functional evidence supporting the conclusion that protein F is capable of mediating entry of *S. pyogenes* into HeLa cells. Using Tn916 insertion mutagenesis or an isogenic *S. pyogenes* strain with a defined mutation in the gene encoding protein F (*prtF*), it was observed that the invasive capacity was affected by the levels of surface-exposed protein F, but not by those of M protein. In addition, heterologous expression of protein F on *Enterococcus faecalis* conferred upon the bacteria an efficient invasive phenotype. Several assays demonstrated that both the fibronectin-binding domains of protein F, UR and RD2, were involved in host-cell invasion. In addition, coinfection experiments of HeLa cells with *S. pyogenes* and an *Escherichia coli* K-12 strain expressing an afimbrial adhesin AFA-I showed that the uptake of *S. pyogenes* did not permit internalization of the *E. coli* cells.

Keywords: streptococcal infection, invasion, transposon mutagenesis, virulence

INTRODUCTION

The Gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus) is one of the most versatile human pathogens with regard to the number of different tissues it can infect and the wide range of different diseases it can cause, including suppurative infections of the throat (pharyngitis) and of the skin and soft tissues (impetigo, erysipelas, cellulitis, necrotizing fasciitis and myositis). Systemic complications can accompany infection of the skin (scarlet fever, toxic-shock-like syndrome and sepsis) (Bisno & Stevens, 1996) and a role for the host immune response to streptococcal antigens has been implicated in rheumatic fever. These diseases result from the ability of *S. pyogenes* to adhere to and colonize on epithelial cells of the pharyngeal mucosa or the skin. Although *S. pyogenes* has generally been considered an extracellular pathogen, it has recently been shown that streptococci can penetrate into several types of cultured human epithelial cells (LaPenta *et al.*, 1994; Greco *et al.*, 1995; Schraner *et al.*, 1996; Molinari *et al.*, 1997).

The initial interaction between bacteria and host cells is mediated by bacterial surface components (adhesins/invasins) and specific receptors on the surface of the host cells. In *S. pyogenes*, several surface components have been shown to participate in the interaction with eukaryotic cells. These include M protein for epidermal keratinocytes (Okada *et al.*, 1994) and cultured HEp-2 cells (Wang & Stinson, 1994), and the fibronectin-binding proteins for respiratory epithelial cells (Hanski & Caparon, 1992; Talay *et al.*, 1992) as well as Langerhans' cells (Okada *et al.*, 1994). It is known that the structure of these streptococcal proteins is analogous to that of internalin A, an invasin protein of *Listeria monocytogenes*, which contains tandem repeats and is anchored to the cell wall peptidoglycan by covalent linkage via the threonine residue of the conserved LPXTG motif (Gaillard *et al.*, 1991). More recently, the involvement of protein SfbI, a protein related to protein F capable of binding to fibronectin, has been implicated in the internalization of the streptococcus by epithelial cells (Molinari *et al.*, 1997).

In *S. pyogenes*, perhaps the best-characterized fibronectin-binding molecules are the closely related proteins protein F (Hanski & Caparon, 1992) and SfbI (Talay *et*

† Present address: Department of Microbiology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.

al., 1991, 1992). These proteins bind fibronectin when expressed in *Escherichia coli* (Hanski & Caparon, 1992; Talay *et al.*, 1992), can inhibit the binding of fibronectin to intact streptococcal cells (Hanski & Caparon, 1992; Talay *et al.*, 1992) and can inhibit the adherence of streptococci to several types of epithelial cells (Talay *et al.*, 1992). Insertional inactivation of the gene encoding protein F (*prtF*) generated mutants of *S. pyogenes* that were completely deficient in binding fibronectin and in their ability to adhere to respiratory epithelial cells (Hanski & Caparon, 1992; Talay *et al.*, 1992). Previous studies have shown that protein F contains two distinct fibronectin-binding domains, a tandem repetitive domain (RD2) and a domain immediately N-terminal to the repetitive domain (UR) (Sela *et al.*, 1994; Ozeri *et al.*, 1996). The minimal functional binding unit of the RD2 repeat domain consists of 44 amino acids located at the junction of two adjacent sequence repeats and is flanked by an 'MGGQSES' motif. This functional unit recognizes the N-terminal fibrin-binding domain of fibronectin (Ozeri *et al.*, 1996). UR contains 49 amino acids and consists of the 43 amino acids located immediately N-terminal to RD2 and the first six amino acids from the first repeat of RD2. This domain binds to a large region of fibronectin that includes both the N-terminal fibrin and adjacent collagen-binding domains (Ozeri *et al.*, 1996). Although UR binds fibronectin with high affinity and is the dominant domain for the binding of soluble fibronectin to protein F (Ozeri *et al.*, 1996), both domains are required for efficient binding to the extracellular matrix (Ozeri *et al.*, 1996) and multimeric fibronectin fibrils (Okada *et al.*, 1997). These fibronectin-binding domains are also involved in streptococcal adherence mediated by protein F to host cells (Talay *et al.*, 1992; Ozeri *et al.*, 1996; Okada *et al.*, 1997).

