INTRODUCTION

Porphyromonas gingivalis is a Gram-negative, anaerobic, non-motile, short rod that resides predominantly in the subgingival crevice, and is a major pathogen in severe manifestations of periodontitis. P. gingivalis can attach to a variety of oral surfaces and can induce its internalization into the normally non-phagocytic gingival epithelial cells (GECs). The invasion of P. gingivalis into GECs is completed in under 20 min, and P. gingivalis accumulates in high numbers in the perinuclear region (Belton et al., 1999). Intracellularly, the organism remains viable and capable of replication. The GECs exhibit morphological changes after prolonged (24 h) association with intracellular P. gingivalis, however, they do not undergo apoptosis and maintain physiological integrity for extended periods (Lamont et al., 1995; Belton et al., 1999; Nakhjiri et al., 2001). Invasion is associated with phosphorylation of c-jun N-terminal kinase (JNK) and down-regulation of extracellular signal-regulated kinase (ERK1/2) (Watanabe et al., 2001). In GECs infected with P. gingivalis, intracellular Ca^{2+} ion levels are transiently elevated but Nuclear Factor kappa B (NF-kB) is not activated and secretion of IL-8 is inhibited (Izutu et al., 1996; Darveau et al., 1998; Huang et al., 2001).

The major fimbriae of P. gingivalis are involved in both adhesion to epithelial cells and in the subsequent signalling events associated with invasion (Weinberg et al., 1997; Njoroge et al., 1997). Our previous studies (Yilmaz et al., 2002) demonstrated that the P. gingivalis fimbriae mediate adhesion to GECs through integrin receptors. In vitro binding and immunochemical analysis revealed a direct physical association between $b_1$ integrins and P. gingivalis fimbrillin (FimA). Moreover, $b_1$ integrin antibodies inhibited P. gingivalis invasion into GECs. In separate studies, Nakagawa et al. (2002) found that antibodies to $z_5b_1$ integrin blocked uptake of FimA-conjugated microspheres by epithelial (HEp-2) cells. At the subcellular level, infection of GECs by wild-type P. gingivalis induces phosphorylation of the focal adhesion protein, paxillin (Yilmaz et al., 2002). Paxillin and focal adhesion kinase (FAK) have emerged as basic signal transducing components for integrin signalling. In many cell types, phosphorylation of paxillin and FAK is followed by the activation of other specific signalling molecules, promoting assembly of focal adhesion complexes subsequent to integrin activation (Clark & Brugge, 1995;
Yamada & Geiger, 1997). Formation of these multiple component complexes generates forces that regulate both actin and microtubule cytoskeleton dynamics and direct intracellular signals to specific targets in the cells. Therefore, the signalling events required for *P. gingivalis* uptake into GECs may emanate primarily from integrin–integrin interactions, although other signal transduction pathways may be operational. In addition, recent findings have demonstrated the direct involvement of integrin-associated signalling and cytoskeletal molecules such as FAK, paxillin, vinculin and talin in the process of invasion of organisms such as uropathogenic *Escherichia coli*, *Shigella flexneri*, streptococci, *Chlamydia pneumoniae* and *Yersinia* spp., into host cells (Watarai et al., 1996; Alrutz & Isberg, 1998; Reddy et al., 2000; Ozeri et al., 2001; Martinez & Hultgren, 2002; Coombes & Mahony, 2002). The subcellular localization of focal adhesion constituents and cytoskeletal components in response to *P. gingivalis* invasion remains to be determined.

This study was directed towards testing the hypothesis that binding of *P. gingivalis* fimbriae to GECs results in the initiation of integrin-associated signalling pathways that ultimately converge on cytoskeletal architecture. As GECs can adapt to, and co-exist with, intracellular *P. gingivalis*, we investigated both short-term (30 min) and longer-term (24 h) responses to isogenic wild-type and fimbriae-deficient *P. gingivalis* strains.

