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Gingipain-dependent degradation of mammalian target of rapamycin pathway proteins by the periodontal pathogen *Porphyromonas gingivalis* during invasion

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SUMMARY

Porphyromonas gingivalis **and** ***Tannerella forsythia*** **are gram-negative pathogens strongly associated with periodontitis. Their abilities to interact, invade and persist within host cells are considered crucial to their pathogenicity, but the mechanisms by which they subvert host defences are not well understood. In this study, we set out to investigate whether** ***P. gingivalis*** **and** ***T. forsythia*** **directly target key signalling molecules that may modulate the host cell phenotype to favour invasion and persistence. Our data identify, for the first time, that** ***P. gingivalis*****, but not** ***T. forsythia*****, reduces levels of intracellular mammalian target of rapamycin (mTOR) in oral epithelial cells following invasion over a 4-h time course, via the action of gingipains. The ability of cytochalasin D to abrogate** ***P. gingivalis*****-mediated mTOR degradation suggests that this effect is dependent upon cellular invasion. We also show that levels of several other proteins in the mTOR signalling pathway are modulated by gingipains, either directly or as a consequence of mTOR degradation including p-4E-BP1. Taken together, our data suggest that** ***P. gingivalis*** **manipulates the mTOR pathway, providing evidence for a potentially novel mechanism by which** ***P. gingivalis*** **mediates its effects on host cell responses to infection.**

INTRODUCTION

Periodontitis is a chronic condition characterized by the inflammation of the ligaments and other structures supporting the teeth. Pathogenesis results from a combination of the host response to microbial challenge and the direct effects of bacterial virulence factors that result in destruction of both hard and soft tissues in the periodontium (Ready *et al*., 2008). Although periodontitis is a polymicrobial disease, the pathogens predominantly responsible are considered to be the red-complex anaerobes *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* (Socransky *et al*., 1998).

Both *P. gingivalis* and *T. forsythia* are gram-negative bacteria harbouring numerous virulence factors, which contribute to tissue destruction and host invasion (Holt & Ebersole, 2005; Tanner & Izard, 2006; Sharma, 2010; Stafford *et al*., 2012). Examples of such virulence factors include lipopolysaccharide, gingipain proteases, lipoproteins and fimbriae in the case of *P. gingivalis* (Holt *et al*., 1999), and the fibronectin-binding protein BspA, a unique glycosylated S-layer (Posch *et al*., 2011; Settem *et al*., 2012) and sialidase for *T. forsythia* (Sharma *et al*., 1998, 2005;

Roy *et al.*, 2011). Lipopolysaccharide on the outer surface of both bacteria as well as BspA have been shown to interact with Toll-like receptor 2 on oral epithelial cells (Burns *et al.*, 2006; Myneni *et al.*, 2011) and trigger interleukin-6 release. The fimbriae of *P. gingivalis* are also involved in cell invasion and bacterial internalization (Weinberg *et al.*, 1997). Other well-known virulence factors are the proteases secreted by both bacteria; *P. gingivalis* secretes a suite of cysteine proteases with trypsin-like activity (Kuramitsu, 1998), whereas *T. forsythia* produces a xenologue of MMP-9 (karilysin) that is known to inhibit components of the complement system, and the protease PrtH, which has been identified as a cytocidal toxin (Cct) (Nakajima *et al.*, 2006; Jusko *et al.*, 2012). Hence, it is evident that periodontal pathogens employ several mechanisms to colonize surfaces, invade host cells and evade immune surveillance.

The mammalian target of rapamycin (mTOR), a serine/threonine kinase, integrates several key processes such as cell growth, proliferation, cell motility, cell survival, protein synthesis and transcription (Hay & Sonenberg, 2004). Mammalian TOR has been implicated in the regulation of proinflammatory cytokine expression following bacterial challenge (Säemann *et al.*, 2009) as well as in the autophagic pathway (Jung *et al.*, 2010). Recent studies have also shown that amino acid starvation induced by bacterial pathogens modulates the mTOR pathway (Tattoli *et al.*, 2012). Previous studies have shown that *P. gingivalis* induces autophagy in endothelial cells (Dorn *et al.*, 2001) and affects both cell proliferation and cell growth in osteoblastic/stromal cells, in human trophoblasts (Kato *et al.*, 2008; Inaba *et al.*, 2009) and gingival epithelial cells (Andrian *et al.*, 2006). Mechanisms underlying these changes however, remain to be elucidated. As mTOR is key to several of the cellular responses elicited by periodontal pathogens such as *P. gingivalis*, this study aimed to determine whether periodontal pathogens are able to influence the mTOR signalling pathway.

METHODS

Reagents and antibodies

Unless otherwise stated all chemical reagents were from Sigma (Poole, UK) and all mTOR pathway antibodies used were from Cell Signalling (New England Biolabs, Hitchin, UK). Mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was from Sigma. Antibodies against *P. gingivalis* were prepared within the Sheffield Antibody Unit (BioServ UK Ltd, Sheffield, UK) by inoculating New Zealand rabbits with formalin-fixed whole *P. gingivalis* (strain NCTC11834).

Cell culture

The oral squamous cell carcinoma-derived cell line H357 (a generous gift from Professor S. Prime, University of Bristol, UK) was grown and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (volume/volume; V/V) fetal bovine serum and 2 mM L-glutamine.

Immortalized human oral keratinocytes (OKF6/Tert2) (Dickson *et al.*, 2000) were kindly provided by Dr J. Rheinwald (Harvard Medical School, Cambridge, MA) and were grown in defined keratinocyte–serum-free media (K-SFM) supplemented with defined growth supplements (Fisher Scientific, Loughborough, UK). Cells were grown to 70–80% confluence and the media were changed every 3–4 days.