We undertook to investigate by transposon mutagenesis the bacterial component(s) involved in host-cell invasion by virulent *S. pyogenes*, and found that less invasive mutants had decreased expression of protein F on their surface compared with the parental strain. Furthermore, in contrast to the wild-type parent strain, an isogenic *prtF* mutant showed greatly diminished invasiveness for HeLa cells. In this study, we provide direct genetic evidence that protein F is a crucial surface component of *S. pyogenes* required for invasion of cultured epithelial cells, and show that HeLa cells coinfecting with *S. pyogenes* and non-invasive *E. coli* permit only the internalization of *S. pyogenes*.

METHODS

Bacterial strains and growth conditions. *S. pyogenes* JRS4 produces a serotype 6 M protein and is a spontaneous streptomycin-resistant derivative of D471 obtained from the Rockefeller University collection (Scott *et al.*, 1986). JRS145 was derived from JRS4 by insertional inactivation of the gene *emm6.1*, which encodes M6 protein (Caparon *et al.*, 1992). Protein F is a major fibronectin-binding protein of JRS4 (Hanski & Caparon, 1992). Insertional inactivation of *prtF* in JRS4 and JRS145 generated SAM1 and SAM2, respectively, which were completely deficient in binding fibronectin

(Hanski & Caparon, 1992). OGIX is a strain of *Enterococcus faecalis* (Ike *et al.*, 1983). *S. pyogenes* and *Ent. faecalis* were cultured in Todd-Hewitt medium (Difco) supplemented with 0.2% yeast extract (THY medium). For coinfection experiments, *E. coli* HB101 carrying pIL14 which encodes the afimbrial adhesin (AFA-I) (Labigne-Roussel *et al.*, 1984), *Salmonella typhimurium* SL1344 (Hoiseth & Stocker, 1981) and *Yersinia pseudotuberculosis* YPIII (Bolin *et al.*, 1982) were used. *E. coli* was grown in Luria-Bertani (LB) broth at 37 °C with agitation. *Salm. typhimurium* was grown under low-oxygen conditions to obtain the efficient invasive phenotype (Lee & Falkow, 1990). Cultures of *Y. pseudotuberculosis* were grown in LB broth with agitation at 28 °C overnight.

Plasmid construction. Replacement of a 1.1 kb *Apal*-*NcoI* fragment of the *E. coli*-streptococcus shuttle vector pLZ12-Km (Hanski *et al.*, 1992) with a 219 bp *Apal*-*PvuII* fragment of pBlueScript II-KS(+) (Stratagene), which contains multicloning sites, generated pLZ12-Km2. Plasmid pLZ12-Km2 was digested with *EcoRI* and *XhoI* and ligated to the 2.9 kb *EcoRI* and *Sall* fragment from pPTF5 (containing the complete *prtF* gene) (Hanski & Caparon, 1992) to generate plasmid pNSP80.

Transposon mutagenesis. Transposon mutagenesis was achieved by conjugation of Tn916 from *Ent. faecalis* CG110 to *S. pyogenes* JRS4, as described previously (Caparon & Scott, 1991). Transconjugants were selected by growth on THY agar containing tetracycline (5 µg ml⁻¹) and streptomycin (1000 µg ml⁻¹) and were confirmed to be *S. pyogenes* by β -haemolysis on sheep blood agar.

Purification of fusion proteins containing the fibronectin-binding domain(s) of protein F and antibody preparation. The fusion proteins were purified from SG13009(pREP4) (Qiagen) containing the appropriate plasmid including pUR-4 (UR), pRD-2 (RD2) and pPTF54 (UR plus five repeats of RD2) as described previously (Hanski *et al.*, 1995). To obtain polyclonal antibodies against the fibronectin-binding domains, rabbits were immunized with the purified fusion proteins containing either UR or RD2. The IgG fraction was separated from sera by affinity chromatography on a protein A-Sepharose column (Pharmacia). The specificity of each antibody was confirmed by Western blotting.

Adherence and internalization assays. Since examination of *S. pyogenes* JRS4 for its invasiveness for A549, HeLa, Hep-2, HaCaT and MDCK cells using a gentamicin protection assay showed that the number of bacteria internalized into HeLa cells was higher than the other epithelial cells (data not shown), HeLa cells were used for the adherence and internalization assays. HeLa cells were cultured in Minimal Eagle's Medium (MEM) containing 10% foetal calf serum (FCS) without antibiotics in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. For adherence assays, bacteria were grown at 37 °C in THY medium overnight and diluted in MEM to a concentration of 1 × 10⁷ bacteria ml⁻¹. A 100 µl aliquot of bacterial suspension was added to cultured cells grown in 24-well plates at a density of 2 × 10⁵ cells per well. After 2 h incubation at 37 °C in 5% CO₂ to allow bacterial adherence, the cells were washed five times and analysed by staining with 0.1% crystal violet and light microscopy as described by Okada *et al.* (1994). Internalization assays were performed in a similar fashion to adherence assays, except that after the 2 h incubation, the monolayers were washed twice, and overlaid with fresh MEM containing gentamicin (200 µg ml⁻¹) to kill extracellular bacteria. The samples were further incubated at 37 °C for 2 h in 5% CO₂. The cells were then washed three times and lysed by adding 500 µl cold distilled water. Viable bacteria released from the cells were counted on

THY agar plates. In some experiments, bacteria were centrifuged onto cells at 380 *g* for 10 min and then assayed for ability to escape gentamicin killing as described above.