**METHODS**

**Bacteria and growth conditions.** *P. gingivalis* ATCC 33277 and its fimbriae-deficient mutant YPF1 were cultured anaerobically for 24 h at 37 °C in trypticase soy broth supplemented with yeast extract (1 mg ml⁻¹), haemin (5 µg ml⁻¹) and menadione (1 µg ml⁻¹). Erythromycin (10 µg ml⁻¹) was also added to the medium for culture of YPF1, which contains an insertional inactivation of the fimA gene (Love et al., 2000). All bacteria, grown from a 24-h culture, were harvested by centrifugation at 6000 × g and 4 °C for 10 min. Bacteria were washed in PBS (Sigma, pH 7.3) and resuspended to an OD₆₀₀ value corresponding to 10⁸ cells ml⁻¹.

**Culture of GECs.** Low passage numbers of GEC cultures were generated as described previously (Lamont et al., 1995). Briefly, healthy gingival tissue was obtained after oral surgery and surface epithelium was separated by overnight incubation with 0.1% dispase. Cells were cultured as monolayers in serum-free keratinocyte growth medium (KGM) (Clonetics) at 37 °C in 5% CO₂. GECs were used at passage four for experimentation and were at 70–75% confluence when reacted with bacterial cells in KGM.

**Epithelial cell invasion assay.** *P. gingivalis* invasion of GECs was determined by the antibiotic protection assay described previously (Lamont et al., 1995). In brief, bacteria in KGM were incubated with GECs in 24-well plates for 30 min at 37 °C. After washing with PBS, remaining external bacteria were killed with metronidazole (200 µg ml⁻¹) and gentamicin (300 µg ml⁻¹) for 60 min. GECs were washed and lysed with sterile distilled water, and intracellular bacteria were enumerated by culture on blood agar supplemented with haemin and menadione.

**Bacterial infection and preparation of GECs for fluorescence labelling.** GECs were seeded onto 4-well chambered coverglass slides (Nalge-Nunc International) at a density of 2 × 10⁵ cells per well and cultured for 48 h. Cells were infected with bacteria at an m.o.i. of 100 at 37 °C in 5% CO₂/95% air. After 30 min or 24 h incubation, the slides were washed four times with PBS containing 0·1% Tween 20 to remove the non-adherent bacteria. Cells were fixed in 10% neutral buffered formalin, rinsed in PBS and permeabilized for 15 min by 0·1% Triton X-100 in PBS at room temperature. Samples were incubated for 30 min in a blocking solution of 5% goat serum/0·1% Tween 20 in PBS to mask non-specific binding sites prior to the fluorescence labelling.

**Fluorescence microscopy.** Labelling of intracellular bacteria and the distribution of paxillin, FAK and microtubules was performed by indirect double immunofluorescence microscopy. Briefly, the coverglass slides were first co-incubated with rabbit polyclonal antibody to *P. gingivalis* 33277 at 1:5000 and mAbs to paxillin, or FAK antibodies (Transduction Laboratories), or monoclonal anti-α-tubulin antibody (Molecular Probes) at 1:200 for 1 h at room temperature. After washing three times with PBS and 0·1% Tween 20, slides were then reacted simultaneously with fluorescein isothiocyanate (FITC)-conjugated Affini-Pure F(ab')₂ fragment goat anti-rabbit IgG (H + L) (Jackson-Laboratories) and Rhodamine red-X-labelled goat anti-mouse IgG (H + L) (Molecular Probes) at 1:500 for 1 h in the dark at room temperature. Similarly, F-actin was labelled with phalloidin-tetramethylrhodamine B isothiocyanate (TRITC) (Sigma) at 1:100 for 45 min. All the antibodies and reagents used for labelling were diluted in the blocking buffer and each incubation step was followed by three washes with PBS and 0·1% Tween 20. No fluorescence staining was observed when the primary antibodies were omitted, and primary *P. gingivalis* antibodies did not react with the uninfected GECs. For microscopy, samples were mounted in mounting medium containing anti-fade agent (Vector Laboratories) and examined using an epifluorescence microscope (Zeiss Axioscope) equipped with long pass optical filter sets appropriate for TRITC (RHODA), FITC and 4',6-diamidino-2-phenylindole (DAPI) dyes. Single exposure images were captured sequentially using a cooled CCD camera (QImaging) and saved by QCAPTURE software version 1394. Collected image layers were superimposed into a single image using Adobe PHOTOSHOP 6.0 software. The images presented are a random representative of four separate experiments wherein at least 10 fields containing an average of 15 cells per sample were studied.