Bacterial strains and growth conditions

Bacterial strains used were *T. forsythia* (ATCC 43037) and the *P. gingivalis* strains NCTC11834 and W50 (ATCC 53978) and the derivative W50 isogenic mutants E8 (*rgpA::Em rgpB::Tet*) and K1A (*kgp*::Em mutant) (Aduse-Opoku *et al.*, 2000). Both the parental strain W50 and the E8 and K1A mutants were kindly supplied by Professor M. Curtis (Barts and The London School of Medicine and Dentistry, London, UK).

All bacterial strains were grown under anaerobic conditions (10% CO_2, 10% H_2 and 80% N_2) at 37°C. Strains of *P. gingivalis* were grown and maintained on Fastidious Anaerobe agar (FA; Lab M, Bury, UK) supplemented with 5% (V/V) oxylated horse blood (Oxoid, Fisher Scientific). For growth in liquid cultures, *P. gingivalis* was grown in brain–heart infusion broth (BHI; Difco Laboratories, East Molesey, Surrey, UK) supplemented with 0.5% (weight/volume; W/V) yeast extract, haemin (5 μg ml^{-1}), vitamin K (0.5 μg ml^{-1}) and cysteine [0.1% (W/V)]. *Tannerella forsythia* was maintained on FA agar supplemented with 5% (V/V) oxylated horse blood and 0.17 mM *N*-acetylmuramic acid. For growth in liquid cultures,

T. forsythia was grown in tryptic soy broth supplemented with 0.5% (W/V) yeast extract, haemin (5 μg ml^{-1}), vitamin K (0.5 μg ml^{-1}), 0.17 mM *N*-acetylmuramic acid and 0.1% (W/V) cysteine.

Construction of an rpgABkgp triple mutant

To create a strain devoid of all gingipains on a *P. gingivalis* W50 background, *kgp*, the gene encoding the lysine-specific gingipain, was deleted in the *rgpA::EmrgpB::Tet* (E8,Δ*rgpA*Δ*rgpB*) strain. Briefly, DNA regions flanking the *kgp* gene were amplified by polymerase chain reaction (PCR) using the primers kgp5′ (CTGCAGAAGTTCACTCTTTC) and kgp5′CatRev (CCAGTGATTTTTTTCTCCACTTTAAAACAATTTATGGTCG) for the 5′ flanking region and primers kgp3′ Cat (ACGACCATAAATTGTTTTAAAGTGGAGAAAAAAATCACTGG) and kgp3′Rev (GGCTTTACACTACCGCGCTT) for the 3′ flanking region and the Phusion® Polymerase (New England Biolabs, Hitchin, UK) according to the manufacturer's instructions. The chloramphenicol resistance cassette was amplified from plasmid pCM18 (a gift from Dr N. Jakubovics, Newcastle, UK) using primers Catfor (ACAGAATTACTTTACAGCGAGTTTCTCTATTACGCCCCGCCCTGCCACTC) and Catrev (ACAGAATTACTTTACAGCGAGTTTCTCTATTACGCCCCGCCCTGCCACTC). The three PCR products were mixed at equimolar ratios, heated to 95°C, annealed for 1 h at 30°C, followed by an extension step at 42°C for 5 min. Overlapping PCR was then performed to produce a PCR product of 2.8 kilobases using Phusion Polymerase according to the manufacturer's instructions. Following purification (QIAquick PCR Purification Kit; Qiagen, Crawley, UK), 200 ng of the PCR product was electroporated into the E8 strain (BioRad micropulser, 2.5 kV; BioRad, Hemel Hempstead, UK) as previously described (Aduse-Opoku *et al.*, 2000). Tc^R Em^R Cm^R colonies were screened by PCR for deletion of the *kgp* gene. The Arg-specific and Lys-specific gingipain activities were determined in chromogenic assays using *N*-benzoyl-DL-Arg-*p*-nitroanilide (BApNA) and *N*-(*p*-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (TGPLNA; Fisher Scientific) as substrates as previously described (Brien-simpson *et al.*, 2001). *Porphyromonas gingivalis* fixed with 4% (W/V) paraformaldehyde and resuspended at an optical density at 600 nm (OD_{600}) of 0.5 were used as a control in both proteinase assays.

Invasion assay

Invasion of *P. gingivalis* strains NCTC118324 and W50, and of *T. forsythia* (ATCC 43037) in both H357 and OK-F6 were quantified by an antibiotic protection assay as previously described (Suwannakul *et al.*, 2010; Honma *et al.*, 2011).

Bacterial challenge and treatment

OK-F6 and H357 were seeded at a density of ~500 cells cm^{-2} and cultured overnight. After replacing the media with either DMEM or K-SFM, both supplemented with 0.5% (V/V) fetal calf serum, the cells were challenged with either *P. gingivalis* NCTC11834 or *T. forsythia* at a multplicity of infection (MOI) of 100 for 4 h. After treatment, the cells were washed twice with phosphate-buffered saline and resuspended in RIPA buffer containing β-mercaptoethanol (1 : 100) and supplemented with Complete EDTA-free protease cocktail inhibitors (Roche, West Sussex, UK) and PhosStop Phosphatase inhibitor cocktail (Roche) for extraction and solubilization of total proteins. Both protease inhibitors were used according to the manufacturer's instructions. Lysed cells were stored at −80°C overnight and after centrifugation (14,000 ***g***, 15 min, 4°C), the supernatants containing proteins were removed and stored at −80°C until used. Unchallenged cells were used as a negative control. For experiments in the presence of cytochalasin D, OK-F6 cells were pretreated for 30 min with 1 μg ml^{-1} cytochalasin D (Kinane *et al.*, 2012), after which they were washed three times with PBS before treatment with *P. gingivalis* as described above. Statistical analysis was performed by Student's *t*-test and a *P* value less than 0.05 was considered significant.

