Involvement of integrins in fimbriae-mediated binding and invasion by *Porphyromonas gingivalis*

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Summary

Interaction between the major fimbriae of *Porphyromonas gingivalis* and gingival epithelial cells is important for bacterial adhesion and invasion. In this study, we identified integrins as an epithelial cell cognate receptor for *P. gingivalis* fimbriae. Immuno-precipitation and direct binding assays revealed a physical association between recombinant fimbrillin and β1 integrins. *In vitro* adhesion and invasion assays demonstrated inhibition of binding and invasion of *P. gingivalis* by β1 integrin antibodies. In contrast, invasion of a fimbriae-deficient mutant of *P. gingivalis* was not affected by integrin antibodies. Infection of gingival epithelial cells with wild-type *P. gingivalis* induced tyrosine phosphorylation of the 68 kDa focal adhesion protein paxillin, whereas the fimbriae-deficient mutant failed to evoke similar changes. Interestingly, activation of paxillin was not accompanied by an increase in the phosphorylation of focal adhesion kinase (FAK). These results provide evidence that *P. gingivalis* fimbriae promote adhesion to gingival epithelial cells through interaction with β1 integrins, and this association represents a key step in the induction of the invasive process and subsequent cell responses to *P. gingivalis* infection.

Introduction

Microbial pathogens have developed an abundance of virulence factors to engage their host in a number of intricate molecular interactions. *Porphyromonas gingivalis*, a Gram-negative anaerobe, recognized widely as a predominant aetiologic agent in the severe forms of adult periodontitis (Socransky and Haffajee, 1992), is one example of a pathogen that can manipulate host cell function. The organism can direct its entry into both epithelial and endothelial cells by exploiting host cell signalling pathways (Lamont et al., 1995; Deshpande et al., 1998; Dorn et al., 2000). The process of invasion of gingival epithelial cells (GEC) is rapid and results in the congregation of high numbers of *P. gingivalis* in the perinuclear area (Belton et al., 1999). After invasion, both host and bacterial cells remain viable for a prolonged period of time (Belton et al., 1999; Nakjiri et al., 2001). Invasion is accompanied by cytoskeletal rearrangements, calcium ion fluxes, modulation of MAP kinase and apoptotic pathways, and downregulation of interleukin 8 (IL-8) expression (Izutsu et al., 1996; Darveau et al., 1998; Nakjiri et al., 2001; Watanabe et al., 2001). Entry of *P. gingivalis* into epithelial cells has been demonstrated in vivo and may contribute to bacterial persistence and to the progression of the chronic manifestations of periodontal diseases (Noiri et al., 1997; Rudney et al., 2001).

Adherence of *P. gingivalis* to epithelial cells is a prerequisite for invasion. *P. gingivalis* possesses a number of adhesins including haemagglutinins, proteases and fimbiae (Lamont and Jenkinson, 2000). The major fimbiae serve as an important adherence-mediating determinant of *P. gingivalis* and participate in almost all the binding activities of the organism, including attachment to epithelial cells, other bacteria, salivary molecules and matrix components (Amano et al., 1996; Murakami et al., 1996; Nagata et al., 1997; Lamont and Jenkinson, 1998). The major fimbiae are constructed from a fimbrillin monomer (FimA ~43 kDa), encoded by a single fimA gene. The *P. gingivalis* fimbiae show no significant homology to fimbiae from other bacteria (Dickinson et al., 1998). In all cases thus far examined, binding activity is associated with discrete peptide domains within the FimA structural subunit protein (Lamont and Jenkinson, 2000). Furthermore, these peptide domains are capable of mediating adherence through protein–protein interactions. FimA-deficient mutants of *P. gingivalis* are attenuated in adherence and invasion (Weinberg et al., 1997; Njoroge et al., 1997), and in their ability to affect phenotypic properties such as downregulation of IL-8 expression (Darveau et al., 1998). Fimbiae-mediated binding may therefore play a significant role in the molecular dialogue that occurs between *P. gingivalis* and epithelial cells. The identity of the GEC receptor that binds to FimA and initiates the uptake process has not been established.

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In this study, we demonstrate that the FimA protein of *P. gingivalis* physically associates with β1 integrins. Moreover, β1 integrin antibodies inhibit *P. gingivalis* attachment to, and invasion of, GEC, indicating that integrins function as the invasion-associated receptor for *P. gingivalis* fimbriae. Consistent with this, interaction of GEC with *P. gingivalis* induces phosphorylation of the integrin-clustering associated protein paxillin. Thus, engagement of the *P. gingivalis* fimbriae with GEC integrins may both mediate attachment and the induction of the invasive process that allows *P. gingivalis* to enter the epithelial cell.