**RESULTS**

**Invasion by a fimbriae-deficient *P. gingivalis* mutant is less efficient than wild-type**

The ability of the fimbriae-deficient mutant strain YPF1 to invade GECs was compared to the parent strain in an antibiotic protection assay (Fig. 1). At m.o.i.s of 10, 100 and 1000, approximately 10-fold fewer YPF1 cells were recovered from GECs than parent *P. gingivalis*. The absence of the major fimbriae, therefore, compromises the invasive capability of *P. gingivalis*.

**Internalized *P. gingivalis* induces redistribution of paxillin and FAK at 30 min**

The subcellular localizations of paxillin and FAK proteins within GECs 30 min following *P. gingivalis* infection were examined by indirect immunofluorescent staining using monoclonal anti-paxillin or anti-FAK antibodies, in conjunction with anti-*P. gingivalis* polyclonal antibody to visualize the internalized bacteria. As shown in Fig. 2, wild-type *P. gingivalis* induced morphological changes characterized...
by major aggregations in the membrane and numerous microspikes and lamellipodial-like protrusions in which focal adhesion components were concentrated. P. gingivalis antibody stained predominantly around the nuclear area consistent with our previous report that the bacterial cells congregate in high numbers in this region of the cell (Belton et al., 1999). A similar pattern of staining was observed with the fimbriae-deficient mutant strain, although the GECs contained fewer bacteria, as determined by decreased fluorescence signal, and consistent with the quantitative invasion assay (Fig. 1). Paxillin was recruited mainly from the cytosol and perinuclear space [locations determined by DAPI (4',6-diamidino-2-phenylindole) labelling of the nuclei (not shown)] to the cell membrane where it displayed uniform fine clusters (dot-like staining patterns), microspikes and lamellipodial-like formations (Fig. 2a). The fimbriae-deficient mutant YPF1 also demonstrated transmembrane aggregation of paxillin, although to a lesser extent than the wild-type and with no pronounced focal contact sites or microspikes (Fig. 2b). Similarly, FAK, which was prominently localized in close proximity to the nucleus in uninfected cells, redistributed to the cell periphery and assembled into numerous focal adhesion points after 30 min infection with the wild-type P. gingivalis (Fig. 2c). In contrast, there was little recruitment of FAK by the fimbriae-deficient mutant YPF1, with only slight lamellipodial-like formations at the cell membrane of GECs (Fig. 2d). Furthermore, some fimbriated P. gingivalis-infected cells became elongated, whereas the non-fimbriated P. gingivalis-infected cells maintained rounded cell morphology. Control, uninfected, cells showed cytoplasmic distribution of paxillin and FAK mainly localized in the cytoplasm and nucleus, with few focal adhesion points containing aggregated proteins at the cell membrane (Fig. 2e, f).

Paxillin and FAK co-localize with P. gingivalis at 24 h infection

In similar co-staining experiments, the spatial relationship between paxillin or FAK and intracellular P. gingivalis was analysed after a prolonged incubation time. Staining of the 24 h-infected samples revealed that paxillin and FAK redistributed back to the cytosol, mainly to the perinuclear area where they co-localized with the internalized P. gingivalis (see yellow staining in Fig. 3a, b and c, d). The formation of peripheral focal paxillin and FAK complexes mediated by the wild-type P. gingivalis was significantly diminished after the 24 h incubation (Fig. 3c). GECs infected with the fimbriae-deficient mutant YPF1 exhibited similar cellular distribution and co-localization patterns for paxillin and FAK, after 24 h infection (Fig. 3b and d), indicating that the departure of the paxillin and FAK from the cell membrane and accumulation back to their original nuclear and cytoplasmic pools are independent of the mechanism of bacterial invasion. The intensity of bacterial staining within GECs increased by 24 h post-infection, as compared to 30 min, for both the wild-type and the fimbriae-deficient P. gingivalis, evidence of an increase in numbers of intracellular organisms (compare Figs 2a, b and c, d with 3a, b and c, d). These results corroborate data from antibiotic protection assays that also demonstrated replication of P. gingivalis within GECs (Lamont et al., 1995). The increase in bacterial numbers observed also correlated with the marked amount of dissociation of paxillin- and FAK-enriched structures at the cell peripheries and their atypical cell membrane assembly and cytoskeletal morphology compared with control cells (Fig. 3e and f). No fluorescence staining was observed when the primary antibodies were omitted, and primary P. gingivalis antibodies did not react with the uninfected GECs.