Assay for intracellular protein degradation with crude gingipain preparations (supernatants and cell-associated gingipains) of *Porphyromonas gingivalis* and extracellular proteases of *Tannerella forsythia*

To prepare crude gingipain preparations, *P. gingivalis* strains W50, E8, K1A and the triple mutant E18 (*rpgABkgp)* were grown as liquid cultures in BHI medium overnight to stationary phase. The cultures were then normalized according to their OD_{600} values (OD_{600} = 1.0) using BHI before removal of whole

cells by centrifugation (14,000 ***g***, 20 min) and filtration through a 0.45-μm filter. Whole bacterial cells were kept on ice for use in assays with OK-F6 cell lysates as 'cell-associated' gingipain preparations. The cleared protein supernatants were assayed for arginine-specific and lysine-specific gingipain protease activity using chromogenic substrates BAPNA and TGPL-pNa as previously described (Brien-simpson *et al.*, 2001) and used immediately. In all assays, the gingipain-containing supernatants were normalized to cell number by dilution with BHI medium and in all cases the cell density of the cultures used differed by no more than 10%.

Oral epithelial cell lysates were prepared from OK-F6 cells using identical growth conditions as described above. The cells were then stored at −80°C for no longer than 1 week before resuspension in PBS. Following centrifugation (14,000 ***g***, 10 min, 4°C), the culture supernatant was transferred into a fresh tube and total protein concentration was determined using the R_CD_C protein assay (Pierce, Thermo Scientific, Cramlington, Northumberland, UK). OK-F6 cell lysate (20 μg) was incubated with normalized *P. gingivalis* culture supernatant (20, 10 or 2 μl) in the presence of either leupeptin (0.2 mM) or sodium-tosyl-lysine choromethyl ketone (TLCK, 0.5 mM) for 30 min after which Laemlli sodium-dodecyl sulphate (SDS) sample buffer was added and samples were denatured at 95°C. Samples were separated by SDS–polyacrylamide gel electrophoresis and immunoblotted for mTOR or other signalling proteins.

Similarly, to determine the effect of cell-associated or secreted gingipains for the wild-type compared with the isogenic *rgp*, *kgp* and *rgpkgp* mutants, OK-F6 cell lysates (20 μg) were incubated with either whole, washed bacterial cells (cell-associated activity) or the supernatants (secreted activity) from the same culture, normalized for cell number. Uninoculated bacterial medium was used as a control (BHI) and mTOR levels were determined by immunoblotting. The effects of any cell-associated and secreted proteases from *T. forsythia* were also investigated, as described above following normalization for cell number.

Western blotting

Total protein was extracted from harvested cells in supplemented RIPA buffer and total protein concentration was determined using the R_CD_C assay (Pierce) according to the manufacturer's instructions. For Western blotting, proteins (20 μg) were subjected to SDS–polyacrylamide gel electrophoresis using 3–8% (W/V) Tris-acetate or Bis-Tris [4–12% (W/V)] gels and transferred to nitrocellulose membrane. Membranes were blocked with TBST [137 mM NaCl, 20 mM Tris–HCl, 0.1% (V/V) Tween, pH 7.6] supplemented with 5% (W/V) powdered skimmed milk for 1 h at room temperature before incubation with primary antibody [1 : 1000 in 5% (W/V) bovine serum albumin in TBST] overnight at 4°C. After overnight incubation, membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit 1 : 1000 or anti-mouse 1 : 3000) in blocking buffer for 1 h. Primary mouse anti-GAPDH antibody (1 : 10,000) was used as a loading control. After washing, protein expression was visualized by incubation with enhanced chemiluminescence (ECL reagents; Pierce) and exposed to CL-Xposure films (Pierce, ThermoScientific). Blots are representative of at least three independent observations and densitometry was performed with Adobe Photoshop.

RESULTS

Porphyromonas gingivalis induces mTOR degradation in oral epithelial cells

To examine the effects of *P. gingivalis* and *T. forsythia* on host cell signalling pathways, we first interrogated their ability to invade oral epithelial cells. Both pathogens invaded both the oral squamous cell carcinoma-derived cell line H357 and the immortalized keratinocyte OK-F6 cell line, although higher invasion levels were consistently observed in the OK-F6 cells (Fig. 1A) using a standard antibiotic protection assay.

Following incubation of both H357 and OK-F6 with either live *T. forsythia* or *P. gingivalis* NCTC11834 at an MOI of 100 for 4 h, changes in mTOR protein were investigated by immunoblotting. Cells challenged with medium only were used as control. Although undegraded mTOR (289 kDa) was the only band detected in both control and *T. forsythia*-treated samples, a marked decrease in mTOR levels together with a characteristic cleavage pattern was observed following treatment with *P. gingivalis* NCTC11834 (Fig. 1B). This degradation was detected both in H357 (left panel; *Pg* lane, Fig. 1B) and OK-F6