**Results**

*Integrins co-precipitate with* *P. gingivalis* *fimbriae*

To identify GEC molecules that interact with *P. gingivalis* fimbriae, GEC membranes were biotinylated and reacted with recombinant fimbriin (rFimA). Precipitation with FimA antibodies, revealed four major bands of 150, 140, 130 and 110 kDa (Fig. 1) that specifically bound to rFimA. The size of these bands was consistent with that of β integrin subunits, which have also been shown to act as the macrophage receptors for *P. gingivalis* fimbriae (Takeshita *et al.*, 1998). Furthermore, β integrins are widely distributed on cells of the gingival epithelium (Hormia *et al.*, 1990; Thorup *et al.*, 1997). Probing the GEC membrane preparation with anti-β1 integrin monoclonal antibodies detected bands of 130 and 140 kDa (Fig. 1), providing support for the concept that some or all of the co-precipitating molecules were β1 integrins. To test this hypothesis, the co-precipitate of the fimbrial-GEC extract was probed with β1 integrin antibodies. As shown in Fig. 2, β1 integrin antibodies detected bands of 110, 130 and 140 kDa, indicating that these fimbriae-binding molecules are β1 integrins. The range of molecular masses displayed by the fimbriae-binding molecules may be the result of splice variation (Languino and Ruoslahti, 1992) or of proteolytic processing. Further support for fimbriae-integrin binding was provided by using β1 integrin antibodies to precipitate the fimbriae-GEC extract reaction. This co-precipitate also displayed the 110, 130 and 140 kDa bands (Fig. 2). In addition, probing of the GEC membrane-fimbriae-anti-integrin precipitates with anti-fimbriae antibodies confirmed the presence of rFimA. The identity of the 150 kDa band is unknown at present. Potentially, it could be the dimerization partner of a β1 integrin subunit, or an unrelated molecule to which *P. gingivalis* fimbriae can bind. Collectively, the data indicate that there is a physical association between *P. gingivalis* fimbriae and β1 integrin subunits on GEC membranes.

*Fimbriae bind to purified* β1 *integrins heterodimers*

To substantiate that an interaction occurs between β1 integrins and *P. gingivalis* fimbriae, we examined the binding between rFimA and commercially available α5β1 integrin subunits in an ELISA. α5 was selected as the dimerization partner because the α5β1 integrin heterodimer can function as a receptor for the integrin-binding proteins of bacteria, such as *Yersinia* spp., *Shigella flexneri*, *Bordetella pertussis* and *Pseudomonas aeruginosa* (Watarai *et al.*, 1996; Roger *et al.*, 1999; Isberg *et al.*, 2000; Ishibashi *et al.*, 2001). rFimA bound in a dose-dependent manner to the integrin subunits and the binding demonstrated saturation kinetics (Fig. 3). Thus, β1 integrin can bind to *P. gingivalis* fimbriae when present in...
the context of a native heterodimer. Whereas α5β1 integrin heterodimers exhibit fimbrillin binding, this does not exclude the possibility that other a dimerization partners can also produce integrin molecules that bind to *P. gingivalis* fimbriae.

Integrin antibodies inhibit *P. gingivalis* adherence to GEC

To assess the functionality of the fimbriae–integrin interaction, the ability of β1 integrin antibodies to inhibit *P. gingivalis* adhesion to GEC was determined. GEC were fixed to prevent internalization by *P. gingivalis*, and binding of whole *P. gingivalis* cells was measured with *P. gingivalis* antibodies and peroxidase conjugated secondary antibodies. β1 integrin antibodies were found to inhibit binding of *P. gingivalis* in a dose-dependent manner, up to a maximum of 79% (Fig. 4). In contrast, control keratin antibodies did not exhibit significant inhibitory activity, and indeed at a dilution of 1:100 there was no detectable effect.
on *P. gingivalis* binding (Fig. 4). This suggests that β1 integrins can function as a cognate receptor for *P. gingivalis* fimbriae. Whereas *P. gingivalis* adhesion may be multi-modal (Du et al., 1997; Chen et al., 2001), and fimbriae themselves may bind to more than one receptor (Weinberg et al., 1997), the fimbriae–integrin interaction would appear to be a significant component of the overall adherence process. Interestingly, RGD peptides did not affect *P. gingivalis*-GEC binding (not shown), demonstrating that the RGD binding motif of integrins (Ruoslahti, 1996) did not contribute to the association. This property of *P. gingivalis* fimbriae is distinct from the integrin-binding ligands of bacteria such as *Yersinia*, *Bordetella* and *Pseudomonas*, all of which involve an RGD-like motif (Leong et al., 1995; Roger et al., 1999; Ishibashi et al., 2001). However, the Ipa proteins of *Shigella flexneri* bind to epithelial cell integrins through an RGD-independent mechanism (Watarai et al., 1996).