For inhibition assays, HeLa cell monolayers were pre-incubated with the purified protein containing various concentrations of either UR, RD2 or UR plus five RD2 repeats at 37 °C for 1 h before the infection. Inhibitions with a rabbit polyclonal antibody specific for the fibronectin-binding domain of protein F were performed by incubation of *S. pyogenes* JRS145 with antibody (25 µg ml⁻¹) at 37 °C for 1 h prior to infection.

For screening of invasion-deficient mutants from the bank of Tn916 insertion mutants, the mutants were grown overnight in THY medium containing tetracycline (5 µg ml⁻¹) and streptomycin (1000 µg ml⁻¹) in 96-well microtitre plates. A 10 µl volume of each bacterial culture was added to HeLa cells grown in 96-well microtitre plates in a volume of 200 µl MEM per well. The gentamicin protection assay was performed as described above. Cells were lysed by adding 100 µl cold distilled water and a portion of 10 µl was plated onto THY agar. The number of viable bacteria was scored after overnight incubation at 37 °C. Mutants for which bacterial growth was markedly reduced were further assayed for entry by quantitative standard internalization assay described above.

DNA sequencing strategy. The Tn916-flanking regions from Tn916 mutants were amplified by using an inverse PCR method as described by Lyrstis *et al.* (1994). DNA fragments amplified were sequenced by the dideoxy chain-termination method with a Sequenase version 2.0 DNA sequencing kit (United States Biochemicals).

Quantitative measurements of protein F expression on the bacterial surface. Bacterial cultures were spotted on a nitrocellulose filter laid on a THY agar plate and incubated at 37 °C for 18 h. The filter was blocked by washing for 30 min in 5% skim milk in PBS containing 0.05% sodium azide and then incubated with anti-RD2 domain antibody at room temperature for 2 h. After washing three times with PBS containing 0.05% Tween 20 (PBS-T), the blots were incubated with alkaline-phosphatase-conjugated goat anti-rabbit IgG antiserum (Sigma) at room temperature for 3 h. The reaction was detected by alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris/HCl, pH 9.5) containing nitro-blue tetrazolium (300 µg ml⁻¹) and bromochloroindolyl phosphate (150 µg ml⁻¹). Data were processed by using a Macintosh-based image analysis program (NIH image).

Coinfection experiments. HeLa cells grown in 24-well plates were inoculated simultaneously with 1 × 10⁷ *E. coli* HB101 harbouring pIL14 and either *S. pyogenes* JRS145, *Salm. typhimurium* or *Y. pseudotuberculosis*, centrifuged at 380 *g* for 10 min and incubated at 37 °C for 1 h in 5% CO₂. The cells were washed and subjected to the gentamicin protection assay to recover intracellular bacteria. The number of gentamicin-resistant bacteria was estimated by colony counting after overnight growth on agar plates; LB agar containing ampicillin (250 µg ml⁻¹) was used for *E. coli* strain HB101(pIL14).

RESULTS

Less invasive mutants of *S. pyogenes* have decreased levels of surface-expressed protein F

To identify the genetic locus of *S. pyogenes* M6 serotype strain JRS4 required for the invasion of epithelial cells, a random insertion mutagenesis with the conjugative

Fig. 1. Surface expression of protein F affects entry of *S. pyogenes* into cultured epithelial cells. (a) Internalization assays for Tn916 mutants were performed as described in Methods. When a total number of 1 × 10⁶ strain JRS4 was added to HeLa cell monolayers, 3–5% of bacteria were recovered from infected cells after treatment with gentamicin for 2 h, which is given the value 100%. Data represent the mean of triplicate determinations, which differed by ≤10% from the mean value. (b) Amount of protein F expressed on the bacterial surface was determined by immunostaining of live bacteria using anti-RD2 domain antibody; 100% represents the amount of protein F expressed by JRS4. Data represent the mean of triplicate determinations, which differed by ≤10% from the mean value. Note that the amounts of M protein expressed from each of the mutants as determined by immunostaining of live bacteria using anti-M6 specific antibody were shown to be less than ±5% different from that of JRS4. The growth rate of each mutant in MEM supplemented with 10% FCS as judged by turbidity measured at 600 nm was also essentially the same.