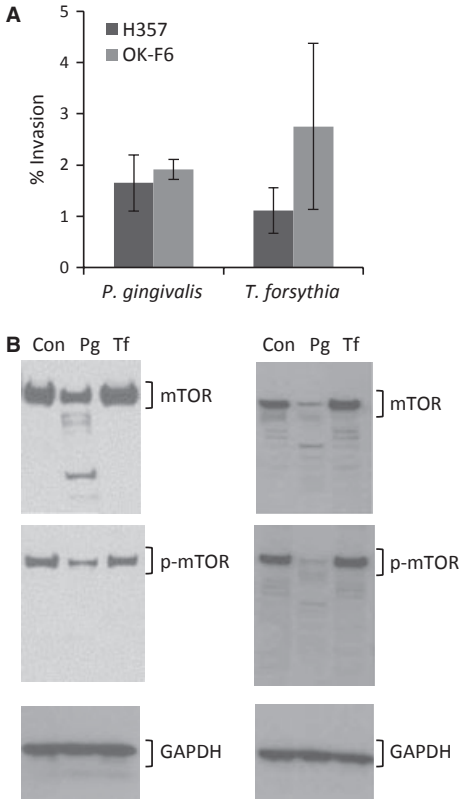

Figure 1 *Porphyromonas gingivalis* degrades mammalian target of rapamycin (mTOR) in both H357 and OK-F6 cells. (A) Invasion efficiencies of H357 and OK-F6 cells by *P. gingivalis* strains NCTC11834 and *Tannerella forsythia* (ATCC 43037) were compared in an antibiotic protection assay and calculated from colony-forming units (CFU) recovered intracellularly as a percentage of the total bacteria inoculated following incubation of the cells with bacteria for 90 min. Error bars represent standard errors and are representative of at least three separate observations. (B) Cells were seeded at a density of 500 cells cm^{-2} and following challenge with either *P. gingivalis* NCTC11834 or *T. forsythia* (ATCC 43037) at an MOI of 100 for 4 h, cells were washed twice in PBS and protein was extracted using RIPA buffer supplemented with protease inhibitors. Twenty micrograms of each sample was immunoblotted with both total and phosphorylated mTOR antibody and visualized by chemiluminescence. GAPDH was used as loading control. Blots are representative of at least three independent observations.

(right panel, *Pg* lane, Fig. 1B) cells using specific antibodies for both total mTOR and phosphorylated mTOR (p-mTOR), while levels of the GAPDH loading control were unaffected (Fig. 1B).

Mammalian TOR degradation is mediated by gingipains

To determine whether the degradation of mTOR was mediated by *P. gingivalis* gingipains, the effects of

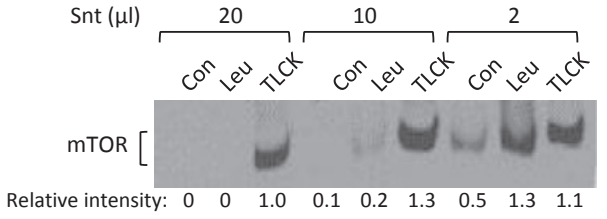

Figure 2 Protease inhibitors impede *Porphyromonas gingivalis*-mediated mammalian target of rapamycin (mTOR) degradation in oral epithelial cells. Total protein was extracted from OK-F6 cells and quantified. The *P. gingivalis* culture supernatants were then incubated with OK-F6 cell lysate in the presence of leupeptin (Leu) or sodium-tosyl-lysine choromethyl ketone (TLCK); mTOR levels were probed for by blotting and visualized by chemiluminescence. Controls (Con) were cell lysates treated with culture supernatants in the absence of inhibitors. Blots are representative of at least three independent observations and relative intensities (compared with the control) are indicated.

protease inhibitors on degradation were examined in a cell-free assay. Briefly, total protein extracted from OK-F6 oral epithelial cells were incubated with decreasing volumes of *P. gingivalis* culture supernatants from a liquid culture of *P. gingivalis* NCTC11834 in the presence and absence of protease inhibitors and mTOR levels were assessed by immunoblotting.

In the absence of these inhibitors, a dose-dependent degradation of mTOR was observed; that is, increasing amounts of full-length mTOR were detectable with decreasing volumes of *P. gingivalis* culture containing secreted gingipains (Fig. 2; lanes 1, 4 and 7). In the presence of both 20 and 10 μl of *P. gingivalis* NCTC11834 culture supernatant, mTOR could not be detected but in the presence of 2 μl supernatant undegraded mTOR could be observed (Fig. 2). In the presence of the Arg-gingipain-specific inhibitor leupeptin, only a small effect on mTOR degradation was seen (lanes 2, 5 and 8), whereas a marked reduction in mTOR degradation was observed in the presence of the inhibitor TLCK (lanes 3, 6 and 9), which is a potent inhibitor of Lys-specific gingipain and a weak inhibitor of Arg-specific gingipain (Fig. 2).

To further investigate the role of gingipains, the effects of crude gingipain preparations from a panel of isogenic *P. gingivalis* W50 gingipain mutants on mTOR were studied. The strains used were wild-type strain W50, the *rgp*-mutant E8, *kgp*-mutant KIA and a newly created *rgpABkgp* triple mutant (EK18) generated in this study. This *rgpABkgp*$^-$ strain

produces neither Arg-gingipain nor Lys-gingipain (Fig. 3A) and does not invade cells, but has an unaltered growth rate in BHI medium (data not shown). OK-F6 cell lysates were incubated with either culture supernatants (containing secreted gingipains) or whole bacterial cells (containing cell-associated gingipains) from bacterial liquid cultures grown overnight and normalized by measurement of OD_{600} and probed for mTOR levels by immunoblotting. As expected, full-length mTOR could not be detected following treatment with culture supernatant from the *P. gingivalis* wild-type strain W50 (Fig. 3B). The degradation was slightly decreased when challenged with the *rgp*⁻ E8 mutant, and greatly reduced with the *kgp*⁻ K1A mutant. No degradation was observed following incubation with supernatant from the triple *rgpABkgp* mutant, which is deficient in both Arg- and Lys-gingipains. Degradation was similarly observed when the epithelial cell lysate was incubated with whole *P. gingivalis* cells (cell-associated activity; Fig. 3C).

Effect of *Porphyromonas gingivalis* on AKT, an upstream modulator of mTOR signalling

To determine the effect upstream of mTOR, the levels of both total and phosphorylated AKT (p-AKT; S473) following invasion with live *P. gingivalis* 4 h postinfection were examined by immunoblotting. A slight reduction in both forms was observed (Fig. 4A) but GAPDH levels remained unchanged.

Further investigations showed that AKT degradation was influenced by cell-free gingipains as a marked reduction was observed following incubation of (20 μg) cell lysate with culture supernatants containing gingipains from the wild-type strain W50 when compared with the triple gingipain mutant *rgp*⁻ *kgp*⁻ (Fig. 4B) whereas GAPDH levels were unaffected (shown in Fig. 3C, as these were performed on the same samples). Cell-associated and secreted protease-containing fractions from *T. forsythia* in the cell-free system under identical conditions did not degrade mTOR (data not shown).