**Fimbriae-mediated invasion of GEC is inhibited by integrin antibodies**

*P. gingivalis* is an invasive organism, rapidly entering epithelial cells and accumulating in the perinuclear area. Fimbriae-mediated adhesion is a requisite first step in the invasion process. We thus investigated the role of integrin binding in *P. gingivalis* invasion. Internalization of *P. gingivalis* 33277 and its fimbriae-deficient mutant YPF1 was measured, in the presence and absence of antibodies, by an antibiotic protection assay. β1 integrin antibodies inhibited *P. gingivalis* 33277 invasion up to 94% (Fig. 5A). In contrast, keratin antibodies had no significant effect on invasion. The invasion efficiency of fimbriae-deficient mutants is approximately one log lower than that of their parent strains as a result of the absence of fimbriae (Weinberg et al., 1997). Nonetheless, about 1% of the input bacterial cell numbers (at a MOI of 100) internalize within GEC, indicating the existence of fimbriae-independent invasion pathways. The inability of β1 integrin antibodies to impede the invasion of YPF1 (Fig. 5B) suggests that the fimbriae-independent pathways do not involve β1 integrins. Taken together, these data support the concept that fimbriae–integrin interactions initiate one pathway that leads to *P. gingivalis* internalization.

**P. gingivalis induces tyrosine phosphorylation of paxillin but not FAK in GEC**

Engagement of integrins by a ligand is associated with subsequent recruitment and activation of a large number of cytoplasmically derived regulatory proteins including FAK and paxillin (Turner, 1998; Vuori, 1998; Giancotti and Ruoslahti, 1999). Hence, we examined the phosphorylation of paxillin and FAK in GEC after infection with *P. gingivalis* 33277 or YPF1. GEC were immunoprecipitated with specific paxillin or FAK antibodies, and tyrosine phosphorylation assessed by immunoblotting with phosphotyrosine antibodies. Paxillin was maximally activated after 20 min infection with *P. gingivalis* 33277 (Fig. 6A). The level of phosphorylation was related to the number of infecting bacteria (Fig. 6A), and densitometric analysis revealed maximal 9.5-fold increase in the ratio of phosphorylated:total paxillin at a MOI of 100 after 20 min 33277–GEC interaction (Fig. 6C). After 45 min, tyrosine phosphorylation returned to near baseline levels. The fimbriae-deficient mutant YPF1 did not produce a signifi-

![Fig. 5. Antibody inhibition of invasion of GEC by *P. gingivalis* 33277 (A) or *P. gingivalis* YPF1 (B). GEC were reacted with integrin or keratin (control) antibodies as indicated. Invasion of *P. gingivalis* strains at MOI 100 was determined by an antibiotic protection assay. Results are expressed as percentage of initial inoculum of *P. gingivalis* recovered intracellularly. Error bars represent standard deviation (n = 3).](image-url)
cant increase in the relative amount of phosphorylated paxillin (Fig. 6B and C).

In contrast to the results with paxillin, neither 33277 nor YPF1 induced significant activation of FAK in GEC (Fig. 7). FAK recruitment and activation is a common occurrence after integrin activation (Alrutz and Isberg, 1998; Parsons et al., 2000; Schaller, 2001) and paxillin can be phosphorylated by activated FAK (Vuori, 1998; Turner, 2000). However, paxillin can also be activated by other phosphotyrosine kinases such as Src (Cary and Guan, 1999), and hence our results would suggest that FAK-independent activation of paxillin may be induced by P. gingivalis. Alternatively, as activation of FAK in the control unstimulated cells was high (Fig. 7), fimbriae-integrin binding may not stimulate further activation of FAK. Unlike the situation in cell lines (Schlaepfer et al., 1999), primary cultures of gingival epithelial cells may therefore have a continual FAK activation that could enable a more rapid response to integrin-mediated signalling.