transposon Tn916 was performed and 5387 insertion mutants were screened by a simplified gentamicin protection assay for their ability to enter HeLa cells. Nine of the resultant mutants were identified with reduced invasive capacity, which was 1–54% of that of JRS4 (Fig. 1a). To investigate the cause of the reduced invasiveness, each of the Tn916-flanking DNA sequences of these mutants was determined and the sites of Tn916 insertions on its chromosome were estimated by using the database of the *S. pyogenes* genome sequence (University of Oklahoma's Advanced Center for Genome Technology). The insertion site for each mutant was assigned as follows: NS3451 possessed Tn916 within a region homologous to the ABC transporter operon of *Bacillus subtilis* (accession number P54719). NS3494 and NS6174 possessed Tn916 in a region homologous to the *spoVM* operon of *Bacillus subtilis* (Levin *et al.*, 1993). NS4807 possessed Tn916 in an

unknown gene encoding an 88 aa protein (accession number U07341). NS4728 possessed Tn916 in the vicinity of a gene encoding a protein homologous to the α -haemolysin of *Aeromonas hydrophila* (accession number L36462). NS5623 possessed Tn916 in a gene encoding a protein similar to the Rgg protein of *Streptococcus gordonii*, known as a positive regulator for glucosyltransferase (Sulavik *et al.*, 1992). NS6180 possessed Tn916 in between two genes within an operon associated with the biosynthesis of D-alanyl-lipoteichoic acid of *B. subtilis* (Perego *et al.*, 1995). Tn916 insertion regions from NS3465 and NS4945 were not homologous to any sequences in the database. Although the precise effect of each mutation on the invasiveness of *S. pyogenes* was still unclear, some surface proteins may be important for determining the level of invasiveness.

To investigate whether the reduced invasiveness of the Tn916 insertion mutants resulted from a defect in their ability to adhere to epithelial cells, the cell-binding capacity to HeLa cell monolayers was measured. Although the adherence ability of each mutant as judged by enumeration of numbers of bacteria associated with the HeLa cells was essentially the same as that of JRS4, the mutant bacteria were distributed more evenly on the cell surface than JRS4. JRS4 bacteria attached to HeLa cells frequently formed aggregates on the cell surface, suggesting that the Tn916 insertion mutations resulted in some change in the surface property of the streptococci (data not shown). We thus examined the mutants for their surface expression of protein F and M protein, since they are the major adhesins on strain JRS4 (Hanski & Caparon, 1992; Okada *et al.*, 1994). The mutants grown on a nitrocellulose membrane were immunostained with either a rabbit anti-RD2 antibody, an antiserum specific for the fibronectin-binding domain of protein F, or a rabbit anti-M6 protein antiserum, and the amount of each protein was quantified. Whereas levels of the surface expression of M protein of the mutants were similar to that of JRS4 (data not shown), the expression of protein F of the mutants was reduced significantly compared with that of JRS4 (Fig. 1). Importantly, the extent of the decreased expression of protein F showed good agreement with the decreased invasiveness of each mutant for HeLa cells (Fig. 1), suggesting that the amount of protein F on the bacterial surface was related to the invasive capacity of *S. pyogenes*.

Protein F is required for streptococcal invasion

To clarify the role of protein F in the expression of invasiveness of *S. pyogenes*, we examined a set of isogenic *S. pyogenes* strains with defined mutations in the genes which encode protein F and M protein for their ability to bind to HeLa cells prior to invasion. Wild-type strain JRS4 (M^+ , F^+) bound 90% of HeLa cells. Strains JRS145 (M^- , F^+) and SAM1 (M^+ , F^-) bound 81% of that bound by JRS4 strain when assayed under identical conditions. By contrast, double-mutant strain SAM2 (M^- , F^-) showed only 27% adherence

Fig. 2. Efficient entry of *S. pyogenes* into HeLa cells requires expression of protein F. Invasiveness of isogenic *S. pyogenes* strains, JRS4 (M^+ , F^+), JRS145 (M^- , F^+), SAM1 (M^+ , F^-) and SAM2 (M^- , F^-) was determined by gentamicin protection assays with (+) or without (-) centrifugation prior to infection (see Methods for details). Data represent the mean of triplicate determinants, which differed by $\leq 10\%$ from the mean value.

compared with the parental strain, confirming previous studies that M protein and protein F served as the major streptococcal adhesions for epithelial cells (Hanski & Caparon, 1992; Okada *et al.*, 1994). Each of the mutant *S. pyogenes* strains was then measured for its invasive capacity by the gentamicin protection assay. As shown in Fig. 2, the invasiveness of the F-deficient mutant SAM1 decreased to 6% of the wild-type (JRS4) level. By contrast, the M-deficient mutant JRS145 showed 90% invasiveness compared with JRS4 (Fig. 2). The invasiveness of the double mutant SAM2 was only 2% of the wild-type level (Fig. 2). Upon mechanical enhancement of attachment of streptococci to the HeLa cell monolayers using centrifugation, the invasiveness of SAM1 increased to 18% of that of the wild-type strain, whereas the invasiveness of SAM2 remained at 2% (Fig. 2). Next, the number of internalized bacteria within HeLa cells was measured at different multiplicities of infection with JRS145 and SAM2. As shown in Fig. 3(b), the number of internalized JRS145 bacteria increased with the infecting dose up to 1×10^7 bacteria per well, whereas the number of internalized SAM2 cells did not increase at all. The results of the series of experiments indicated that although adherence directed by protein F could directly promote uptake of streptococci by HeLa cells, neither the bacterial binding mediated by M protein nor mechanical contact was involved in the invasiveness.