Actin and microtubule cytoskeletal architecture is reorganized by P. gingivalis invasion

In parallel experiments, the actin and microtubule rearrangements induced by P. gingivalis invasion were investigated. Subcellular structures in fixed and permeabilized GECs were analysed by fluorescence microscopy. Filamentous actin was identified with phalloidin–tetramethylrhodamine B isothiocyanate. Microtubules were labelled with anti-α-tubulin antibody. After 30 min infection with either wild-type or fimbriae-deficient P. gingivalis, GECs demonstrated distinct nucleation sites for filament growth and polymerization of monomeric actin (Fig. 4a, b). Wild-type P. gingivalis induced nucleation of actin filaments, which appeared to be thin microspike-like protrusions forming long stable microfilaments distributed throughout the cells (see arrows in Fig. 4a), whereas the fimbriae-deficient P. gingivalis promoted a widespread cortical actin meshwork accompanied by extensions resembling lamellipodia (see arrows in Fig. 4b). After infection for 24 h, wild-type P. gingivalis induced significant depolymerization of the global actin network and the stress fibres and their condensation around the cell periphery. The actin cytoskeletal organization of GECs was also disrupted after 24 h incubation with the
fimbriae-deficient mutant, as evidenced by the formation of numerous, sparse actin filaments (stress fibres) and short actin cables radiating from the perinuclear region to the edges of the cell (Fig. 4d, arrows).

Immunostaining of the microtubule cytoskeleton in GECs after 30 min infection with wild-type or mutant P. gingivalis displayed minimal alterations in the tubulin cytoskeletal structure (Fig. 5a, b). However, after 24 h the GECs infected with wild-type P. gingivalis showed significant microtubule network disassembly and nucleation to the microtubule organization centre (Fig. 5c, arrows). GECs containing the fimbriae-deficient mutant showed some degree of microtubule disassembly and nucleation (Fig. 5d). In contrast,

**Fig. 2.** Relocalization of paxillin and FAK in GECs induced by P. gingivalis after 30 min infection. GECs were double-labelled with paxillin (a, b, e) or FAK (c, d, f) mAbs (shown in red) and P. gingivalis polyclonal antibodies (shown in green). Stained cells were viewed by indirect fluorescence microscopy. Paxillin (a) and FAK (c) localized and clustered in focal adhesions at the peripheries of GECs infected with strain 33277. GECs infected with strain YPF1 showed more moderate relocalization of paxillin (b, d). Uninfected GECs were used as a control (e, f).
control cells for the two different time intervals demonstrated a dense microtubule network assembled in parallel and with radial arrays originating from the microtubule organization centre (Fig. 5e, f). Overall, infection of GECs with the wild-type *P. gingivalis* exhibited substantial effects on the epithelial integrity, whereas the non-fimbriated mutant’s effects on the GECs were similar but moderate. This may be a result both of fewer internal bacteria and of differential stimulation of epithelial cell signalling pathways.

**DISCUSSION**

*P. gingivalis*, a periodontal pathogen and successful colonizer of oral surfaces, can invade GECs rapidly and
Fig. 4. Reorganization of GEC actin filaments by *P. gingivalis* at 30 min (a, b, e) and 24 h (c, d, f) infection. GECs were double-labelled with phalloidin (shown in red) and *P. gingivalis* polyclonal antibodies (shown in green). Stained cells were viewed by indirect fluorescence microscopy. At 30 min infection, microspike-like protrusions forming long stable microfilaments (arrows) distributed throughout the cells are induced by strain 33277 (a). Strain YPF1 promoted a widespread cortical actin meshwork accompanied by lamellipodia-like extensions (arrows) (b). Both strain 33277 (a) and strain YPF1 (b) co-localized (yellow) with the actin microfilaments. After 24 h infection, 33277 induced significant depolymerization of the actin network and stress fibres and their condensation around the cell periphery (c). YPF1 disrupted the actin cytoskeletal organization causing the formation of numerous, sparse actin filaments and short actin cables radiating from the perinuclear region to the edges of the cell (arrows) (d). Uninfected GECs were used as a control (e, f).
in high numbers. Interaction between the major fimbrial structure of *P. gingivalis* and GECs mediates both bacterial adhesion and invasion. Our previous studies indicated that the targeting of *P. gingivalis* to GECs occurs through a specific interaction between the major fimbriae and β1 integrins on the GECs, following which the bacterial cells direct their uptake into the epithelial cells by a process that requires both microfilament and microtubule activity (Lamont *et al.*, 1995; Belton *et al.*, 1999; Yilmaz *et al.*, 2002). The molecular dialogue between *P. gingivalis* and GECs