Discussion

Fimbriae are an essential virulence factor for many pathogenic bacteria. A primary function of these structures is in the initial attachment to host surfaces (Smyth et al., 1996; Abraham et al., 1998). However, in recent years, a novel role for fimbriae has emerged, that of direct mediators of signalling between bacteria and host cells during invasion. For example, the fimbriae of uropathogenic Escherichia coli, Neisseria and Salmonella have been found to stimulate host cell signalling pathways that involve protein phosphorylations, calcium ion fluxes and culminate in rearrangements of actin and other cytoskele-

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are also initiators of signal transduction pathways that lead to a variety of biological processes, such as cell migration, proliferation, transformation, differentiation, apoptosis and inflammatory responses (Hynes, 1992; Sheppard, 1996; Schoenwaelder and Burridge, 1999). Many bacteria, such as Yersinia enterocolitica, Shigella flexneri, Neisseria gonorrhoeae, and Pseudomonas aeruginosa exploit integrins to direct their uptake into non-phagocytic host cells (Isberg and Van Nhieu, 1994; Watarai et al., 1996; Dehio et al., 1998; Roger et al., 1999). To this list can now be added P. gingivalis, which engages integrins on gingival epithelial cells through the major fimbriae. This result is consonant with previous studies demonstrating that P. gingivalis fimbriae bind to β2 integrins on macrophages (Takeshita et al., 1998). Moreover, Nakagawa et al. (2002) reported that antibodies to α5β1, integrin blocked uptake of rFimA-conjugated microspheres by epithelial (HEp-2) cells. Collectively, these results support the concept that fimbriae-integrin binding is sufficient to initiate a signal transduction cascade that allows internalization to occur. However, the data are not incompatible with a role for other epithelial cell molecules as fimbrial receptors. For example, Sojar et al. (2002) found that P. gingivalis fimbriae can interact with cytokeratin 14 on the surface of KB cells. Bacterial invasion mediated through integrin binding is usually associated with minimal and transient cytoskeletal remodeling (Young et al., 1992). Consistent with this, while microfilament and microtubule reorganization is required for P. gingivalis invasion, major rearrangements of actin microfilaments or of microtubules are not observed during P. gingivalis entry into GEC (Belton et al., 1999). 

Events that follow integrin-ligand binding generally involve integrin clustering and assembly of a focal adhesion complex containing structural and regulatory proteins such as FAK, paxillin, tensin, talin, vinculin, α-actinin, Src family kinases, PI3 kinase, P130Cas and small GTP-binding proteins. The β-cytoplasmic domains of integrins are required for recruitment of these proteins which effectuate the linkage between integrins and the actin-based cytoskeleton, along with other downstream signalling pathways, through a cascade of tyrosine phosphorylations (Clarke and Brugge, 1995; Yamada and Geiger, 1997). Fimbriated P. gingivalis can stimulate activation of paxillin, indicating that the integrin binding event does initiate signal transduction. Further, inhibition of actin polymerization by cytochalasin D impedes P. gingivalis invasion (Lamont et al., 1995), and so a classical integrin signalling pathway may be activated by P. gingivalis. The full nature of the focal adhesions assembled in GEC in response to P. gingivalis remains to be determined. 

Integrin-mediated uptake and signalling is consistent with a number of additional aspects of the P. gingivalis invasive process. Integrin signalling can result in activation of JNK, a stress-activated protein kinase that has
recently been demonstrated to be associated with P. gingivalis invasion (Watanabe et al., 2001). Integrin focal adhesions can be physically linked through paxillin to tubulins in the perinuclear microtubule organizing center (Herreros et al., 2000; Turner, 2000). P. gingivalis activation of integrin could thus provide the stimulus to cause the microtubule reorganization that is required for entry as has been demonstrated for other bacteria such as uropathogenic E. coli (Guignot et al., 2001). Recently, integrin-mediated adhesion has been demonstrated to modulate the activity and physical location of apoptosis-related proteins. In intestinal epithelial and CHO cells, the anti-apoptotic molecule Bcl-2 protein can be upregulated by integrin binding, thus resulting in suppression of apoptotic cell death (Gauthier et al., 2001; Matter and Ruoslahti, 2001). Similarly, in primary mouse mammary cells, loss of integrin adhesion induced rapid translocation of the pro-apoptotic molecule BAX from the cytosol to the mitochondria, initiating an early event in the apoptotic pathway (Gilmore et al., 2000). Interestingly, P. gingivalis invasion of GEC blocks camtothecin mediated apoptosis and elevates Bcl-2 at both the protein and mRNA level. In contrast, BAX levels are downregulated by P. gingivalis (Nakhjiri et al., 2001). Both these events may thus depend, to some extent, on integrin activation by the P. gingivalis fimbriae.