To better understand the role of protein F in the invasiveness of streptococci, we introduced plasmid pNSP80, which contains the complete *prtF* gene, into *Ent. faecalis* strain OG1X, which lacks a *prtF*-related gene (Hanski *et al.*, 1992), and the resulting *Ent. faecalis* transformant OG1X(pNSP80) was examined for its adhesive and invasive capacity for HeLa cells. Strain

Fig. 3. Protein F mediates entry of *S. pyogenes* into cultured epithelial cells. HeLa cell monolayers were infected with increased numbers of various *Ent. faecalis* and *S. pyogenes* strains, and subjected to the gentamicin protection assays. (a) Structure of protein F (Sela *et al.*, 1994; Ozeri *et al.*, 1996). (b) Invasiveness of JRS145 and SAM2 at various infection dosages. (c) Invasiveness of *Ent. faecalis* OGIX harbouring pNSP80 which encodes protein F and OGIX strain harbouring the vector plasmid pLZ12-Km2. Under the same conditions, the level of JRS145 invasiveness was similar to that presented in (b). Data represent the mean of triplicate samples from duplicate experiments and standard error of the mean.

OGIX(pNSP80) showed increased binding (32% of HeLa cells) compared with OGIX(pLZ12-Km2: vector only) which did not bind at detectable levels to HeLa cells. The invasiveness of OGIX(pNSP80) was also increased compared with that of OGIX(pLZ12-Km2) (Fig. 3c). Interestingly, the invasive efficiency of OGIX(pNSP80) was significantly lower than that of *S. pyogenes*; invasiveness of OGIX(pNSP80) at an inoculum of 1×10^7 was only 15% of the JRS145 level (data not shown). Since the level of protein F expressed on the surface of *Ent. faecalis* has been shown to be less than that in *S. pyogenes* strains (Hanski *et al.*, 1992), we measured the level of protein F expressed by *Ent. faecalis* OGIX(pNSP80) by immunostaining with anti-RD2 antibody. The results showed that the protein F level on OGIX(pNSP80) was 18% of that on JRS145, consistent with the low invasiveness of the *Ent. faecalis* strain. Based on all the above results, we concluded that protein

F plays a major role in facilitating the invasiveness of *S. pyogenes*.

Two distinct fibronectin-binding domains of protein F, UR and RD2, are essential for invasion

Previous studies demonstrated that protein F contained two distinct fibronectin-binding domains, termed UR and RD2 (Sela *et al.*, 1994; Ozeri *et al.*, 1996), that are responsible for bacterial adhesion to epithelial cells (Fig. 3a). To determine which of the fibronectin-binding domains was involved in streptococcal invasion of cultured epithelial cells, purified proteins containing either UR, RD2, or UR plus five RD2 repeats were tested for their ability to inhibit competitively the invasion of HeLa cells by *S. pyogenes*. Pretreatment with the purified protein containing UR plus five RD2 repeats at 1000 nM reduced the invasiveness of JRS145 by 72%, whereas purified proteins containing either UR or RD2 alone inhibited the invasiveness by 51% and 60%, respectively (Fig. 4a). To confirm this, we produced polyclonal antibodies against each of UR and RD2 domains and examined their abilities to block bacterial entry into HeLa cells. Each of anti-UR and anti-RD2 antibodies at $25 \mu\text{g ml}^{-1}$ inhibited the entry of JRS145 by 10% and 38%, respectively, whereas the mixture of the same amounts of anti-UR and anti-RD2 antibodies inhibited the entry by 78% (Fig. 4b), indicating that the two domains participate in the invasion of epithelial cells.

Streptococcal invasion strategy

To assess the internalization strategies adopted by *S. pyogenes*, HeLa cell monolayers were infected with JRS145 and the non-invasive *E. coli* strain HB101 harbouring pIL14, which encodes the afimbrial adhesin AFA-I, and assayed for the uptake of HB101(pIL14). As shown in Table 1, uptake of HB101(pIL14) by JRS145 was not significantly promoted as compared with that by *Salmonella*; rather the efficiency of uptake of HB101(pIL14) by JRS145 was similar to that by *Yersinia*.