Effect of *Porphyromonas gingivalis* on downstream mTOR signalling pathway proteins

To explore whether invasion by *P. gingivalis* affected mTOR-associated proteins and signalling pathways

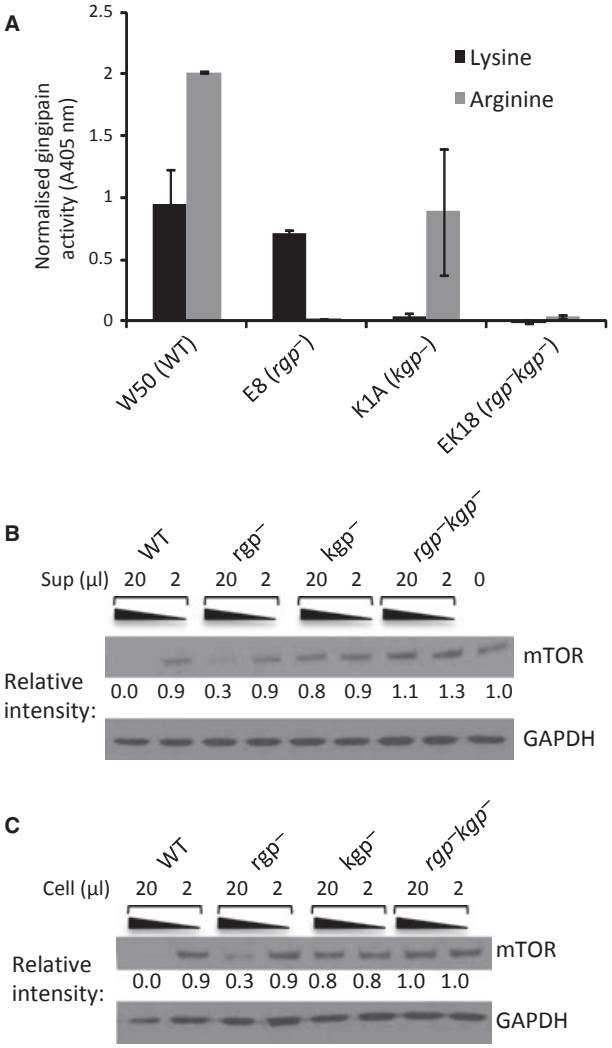

Figure 3 Mammalian target of rapamycin (mTOR) degradation is mediated by gingipains. (A) A triple *rgp*⁻ *kgp*⁻ gingipain mutant was created in *Porphyromonas gingivalis* W50 *rgpA::EmrgpB::Tet* (E8, Δ*rgpA*Δ*rgpB*) strain by introducing a chloramphenicol cassette within the *kgp* gene. The *P. gingivalis* colonies were screened for Tc^R Em^R Cm^R resistance to confirm *kgp* gene deletion and gingipain activities were determined in chromogenic assays using *N*-benzoyl-DL-Arg-*p*-nitroanilide (BApNA) and *N*-(*p*-Tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt (TGPLNA) as substrates. (B) OK-F6 cells were seeded overnight in K-SFM containing 0.5% fetal calf serum (V/V) and after washing the cells were harvested and lysed into phosphate-buffered saline. Cell lysates (20 μg) were treated with either culture supernatants containing secreted gingipains (cell-free gingipains) or with (C) whole bacterial cells from the same culture harbouring cell-associated gingipains from the *P. gingivalis* strains W50, E8 (*rpg*⁻), KIA (*kgp*⁻) and E18 (*rgp*⁻*kgp*⁻) for 30 min in the presence of cysteine after which mTOR levels were determined by blotting and visualized by chemiluminescence. GAPDH was used as a loading control. Cells treated with brain–heart infusion broth were used as control. Blots are representative of at least three independent observations and relative intensities (compared with the control) are indicated.

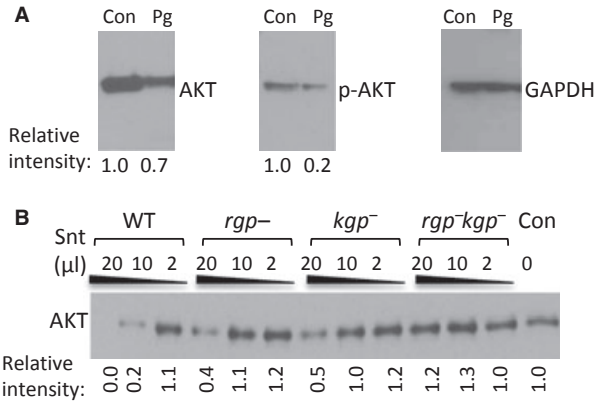

Figure 4 AKT is degraded following *Porphyromonas gingivalis* treatment by both lysine-specific and arginine-specific gingipains. (A) Cells were seeded at a density of 500 cells cm^{-2} and following challenge with *P. gingivalis* NCTC11834 (Pg; MOI: 100) for 4 h, cells were washed twice in phosphate-buffered saline (PBS), scraped in RIPA buffer supplemented with protease inhibitors and total proteins were determined. Cells challenged with media only were used as control (Con). Twenty micrograms of each sample was immunoblotted with both total and phosphorylated-AKT (S473) antibodies and visualized by chemiluminescence. GAPDH was used as a loading control. (B) OK-F6 cells were seeded overnight in K-SFM containing 0.5% (V/V) fetal calf serum and after washing the cells were scraped into PBS. Cell lysates (20 μg) were treated with culture supernatants from the *P. gingivalis* strains W50, E8 (*rpg*⁻), KIA (*kgp*⁻) and E18 (*rgp*⁻*kgp*⁻) for 30 min in the presence of cysteine after which mammalian target of rapamycin (mTOR) levels were determined by blotting and visualized by chemiluminescence. Cell lysate treated only with culture media was included as control (Con). GAPDH was used as a control but is shown in Fig. 3B, because identical samples were used for these experiments (Figs 3B and 4B). Blots are representative of at least three independent observations and relative intensities (compared with the control) are indicated.