In the human oral cavity, the major site of P. gingivalis colonization and pathogenicity is the gingival sulcus, the crevice between surfaces of the tooth and the gingiva. The gingival sulcus is lined with junctional epithelial cells that are characterized by a lack of keratinization, limited differentiation and a relatively permeable structure. These junctional epithelial cells express an overall surface distribution of integrins (Hormia et al., 1990; Thorup et al., 1997). Thus the receptors for P. gingivalis fimbriae are available on the epithelial cells that provide the primary barrier to P. gingivalis intrusion. Integrin-mediated attachment and invasion may then contribute to the colonization of the organism, and to the successful avoidance of host defences and recalcitrance to elimination by physical and chemical means.

In summary, in this study we have identified β1 integrins as a GEC receptor for the major fimbriae of P. gingivalis. Fimbriae-integrin binding is involved in both attachment and invasion of the organism. Downstream signalling events resulting from fimbriae binding include tyrosine phosphorylation of paxillin. However, the precise biological and biochemical roles of the individual components of the interaction and of the signalling hierarchy mediated by integrins during the invasion of GEC remains to be determined. Future efforts to establish and decipher the interactions between P. gingivalis and the other signalling molecules will continue to enhance our knowledge of P. gingivalis induced diseases.

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Experimental procedures

Bacterial strains and culture conditions

P. gingivalis ATCC 33277 and its fimbriae-deficient mutant YPF1 (Love et al., 2000) 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 media for culture of YPF1, which contains an inserional inactivation of the fimA gene. All bacteria were grown for 24 h, harvested by centrifugation, washed and resuspended in PBS. The number of bacteria was determined in a Klett–Summerson photometer.

Culture of gingival epithelial cells

Primary cultures of GEC 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 0.4% dispase. Cells were cultured as monolayers in serum-free keratinocyte growth medium (KGM) (Clonetics) at 37°C in 5% CO₂. GEC were used for experimentation at 80% confluence and reacted with bacterial cells or other test reagents in KGM.

Preparation of recombinant fimbrillin and fimbrillin antibodies

Recombinant fimbrillin (rFimA) was produced by cloning the PCR-amplified fimA coding sequences from P. gingivalis chromosomal DNA into the pET30 expression system (Novagen). After induction in E. coli, rFimA was purified by chromatography over a Ni²⁺ metal chelation resin and eluted with imidazole. Upstream vector-derived sequences were then removed by cleavage with enterokinase. The recombinant product is a full-length mature fimbrillin without the leader amino acid sequence. Monospecific polyclonal antibodies to rFimA were produced in rabbits by Covance.

Cell surface labelling of gingival epithelial cells and immunoprecipitation

GEC monolayers were washed twice in PBS and surface labelled with N-hydroxysuccinimibiotin (2.5 mg/10⁶ cells) in 0.1M NaHCO₃ (pH 8.1) for 2 h at room temperature. After biotinylation, GEC were washed in ice-cold PBS and lysed in RIPA buffer (PBS pH 7.4 containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μg ml⁻¹ aprotinin). The soluble fraction was collected by centrifugation at 15000 g for 15 min and the protein concentration determined by the Bio-Rad protein assay.

Crude GEC extracts containing biotinylated membrane proteins were incubated with 3 ng of rFimA for 1 h at 4°C. Immunoprecipitation was accomplished by reacting for 1 h with anti-rFimA antibodies (1:100) or with anti-β1 integrin monoclonal antibody (Santa Cruz) 1:100. The immunocomplexes were then pelleted with Protein A-Sepharose beads (Amersham Pharmacia) at 1:1 by volume, washed in RIPA buffer and boiled in SDS–PAGE sample buffer.
**Western blot analysis**

Biotin-labelled samples and precipitates were resolved by 10% SDS–PAGE, and transferred to nitrocellulose membranes. After blocking with PBS containing 0.1% Tween 20, membranes were incubated with streptavidin peroxidase (1:3000) for 1 h. Biotin–streptavidin reactions were developed with diaminobenzidine tetrahydrochloride. For antibody probing, anti-β1 integrin monoclonal antibody (Transduction Laboratory; 1:1000) or anti-rFimA (1:1000) were used with peroxidase-conjugated secondary antibody (Bio-Rad; 1:3000).