DISCUSSION

Recent studies have indicated that, although *S. pyogenes* has long been considered a highly adhesive extracellular pathogen, the bacteria can also invade various human epithelial cells (LaPenta *et al.*, 1994; Greco *et al.*, 1995; Schraner *et al.*, 1996; Molinari *et al.*, 1997). However, neither the direct genetic evidence nor the underlying invasive strategy leading to uptake of streptococci by mammalian cells has been reported. In this study, we have provided genetic evidence that the ability of *S. pyogenes* to enter epithelial cells is mediated by protein F. These conclusions were drawn from the following results: (i) the amounts of protein F expressed on *S. pyogenes* can affect the invasion efficiency; (ii) the inactivation of a *prf* gene from *S. pyogenes* results in a remarkable decrease in invasiveness; (iii) the heterologous expression of protein F in *Ent. faecalis* renders

Fig. 4. Both fibronectin-binding domains of protein F are required for efficient invasion by *S. pyogenes*. Inhibition by purified proteins containing fibronectin-binding domains of protein F (a) or antibody specific for either UR or RD2 domain (b) is shown. (a) Semiconfluent HeLa cell monolayers were pretreated with various concentrations of purified protein and then incubated for 2 h at 37 °C with 1×10^6 bacteria. For the control, BSA (1000 nM) was used. (b) Antibodies at $25 \mu\text{g ml}^{-1}$ were preincubated with bacteria at 37 °C for 1 h prior to infection. Purified IgG from normal rabbit serum at the same concentration was used as a control. The number of intracellular bacteria was quantified by gentamicin protection assay. The data are expressed as the percentage of inhibition relative to the control experiments. Data represent the mean of triplicate determinants, which differed by $\leq 5\%$ from the mean value.

Table 1. Coinfection of HeLa cells with *S. pyogenes* JRS145 and AFA-I expressing *E. coli*

Both the strain indicated and 1×10^7 *E. coli* HB101(pIL14) were added to the same wells and centrifuged at $380 \times g$ for 10 min. Samples were incubated for 1 h at 37 °C in 5% CO₂ and then were incubated for a further 2 h at the presence of gentamicin. The number of gentamicin-resistant bacteria was determined by counting viable cells using selective plates. Data represent the means of triplicate samples from duplicated assays.

Strain	Inoculum (c.f.u. per well)	Intracellular survival (c.f.u. per well)	
		Bacteria recovered	HB101(pIL14) when coinfectd
<i>S. pyogenes</i> JRS145	2.0×10^5	2.6×10^2	4.1×10^1
	2.0×10^6	1.1×10^4	4.5×10^1
	2.0×10^7	5.3×10^4	4.0×10^1
<i>Salm. typhimurium</i>	2.1×10^7	2.6×10^4	2.5×10^3
<i>Y. pseudotuberculosis</i>	2.6×10^6	6.0×10^4	4.0×10^1
<i>E. coli</i> HB101(pIL14)	1.0×10^7	1.9×10^1	

bacteria invasive; and (iv) the invasive capacity of *S. pyogenes* is inhibited by the addition of purified protein F portions or specific antibodies.

In this study, we isolated nine mutants of *S. pyogenes* with reduced invasiveness by using random Tn916 insertion mutagenesis and noted that the Tn916 insertion mutants were all significantly decreased in the expression of protein F. Importantly, the levels of M protein, another major surface protein involved in adherence to epithelial cells, were not affected by the Tn916 insertions at all. Rather, the decreased levels of invasiveness showed good correlation with the surface levels of protein F. The defined *prtF* mutant strains of *S. pyogenes* clearly demonstrated that the absence of *prtF* affects invasive capacity. Furthermore, the heterologous

expression of protein F in *Ent. faecalis* can confer on the bacteria the ability to invade cultured epithelial cells. The induced invasive capacity in *Ent. faecalis* was less than 15% of the *S. pyogenes* level. It has been shown that the heterologous expression of protein F on *Ent. faecalis* was weaker than that on *S. pyogenes* (Hanski *et al.*, 1992); the observed decrease in the invasiveness of *Ent. faecalis* is consistent with the less efficient expression of the protein F on the surface.

The genes that encode fibronectin-binding proteins, known as the protein F/SfbI family, are widely distributed among diverse isolates of *S. pyogenes*, in which the two distinct fibronectin-binding domains are highly conserved (Talay *et al.*, 1994; Natanson *et al.*, 1995). DNA sequencing analysis has revealed that whereas SfbI

protein contains only four RD2-type repeats, the amino acid sequences of the fibronectin-binding domains of SfbI are similar to that of protein F, namely UR and RD2 (Talay *et al.*, 1992; Sela *et al.*, 1994). These fibronectin-binding domains of both proteins have been shown to be essential for binding to fibronectin and adherence to host cells (Talay *et al.*, 1992; Sela *et al.*, 1994; Ozeri *et al.*, 1996; Okada *et al.*, 1997). Recently, Molinari *et al.* (1997) reported that SfbI mediates streptococcal attachment to, and internalization by, cultured HEP-2 cells via fibronectin-binding domains. In the present study we found that either purified UR or RD2 portions of protein F or antibodies specific for UR and RD2 could inhibit the entry of bacteria into cultured epithelial cells. The fact that streptococci expressing either UR or RD2, or both, on their surface possess a potency to adhere to, as well as to invade mammalian cells argues strongly that a direct link of streptococci to the host-cell receptor is involved in uptake of bacteria.