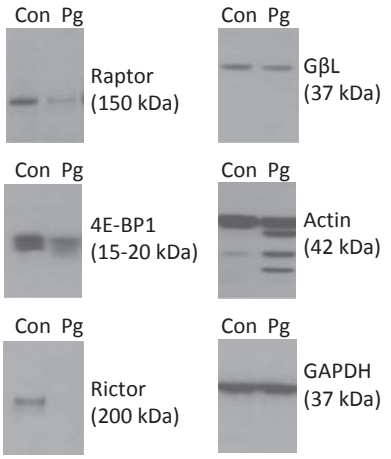

Figure 5 *Porphyromonas gingivalis* alters the levels of signalling proteins in the mammalian target of rapamycin (mTOR) pathway. OK-F6 cells (~500 cell cm^{-2}) seeded in K-SFM supplemented with 0.5% fetal calf serum (V/V) were challenged with *P. gingivalis* NCTC11834 (Pg; MOI: 100) for 4 h (Pg). Unchallenged cells were included as control (Con). The media were discarded and after washing the cells twice in phosphate-buffered saline, the cells were scraped in RIPA buffer supplemented with protease inhibitors. Twenty micrograms of each sample was immunoblotted with antibodies against the mTORC1 associated protein Raptor, the mTORC2 associated protein Rictor, GβL (associated with both mTOR complexes), phosphorylated-4E-BP1 [a transcription factor which acts downstream of mTOR; (T37/46)] and β-actin. GAPDH was included as a loading control. Blots were visualized by chemiluminescence. Blots are representative of at least three independent observations.

downstream of mTOR, total protein extracts from OK-F6 cells were challenged with *P. gingivalis* NCTC11834 and then probed with antibodies to a panel of proteins involved in the mTOR signalling pathway using immunoblotting.

The levels of the mTOR complex (mTORC) 1-associated protein, raptor, and the mTORC2-associated protein, rictor, were reduced 4 h after *P. gingivalis* infection when compared with the control, whereas GβL, a positive regulator associated with both complexes, remained unchanged (Fig. 5). In addition, we probed for the phosphorylated form of the downstream target of mTORC1, 4E-BP1 (p-4E-BP1 T37/46) showed that levels of p-4E-BP1 protein expression were also reduced (Fig. 5) compared with uninfected control. Similarly, the levels of β-actin were markedly decreased when compared with the untreated control sample (Fig. 5).

Porphyromonas gingivalis internalization is required for mTOR degradation

To determine whether mTOR degradation was dependent on the internalization of *P. gingivalis* or simply caused by the action of secreted gingipains entering the cell, OK-F6 cells pretreated with cytochalasin D for 30 min were challenged with *P. gingivalis* for 4 h. Treatment of OK-F6 cells with cytochalasin D caused a fivefold reduction in *P. gingivalis* invasion when compared with untreated controls (Fig. 6A). In the absence of cytochalasin D (Fig. 6B), mTOR degradation in response of *P. gingivalis* was observed. However, treatment with cytochalasin D markedly inhibited mTOR degradation, suggesting that invasion is required for degradation of mTOR (Fig. 6A).

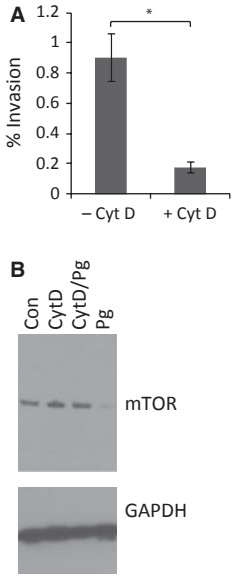

Figure 6 Cytochalasin D inhibits invasion of oral epithelial cells by *Porphyromonas gingivalis*. (A) Invasion efficiencies of H357 and OK-F6 cells by *P. gingivalis* strains NCTC11834 in the presence of cytochalasin D were compared in an antibiotic protection assay. Colony-forming units (CFU) recovered intracellularly as a percentage of the total bacteria inoculated were calculated following incubation of the cells with bacteria for 90 min. Error bars represent standard errors and are representative of at least three separate observations. Percentage invasion is shown and reduction in invasion was found to be statistically significant ($*P < 0.05$). (B) Cytochalasin D inhibits *P. gingivalis*-mediated mammalian target of rapamycin (mTOR) degradation. OK-F6 cells were seeded overnight at a density of ~500 cells cm^{-2} and the following day the medium was replaced with K-SFM containing 0.5% (V/V) fetal calf serum. The cells were pretreated with cytochalasin D (1 μg ml^{-1}) for 30 min after which they were challenged with *P. gingivalis* (MOI 100) for 4 h (CytD/Pg). Total proteins were extracted in RIPA buffer supplemented with protease inhibitors and β-mercaptoethanol and 20 μg of each sample was probed for mTOR and visualized by chemiluminescence. Cells were treated with *P. gingivalis* only in the absence of cytochalasin D (Pg), cells treated with media (Con) and cells treated with cytochalasin D only (Cyt D). GAPDH was included as a loading control. Blots are representative of at least three independent observations.