**ELISA of fimbrillin binding to integrin**

Purified human αβ3 integrin subunits (Chemicon International; 0.5 μg protein) were immobilized onto 96-well microtitre plates in 50 mM carbonate/bicarbonate buffer pH 9.6 overnight at 4°C. After removal of unbound proteins by washing with PBS, the plate was blocked for 2 h with 2% BSA in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM MgCl2, 0.1 mM CaCl2, 0.1% Triton X-100. rFimA at varying concentrations was then reacted with the integrins for 1 h at 37°C. Binding was detected by sequential reactions (1 h, 37°C) with FimA antibodies (1:1000), peroxidase-conjugated anti-rabbit IgG (Bio-Rad; 1:5000) and TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate (Bio-Rad), and absorbance at 450 nm was recorded. Controls without rFimA were included in all experiments and were less than 10% of specific values.

**Epithelial cell adherence inhibition assay**

GEC were seeded onto a 96-well plate at a density of 5 × 10³ cells per well. After culture for 48 h, cells were fixed with 4% paraformaldehyde in PBS and reacted with anti-β1 antibodies (Santa Cruz) or anti-keratin 5/6 antibodies (Chemicon International) at 1:10 and 1:100. In separate experiments, RGD (Arg-Gly-Asp-Ser) tetrapeptides (Sigma; 10 μM) were added to the GEC. After 1 h at room temperature, cells were washed (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM MgCl2, 0.1 mM CaCl2, 0.1% Triton X-100) and exposed to varying concentrations of P. gingivalis cells for an additional hour. Unattached bacteria were removed by washing, and binding of P. gingivalis determined with anti-whole cell P. gingivalis antibody (Jackson Laboratory) and chromogenic substrate as described for the ELISA above. Controls without P. gingivalis or blocking antibody/peptide were included in all experiments and values subtracted from the experimental results.

**Epithelial cell invasion inhibition assay**

Antibody-mediated inhibition of invasion of GEC by P. gingivalis 33277 and YPF1 was measured by an antibiotic protection assay as modified for P. gingivalis. GEC were preincubated with anti-β1 integrin antibodies or anti-keratin 5/6 antibody (1 h, 37°C). Bacteria, at a multiplicity of infection (MOI) of 100, were incubated with GEC for 90 min at 37°C. After washing with PBS, remaining external bacteria were killed with metronidazole (200 μM) and gentamicin (300 μM) for 60 min. GEC were washed and lysed with sterile distilled water, and intracellular bacteria were enumerated by culture on blood agar supplemented with haemin and menadione.

**Assay of paxillin and FAK activation**

GEC were infected with P. gingivalis 33277 or YPF1 at a MOI of 100 for 5, 20 or 45 min. Cells were washed twice with cold PBS and solubilized in lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF and 0.1% aprotinin). Lysates were clarified by centrifugation at 15000 g for 15 min and protein concentration was determined by the Bio-Rad protein assay. Paxillin and FAK were precipitated from the cell lysates with the specific antibodies: anti-paxillin (H-114, Santa Cruz) or anti-FAK (A-17, Santa Cruz) at 2 μg/ml overnight at 4°C. The protein–antibody complexes were collected with Protein A-Sepharose beads at 1:1 by volume and washed three times with lysis buffer. Samples (5 μg of protein) were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Transient staining with Ponceau S was used to verify that equivalent amounts of protein were loaded. Tyrosine phosphorylation of FAK and paxillin were assessed by reacting with a 1:200 dilution of peroxidase-conjugated anti-phosphotyrosine antibodies (RC 20, Transduction Laboratory). Results were visualized by the enhanced chemiluminescence detection system (Amersham Pharmacia), analysed by scanning densitometry and quantitated using NIH Image. Blots were then stripped and reprobed with a 1:250 dilution of anti-FAK (C-20, Santa Cruz) or a 1:1000 dilution of anti-paxillin (Transduction Laboratory) to determine total paxillin and FAK in the samples.

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