Since protein F binds fibronectin at high affinity, it would be reasonable to speculate that a cellular fibronectin receptor such as $\alpha_5\beta_1$ integrin serves as a receptor for protein F, in which fibronectin may be required as the adapter for connection between protein F and the integrin. Our preliminary data support this hypothesis, since invasive efficiency of *S. pyogenes* was significantly increased for CHO-HFR5 cells (Watarai *et al.*, 1996), a high $\alpha_5\beta_1$ -integrin-expressing CHO cell line, compared with that for the parental CHO cells. Although no direct evidence supporting our hypothesis has been obtained, it is most likely that the ability of protein F to bind fibronectin plays a crucial role in promoting the internalization of *S. pyogenes*. In any case, further studies must be required for elucidating the mechanisms for streptococcal invasion of epithelial cells.

Although none of the Tn916 insertion sites on the chromosome of the mutant was in the *prtF* region, the decrease in the protein F expression on the bacterial surface appears to have arisen from different defects in each mutant. For example, protein F secretion might be impaired in NS3451, since the Tn916 was inserted in a DNA region homologous to the ABC transporter operon of *B. subtilis*. An alteration on the bacterial surface might occur in NS6180, which may affect the linkage of protein F to the peptidoglycan in the streptococcal cell wall, since the insertion of Tn916 was located within an operon encoding genes involved in the biosynthesis of D-alanyl-lipoteichoic acid of *B. subtilis*. Although the precise functional roles of each mutated gene in the nine Tn916 insertion mutants in terms of their expression of protein F remain to be elucidated, it is likely that a number of functions could directly and indirectly be involved in the surface expression of protein F, thereby eventually affecting the invasiveness of *S. pyogenes*.

ACKNOWLEDGEMENTS

We would like to thank Ben Adler for critical reading of this manuscript and Miki Yokota for skilled technical assistance. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture

of Japan and the 'Research for the Futures' program of the Japanese Society for the Promotion of Science.

REFERENCES

- Bisno, A. L. & Stevens, D. L. (1996). Streptococcal infections of skin and soft tissues. *N Engl J Med* **334**, 240–245.
- Bolin, I., Norlander, L. & Wolf-Watz, H. (1982). Temperature-inducible outer membrane proteins of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are associated with the virulence plasmid. *Infect Immun* **37**, 506–512.
- Caparon, M. G. & Scott, J. R. (1991). Genetic manipulation of the pathogenic streptococci. *Methods Enzymol* **204**, 556–586.
- Caparon, M. G., Geist, R. T., Perez-Casal, J. & Scott, J. R. (1992). Environmental regulation of virulence in group A streptococci: transcription of the gene encoding M protein is stimulated by carbon dioxide. *J Bacteriol* **174**, 5693–5701.
- Gaillard, J.-L., Berche, P., Frehel, C., Gouin, E. & Cossart, P. (1991). Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigen from gram-positive cocci. *Cell* **65**, 1127–1141.
- Greco, R., De Martino, L., Donnarumma, G., Conte, M. P., Seganti, L. & Valenti, P. (1995). Invasion of cultured human cells by *Streptococcus pyogenes*. *Res Microbiol* **146**, 551–560.
- Hanski, E. & Caparon, M. G. (1992). Protein F, a fibronectin-binding protein, is an adhesin of the group A streptococcus, *Streptococcus pyogenes*. *Proc Natl Acad Sci USA* **89**, 6172–6176.
- Hanski, E., Horwitz, P. A. & Caparon, M. G. (1992). Expression of protein F, the fibronectin-binding protein of *Streptococcus pyogenes* JRS4, in heterologous streptococcal and enterococcal strains promotes their adherence to respiratory epithelial cells. *Infect Immun* **60**, 5119–5125.
- Hanski, E., Fogg, G., Tovi, A., Okada, N., Burstein, I. & Caparon, M. (1995). Molecular analysis of *Streptococcus pyogenes* adherence. *Methods Enzymol* **253**, 269–305.
- Hoiseth, S. K. & Stocker, B. A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238–239.
- Ike, Y., Craig, R. A., White, A. B., Yagi, Y. & Clewell, D. B. (1983). Modification of *Streptococcus faecalis* sex pheromones after acquisition of plasmid DNA. *Proc Natl Acad Sci USA* **80**, 5369–5373.
- Labigne-Roussel, A. F., Lark, D., Schoolnik, G. & Falkow, S. (1984). Cloning and expression of an afimbrial adhesin (AFA-I) responsible for P blood group-independent, mannose-resistant hemagglutination from a pyelonephritic *Escherichia coli* strain. *Infect Immun* **46**, 251–259.
- LaPenta, D., Rubens, C., Chi, E. & Cleary, P. P. (1994). Group A streptococci efficiently invade human respiratory epithelial cells. *Proc Natl Acad Sci USA* **91**, 12115–12119.
- Lee, C. A. & Falkow, S. (1990). The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc Natl Acad Sci USA* **87**, 4304–4308.
- Levin, P. A., Fan, N., Ricca, E., Driks, A., Losick, R. & Cutting, S. (1993). An unusually small gene required for sporulation by *Bacillus subtilis*. *Mol Microbiol* **9**, 761–771.
- Lyristis, M., Bryant, A. E., Sloan, J., Awad, M. M., Nisbet, I. T., Stevens, D. L. & Rood, J. I. (1994). Identification and molecular analysis of a locus that regulates extracellular toxin production in *Clostridium perfringens*. *Mol Microbiol* **12**, 761–777.
- Molinari, G., Talay, S. R., Valentin-Weigand, P., Rohde, M. & Chhatwal, G. S. (1997). The fibronectin-binding protein of *Strep-*