DISCUSSION

The aims of this study were to determine the effects of periodontal pathogens *P. gingivalis* and *T. forsythia* on mTOR and its signalling pathways. Having established that both *P. gingivalis* and *T. forsythia* invaded the oral squamous cell carcinoma-derived cell line H357, in keeping with our previous findings (Suwannakul *et al.*, 2010) and the immortalized oral keratinocyte OK-F6 cell line, levels of mTOR were assessed by immunoblotting. The degradation of both total and p-mTOR in the H357 and OK-F6 cell lines indicates that this phenomenon was cell-line independent and, importantly, *P. gingivalis* dependent as no degradation was observed with *T. forsythia*. A 4-h incubation period was chosen to allow sufficient time for invasion [known to occur within 90 min; (Lamont *et al.*, 1995)]. Infection was carried out at an MOI of 100 as this has previously been shown to be optimal for invasion (Lamont *et al.*, 1995) and is frequently used in studies of modulation of host cell phentotype by *P. gingivalis* (Stathopoulou *et al.*, 2009a,b). This MOI does not affect the viability of the epithelial cells following infection with *P. gingivalis* (Fravalo *et al.*, 1996; Madianos *et al.*, 1996). Stathopoulou *et al.* (2009a,b) have also shown that there is no evidence of apoptosis when cells were infected with *P. gingivalis* (MOI 100) for 4 h. Hence, in our study, *P. gingivalis* challenge at an MOI 100 for 4 h will allow the early cell responses following invasion to be determined without time for apoptosis to influence cell signalling responses.

The differential effect of *T. forsythia* and *P. gingivalis* on mTOR was unexpected as *T. forsythia* is known to secrete proteases such as karilysin (Karim *et al.*, 2010; Cerdà-Costa *et al.*, 2011; Jusko *et al.*, 2012) and *prtH/cct* (Nakajima *et al.*, 2006). Our findings suggest that although *T. forsythia* invaded H357 and OK-F6 cells as efficiently as *P. gingivalis*, under our experimental conditions, it is unlikely that mTOR is a substrate for the proteases secreted by *T. forsythia*. Another explanation might be that the proteases are not expressed by *T. forsythia* inside epithelial cells. Although we have no direct evidence for this, it is noteworthy that both karilysin and PrtH act extracellularly with karilysin inhibiting several pathways of the complement system (Jusko *et al.*, 2012) and inactivating the anti-bacterial peptide LL-37 (Koziel *et al.*, 2010), whereas PrtH is able to detach epithelial cells from surfaces (Nakajima *et al.*, 2006; Pei & Grishin, 2009). These findings also support previously published data that *P. gingivalis* and *T. forsythia* trigger different signalling pathways following invasion to modulate host cell responses (Bodet *et al.*, 2006). As changes in mTOR levels were only observed with *P. gingivalis*, the rest of the study focused on investigating changes in mTOR signalling after *P. gingivalis* challenge.

We next set out to investigate the mechanisms underlying the mTOR degradation elicited by

P. gingivalis in a cell-free assay. The dose-dependent degradation observed in the presence of increasing volumes of *P. gingivalis* NCTC11834 supernatants suggests that this might be mediated by *P. gingivalis*-secreted gingipains. This hypothesis was further corroborated by data obtained from examining degradation in the presence of protease inhibitors. The slight decrease in mTOR degradation in the presence of the Arg-gingipain-specific inhibitor leupeptin compared with the strong inhibition in degradation in the presence of Lys-specific gingipain inhibitor TLCK, confirmed that degradation of mTOR was gingipain-dependent and suggested that Lys-gingipain might be predominantly responsible for this phenomenon.

We hypothesized that the observed degradation was primarily mediated by Lys-specific gingipain; this was corroborated in a cell-free assay using a panel of isogenic *P. gingivalis* W50 gingipain mutants. A cell-free assay was chosen to investigate these changes as levels of invasion between the *rgpAB* and *kgp* strains are not comparable (Suwannakul *et al.*, 2010) and these mutants have pleiotropic phenotypes such as alterations in fimbrial maturation that may also influence host–bacteria interactions, which is probably why the *rgpABkgp* triple mutant is non-invasive (data not shown). The degradation mediated by the wild-type strain W50 shows that this phenomenon is not strain-specific whereas inhibition of degradation with the triple mutant *rgpABkgp*$^{-}$ substantiates the hypothesis that gingipains are fundamental for this process. As degradation of mTOR in epithelial cell lysate was observed both with bacterial culture supernatants (secreted gingipains) and whole bacterial cells (cell-associated activity), the data taken together confirmed that mTOR is degraded by both *P. gingivalis* cell-associated and secreted gingipains.

Upstream of mTOR signalling is the serine/threonine kinase AKT, also known as protein kinase B (Peng *et al.*, 2003). The mTOR activation is dependent on the binding of phosphatidylinositol 3-kinase (PI3K) and subsequent phosphorylation of AKT (Peng *et al.*, 2003). The PI3K/AKT signalling pathway is involved in several processes including cell proliferation, survival, growth and motility, some of which are mTOR-mediated. The slight reduction in the levels of both total and phosphorylated AKT demonstrated here was also attributed to cell-free gingipains. Our results are in contrast to previous studies that showed that *P. gingivalis* lipopolysaccharide activates the PI3K-AKT pathway in a Toll-like receptor 2-dependent manner in human monocytes (Martin *et al.*, 2003), whereas increases in AKT after 24 h of infection with live *P. gingivalis* have been observed in gingival epithelial cells (Yilmaz *et al.*, 2004). The reasons for this difference are unclear but may be due to both the time point at which the observations were made and the cells used.

As *P. gingivalis* has an effect on mTOR levels, we explored whether invasion of cells by *P. gingivalis* affected both mTOR-complex-associated proteins and proteins downstream of mTOR. The mTOR pathway comprises two distinct multi-protein complexes known as mTORC1 and mTORC2, with the specific functions of the complexes determined by the regulatory proteins they contain. The mTORC1 specifically contains raptor (regulatory-associated protein of mTOR) and is essentially involved in cell growth, proliferation and autophagy (Kim *et al.*, 2002; Laplante & Sabatini, 2009). Although the exact function of most of the mTOR-binding proteins remains to be elucidated, it has been proposed that raptor regulates the assembly of the complex, recruits mTORC1 substrates, such as 4E-BP1 and p70SO6, and catalyses their phosphorylation (Hara *et al.*, 2002) so is essential for mTOR signalling *in vivo* (Nojima *et al.*, 2003).