**Fig. 5.** Microtubule structure in GECs infected with *P. gingivalis* for 30 min (a, b, e) and 24 h (c, d, f). GECs were double-labelled with tubulin mAb (shown in red) and *P. gingivalis* polyclonal antibodies (shown in green). Stained cells were viewed by indirect fluorescence microscopy. Neither 33277 (a) nor YPF1 (b) induced significant changes in microtubule organization at 30 min infection. After 24 h infection, strain 33277 induced pronounced nucleation and degradation in the tubulin network (arrows) (c). Strain YPF1 evoked a lesser degree of condensation and nucleation of the tubulin network (d). Both strain 33277 and strain YPF1 co-localized (yellow) with the region that contains the minus ends of the microtubule network. Uninfected GECs were used as a control (e, f).
Involves modulation of the activity of MAP kinase family proteins along with transient increases in intracellular Ca\(^{2+}\) levels, and ultimately leads to down-regulation of IL-8 secretion and to inhibition of apoptotic GEC death (Izutsu et al., 1996; Darveau et al., 1998; Huang et al., 2001; Nakajiri et al., 2001, Watanabe et al., 2001). Moreover, the focal adhesion signalling component paxillin is phosphorylated following binding of P. gingivalis fimbriae to integrins on the GECs surface (Yilmaz et al., 2002). Paxillin, along with other focal adhesion constituents such as FAK, are considered essential integrin-associated signalling molecules. Phosphorylation of paxillin and FAK coordinate the formation of focal adhesions and stress fibres, creating a platform for transduction of a variety of signalling information to specific targets via hierarchical and synergistic molecular interactions (Gilmore & Burridge, 1996; Schlaepfer et al., 1999; Turner, 2000; Schaller, 2001). We have investigated the temporal and spatial distribution of paxillin and FAK, along with microfilament and microtubule components of the GEC cytoskeleton, following infection with P. gingivalis.

To examine fimbriae–integrin-based signalling events, we utilized a fimbriae-deficient mutant of P. gingivalis 33277, designated YPF1. Strain YPF1 contains an insertional inactivation of the fimA gene and hence is unable to produce FimA protein and lacks the major fimbriae (Love et al., 2000). In antibiotic-protection-based invasion assays, strain YPF1 showed a 10-fold reduction in internalization within GECs. These data, along with immunofluorescence of YPF1 within GECs, demonstrated that the mutant has impaired invasion capabilities; however, there remains a degree of residual, albeit less-efficient, invasion and the mutant is competent in non-fimbriae-dependent interactions with GECs.

Uptake of wild-type P. gingivalis into GECs is complete after approximately 20 min (Belton et al., 1999); therefore, 30 min P. gingivalis infection will reveal properties of GECs modulated concurrent with the invasion process. Visualization of the intracellular distribution of paxillin and FAK 30 min after P. gingivalis infection demonstrated a substantial amount of aggregation at the plasma membrane and the formation of microspikes and lamellipodial-like extensions along the edges of the cells. Paxillin activation and assembly into focal adhesions by P. gingivalis is consistent with our previous observations demonstrating phosphorylation of paxillin during P. gingivalis invasion (Yilmaz et al., 2002). However, these earlier immunoprecipitation experiments did not demonstrate an increase in FAK activity triggered by P. gingivalis. This discrepancy is likely to originate from the use of only the Triton X-100 soluble cell lysate fractions in the prior study. Invasion by fimbriate P. gingivalis thus evokes the formation of integrin-associated focal adhesions and will likely stimulate the cascade of events that normally follows integrin activation (Alrutz & Isberg, 1998; Metheniti et al., 2001; Ozeri et al., 2001; McGee et al., 2003).