Staphylococcus pyogenes, SfbI, is involved in the internalization of group A streptococci by epithelial cells. *Infect Immun* 65, 1357–1363.

Natanson, S., Sela, S., Moses, A. E., Musser, J. M., Caparon, M. G. & Hanski, E. (1995). Distribution of fibronectin-binding proteins among group A streptococci of different M-types. *J Infect Dis* 171, 871–878.

Okada, N., Pentland, A., Falk, P. & Caparon, M. (1994). M protein and protein F act as important determinants of cell-specific tropism of *Streptococcus pyogenes* in skin tissue. *J Clin Invest* 94, 965–977.

Okada, N., Watarai, M., Ozeri, V., Hanski, E., Caparon, M. & Sasakawa, C. (1997). A matrix form of fibronectin mediates enhanced binding of *Streptococcus pyogenes* to host tissue. *J Biol Chem* 272, 26978–26984.

Ozeri, V., Tovi, A., Burstein, I., Natanson-Yaron, S., Caparon, M. G., Yamada, K. M., Akiyama, S., Vlodavsky, I. & Hanski, E. (1996). A two-domain mechanism for group A streptococcal adherence through protein F to the extracellular matrix. *EMBO J* 15, 989–998.

Perego, M., Glaser, P., Minutello, A., Strauch, M. A., Leopold, K. & Fischer, W. (1995). Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*. *J Biol Chem* 270, 15598–15606.

Schranger, H. M., Rheinwald, J. G. & Wessels, M. R. (1996). Hyaluronic acid capsule and the role of streptococcal entry into keratinocytes in invasive skin infection. *J Clin Invest* 98, 1954–1958.

Scott, J. R., Guenther, P. C., Malone, L. M. & Fischetti, V. A. (1986). Conversion of an M⁻ group A streptococcus to M⁺ by

transfer of a plasmid containing an M6 gene. *J Exp Med* 164, 1641–1651.

Sela, S., Arvik, A., Burstien, I., Tovi, A., Caparon, M. G. & Hanski, E. (1994). Protein F, an adhesin of *Streptococcus pyogenes*, binds fibronectin via two distinct domains. *Mol Microbiol* 10, 1049–1055.

Sulavik, M. C., Tardif, G. & Clewell, D. B. (1992). Identification of a gene, *rgg*, which regulates expression of glucosyltransferase and influences the Spp phenotype of *Streptococcus gordonii* Challis. *J Bacteriol* 174, 3577–3586.

Talay, S. R., Ehrenfeld, E., Chhatwal, G. S. & Timmis, K. N. (1991). Expression of the fibronectin-binding components of *Streptococcus pyogenes* in *Escherichia coli* demonstrates that they are proteins. *Mol Microbiol* 5, 1727–1734.

Talay, S. R., Valentin-Weigand, P., Jerlström, P. G., Timmis, K. N. & Chhatwal, G. S. (1992). Fibronectin-binding protein of *Streptococcus pyogenes*: sequence of the binding domain involved in adherence of streptococci to epithelial cells. *Infect Immun* 60, 3837–3844.

Talay, S. R., Valentin-Weigand, P., Timmis, K. N. & Chhatwal, G. S. (1994). Domain structure and conserved epitopes of Sfb protein, the fibronectin-binding adhesin of *Streptococcus pyogenes*. *Mol Microbiol* 13, 531–539.

Wang, J.-R. & Stinson, M. W. (1994). M protein mediates streptococcal adhesion to HEp-2 cells. *Infect Immun* 62, 442–448.

Watarai, M., Funato, S. & Sasakawa, C. (1996). Interaction of Ipa proteins of *Shigella flexneri* with $\alpha_5\beta_1$ integrin promotes entry of the bacteria into mammalian cells. *J Exp Med* 183, 991–999.

Received 20 April 1998; revised 15 June 1998; accepted 24 July 1998.