Following *P. gingivalis* treatment, the levels of the mTORC1-associated protein raptor were reduced. The combined reduction in levels of both mTOR and raptor suggest that *P. gingivalis* is likely to have a downstream effect in the mTORC1 signalling pathway and this was further corroborated by the fact that the levels of phosphorylated 4E-BP1 decreased. The mTOR substrate 4E-BP1 is important in mTORC1-mediated cell proliferation but not cell growth (Dowling *et al.*, 2010) with its phosphorylation being linked to increased cellular proliferation as well as elevated levels of cyclin D1(CCND1) mRNA; CCND1 encodes a protein normally required for G1/S transition in the cell cycle (Barnhart *et al.*, 2008). Previous studies have reported an increase in cell proliferation following *P. gingivalis* infection in gingival epithelial cells (Kuboniwa *et al.*, 2008) and a decrease in cell proliferation in the extravillous trophoblast cell line, HTR-8 (Inaba *et al.*, 2009). Our observation that levels of phospho-4E-BP1 are reduced could therefore be a plausible explanation for the decrease in cell proliferation reported (Inaba *et al.*, 2009). This suggestion is also supported by previous findings that a

decrease in CCND1 is observed following *P. gingivalis* invasion (Kato *et al.*, 2008; Inaba *et al.*, 2009). This, coupled with the gingipain-mediated degradation of mTOR, suggests that following infection, the mTORC1 signalling pathway is severely impacted.

GβL, a positive regulator of mTOR, is associated with both mTORC1 and mTORC2 (Kim *et al.*, 2003). Within mTORC1, GβL interacts directly with mTOR and increases its kinase activity. Following bacterial challenge, levels of GβL remained unchanged, showing that the *P. gingivalis* proteolytic effects are specific to a subset of substrates within the mTOR pathway.

Having examined potential effects on mTORC1, we also investigated whether the gingipains were having an effect on the much less well characterized mTORC2 complex. The composition of this complex differs from mTORC1, with rictor, the rapamycin-insensitive companion of mTOR, being a key component that is essential for its function as highlighted by its role in the modulation of the phosphorylation of protein kinase Cα and the actin cytoskeleton (Sarbassov *et al.*, 2004). The significant reduction in rictor levels suggests that the functions mediated by mTORC2 might also be dysregulated. The levels of β-actin were also found to be altered, corroborating the recent findings of Kinane *et al.* (2012) who, using a similar approach, showed actin degradation by gingipains.

To determine whether mTOR degradation was dependent on internalization of *P. gingivalis* or was a consequence of secreted gingipains, the effect of cytochalasin D on mTOR degradation was investigated. Cytochalasin D is a fungal metabolite that inhibits actin polymerization (Casella *et al.*, 1981) and is known to inhibit invasion of both oral epithelial cells (Nakagawa *et al.*, 2006) and endothelial cells (Deshpande *et al.*, 1998; Dorn *et al.*, 2001) by *P. gingivalis.* In addition the recent work of Kinane and co-workers showed that intracellular degradation of actin was abrogated following incubation with cytochalasin D in human gingival epithelial cells (Kinane *et al.*, 2012). In our study, the inhibition of mTOR degradation together with a fivefold reduction in invasion indicates that invasion is required for degradation of mTOR. In addition, we also confirmed the observation by Kinane *et al.* (2012) that actin degradation by *P. gingivalis* is inhibited by cytochalasin D. Our data indicate that the gingipain-dependent degradation of mTOR requires the action of *P. gingivalis* cell-associated or secreted gingipains deployed after invading host cells.

In conclusion, in this study we have highlighted for the first time a direct effect of the periodontal pathogen *P. gingivalis* on the mTOR signalling pathway. In contrast, this was not the case for its fellow red-complex pathogen *T. forsythia*. We have further highlighted the fact that these pathogens stimulate differential host cell responses following infection. Our data demonstrate that mTOR degradation by *P. gingivalis* is mediated by both secreted and cell-associated gingipains, again illustrating that deployment of gingipains is a central strategy used by *P. gingivalis* to manipulate host cell responses (Sheets *et al.*, 2005; Stathopoulou *et al.*, 2009a,b). We further showed that *P. gingivalis* invasion of oral epithelial cells is required for mTOR degradation. We also observed changes in the levels of mTOR-associated proteins such as raptor (mTORC1) and rictor (mTORC2) and observed alterations in downstream signalling proteins such as 4E-BP1 (mTORC1-dependent pathway) and actin, suggesting that *P. gingivalis*-mediated mTOR degradation is one facet of how cellular responses are influenced by infection.

Our study adds to the mechanistic picture of how *P. gingivalis*, and specifically its gingipains, exerts its many cellular responses. Specifically, degradation of mTOR may contribute to the modulation of some of the cellular changes such as proliferation, survival and induction of autophagy, which are observed following *P. gingivalis* invasion and internalization (Dorn *et al.*, 2001; Yamatake *et al.*, 2007; Jung *et al.*, 2010; Mizushima & Komatsu, 2011; Yuk *et al.*, 2012). Although a link between *P. gingivalis* and autophagy in other cells such as the coronary artery endothelial cells has been established (Dorn *et al.*, 2001; Bélanger *et al.*, 2006), recent studies have also documented autophagy in the gingival fibroblasts of periodontal patients (Bullon *et al.*, 2012). It is therefore tempting to speculate that the data presented here indicate that there may be a link between *P. gingivalis* internalization and persistence in oral epithelial cells that is related to its ability to engage autophagy pathways. Furthermore, it is becoming clear that autophagy may play a role in a wide range of infections including the alterations in the intracellular defence pathway following infection by bacteria such as *Mycobacterium tuberculosis* (Gutierrez *et al.*,

2004) or by viruses (Grose, 2010). Work is ongoing in our laboratories to further investigate the influence of *P. gingivalis*-induced mTOR degradation on cellular processes, including cell proliferation and autophagy.

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