One downstream target of integrin signalling is the cellular cytoskeleton (Schoenwaelder & Burridge, 1999). Sensitivity of the invasion process to the inhibitors nocodazole and cytochalasin D signifies the necessity of both actin and microtubule cytoskeletal reassembly for the internalization of P. gingivalis (Lamont et al., 1995). The present microscopic study demonstrated that wild-type P. gingivalis triggers distinct actin rearrangements during infection and promotes the formation of thin filamentous microspike-like structures emanating from the cell cortex. Microtubule rearrangements were less dramatic. However, microtubule dynamics can occur rapidly and may not be observable by a single technique (Waterman-Storer, 1998). Therefore, our fixed point observations may not reflect the complete range of tubulin remodelling induced by P. gingivalis in GECs.

Following invasion, both P. gingivalis and epithelial cells remain viable for extended periods. Hence, GEC signal transduction pathways can be expected to be modulated both in the short term during the entry process and in the longer term as the bacteria and GECs adapt to their new conditions. After 24 h of P. gingivalis association with GECs, focal adhesions began to disassociate and paxillin and FAK redistributed back from the membrane and into the cytoplasm where there was a significant degree of co-localization with P. gingivalis cells in the perinuclear area. The early morphological alterations of both the actin filaments and the microtubules culminated in the significant amount of depolymerization and nucleation. Long-term stable changes in cell structure thus appear to accompany the adaptation of GECs to the burden of large numbers of intracellular P. gingivalis. This could also result in intracellular P. gingivalis interference with epithelial cell migration, proliferation and modulation of cell-matrix adhesion, along with disruption of cell-matrix remodelling over longer periods.

Collectively, the results presented here, along with our previous study demonstrating that integrin antibodies do not inhibit invasion of the non-fimbriated YPF1 (Yilmaz et al., 2002), show that P. gingivalis possesses mechanisms of entry into GECs that are independent of fimbriae–integrin binding. While less efficient than fimbriae-dependent pathways, the YPF1 cells remain viable and accumulate in the perinuclear area. The GEC receptor for YPF1 invasins is currently under investigation. Although paxillin and FAK were only minimally redistributed during the fimbriae-independent invasion process, paxillin and FAK did co-localize with both parent and mutant strains after 24 h infection. This result indicates that redistribution of paxillin and FAK at later time points is a property of internalized bacteria and does not require the presence of fimbriae. Indeed, proteomic analysis (Wang et al., 2002) has demonstrated that levels of FimA are reduced shortly after contact between P. gingivalis and GECs, thus internalized wild-type bacteria are likely to be only sparsely fimbriated. Actin rearrangements induced by invasion of the fimbriae-deficient mutant were also different from those of the parent. YPF1 induced actin condensation distributed throughout the cells as opposed to the filamentous structures induced by...
the wild-type. These findings are consistent with reports that bacterial invasion mediated through integrin binding is usually associated with minimal and transient cytoskeletal remodelling (Young et al., 1992). YPF1 may, therefore, engage epithelial cells in a manner that requires major actin cytoskeletal rearrangements. As the fimA gene of P. gingivalis can be regulated by a number of environmental conditions (Xie et al., 1997, 2000), the ability to invade epithelial cells by fimbiae-independent mechanisms may be beneficial to the organism in situations where the fimbiae are down-regulated. Furthermore, the presence of fimbiae-dependent and independent invasion mechanisms may account, at least partially, for the differences in invasive pathways utilized by P. gingivalis in non-transformed GECs (Lamont et al., 1995), transformed epithelial cells (Chen et al., 2001) and endothelial cells (Dorn et al., 2000).

This work demonstrates that invasive P. gingivalis modifies both the GEC membrane-associated structural and signalling protein arrangements and the cytoskeletal organization. Thus, P. gingivalis is capable of targeting specific epithelial cell pathways during invasion and adaptation to an intracellular lifestyle. Such complex and multi-threaded interactions point toward a long evolutionary relationship between P. gingivalis and host cells, resulting in a balanced association whereby the organism can survive within epithelial cells without causing excessive harm. P. gingivalis-induced diseases may then ensue from a disruption of this balance by factors that may trigger virulence or lead to host-immune-mediated tissue damage.

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