Translocation of *Porphyromonas gingivalis* Gingipain Adhesin Peptide A44 to Host Mitochondria Prevents Apoptosis[▽]

Heike Boisvert* and Margaret J. Duncan

Department of Molecular Genetics, The Forsyth Institute, Boston, Massachusetts 02115

Received 23 February 2010/Returned for modification 17 March 2010/Accepted 3 June 2010

Porphyromonas gingivalis, a Gram-negative oral anaerobe, is associated with periodontal diseases that, in some form, affect up to 80% of the U.S. population. The organism is highly proteolytic, and noncatalytic adhesin domains of the major proteases, gingipains, are involved in bacterium-host interactions. Recently, we showed that gingipain adhesin peptide A44 hijacks the host's clathrin-dependent endocytosis system, allowing the peptide and whole bacteria to be internalized by epithelial cells. In the present study, we found by cell fractionation assays and confocal microscopy that peptide A44 translocated to host mitochondria. Cell viability assays and quantitative real-time PCR showed that the peptide interacted with the cell death machinery by triggering upregulation of antiapoptotic factors *bcl-2* and *bcl-XL* and prevented staurosporine-induced apoptosis for up to 12 h. We confirmed these findings with Western blot analyses of caspase-9 activation in time course experiments with staurosporine. Finally, we verified a similar antiapoptotic effect for *P. gingivalis*, showing for the first time that the organism manipulated mitochondrial functions during the first hours of infection, thus resisting host cell clearance by apoptosis of infected cells. This mechanism may enable the bacteria to persist in the protected cellular environment until the next step in pathogenesis, progression or resolution of infection.

The Gram-negative oral anaerobe *Porphyromonas gingivalis* is a causative agent of chronic periodontitis in adults. The disease is episodic in nature, characterized by periods of acute infection accompanied by a strong host inflammatory response. Following treatment, the organism chronically persists in low numbers in subgingival plaque and has also been detected *in vivo* within epithelial cells (44). While the latter sequester bacteria from the host immune response, in the intracellular environment bacteria must adjust to and modulate conditions to ensure their survival.

Many *in vitro* studies have demonstrated *P. gingivalis* adherence to and entry into host cells mediated by bacterial surface fimbriae (24, 46) and gingipain cysteine proteases (4, 34, 35). Gingipains are a family of cysteine proteases found on the cell surface, in membrane vesicles, and in culture supernatants of *Porphyromonas gingivalis*. Arg-gingipain (Rgp) and Lys-gingipain (Kgp) both have catalytic domains that autoprocess the adhesin domains into smaller adhesin peptides. Recently, we showed that peptide A44 from the adhesin domain of RgpA and live *P. gingivalis* cells were internalized by HEP-2 cells by a clathrin-dependent mechanism (4). Analyses of host cell signaling and survival following *P. gingivalis* infection have demonstrated the induction of both pro- and antiapoptotic events (25, 26, 45), suggesting direct or indirect involvement with host mitochondria. In addition to generating most of the cell's energy, mitochondria play a role in other cell functions such as differentiation, cell signaling, control of the cell cycle and growth, and cell death (11, 32). Apoptosis, or programmed cell death, occurs in selected cells and is characterized by DNA

fragmentation and chromatin condensation and shrinkage, while sparing neighboring cells from damage. Bacteria manipulate host cell pathways to ensure their own survival, and pathogens have the ability to induce or to block apoptosis (13, 14, 41). Mitochondria, therefore, are prime targets for pathogens to manipulate to ensure their survival (reviewed in reference 2).

In the present study, we investigated the events that followed entry of RgpA adhesin peptide A44 into epithelial cells. Using several approaches we first determined that A44 rapidly trafficked to mitochondria, suggesting that the peptide interacted directly with the organelle and its pathways. In the first hours after entry and targeting, A44 appeared to have a protective effect and also protected cells from staurosporine-induced apoptosis, but after prolonged incubation, A44 was no longer effective at preventing the pathway.

MATERIALS AND METHODS

Bacterial strains, cell culture, and chemicals. Bacterial strains and HEP-2 cells were cultured as described previously (4). All chemicals were purchased from either Sigma-Aldrich (MO) or Fisher Scientific (PA), unless specifically stated.

Purification and FITC labeling of recombinant peptide A44. Construction and purification of recombinant hexahistidyl-tagged A44 peptide (r-A44) was described previously (4). For fluorescein isothiocyanide (FITC) labeling, r-A44 was dialyzed overnight against 50 mM Na₂CO₃ buffer (pH 9.3), followed by addition of a 10 molar ratio of FITC in dimethyl sulfoxide (DMSO) and incubation for 1 h in the dark. To purify labeled peptide from free FITC, dialysis was performed at 4°C with several changes of phosphate-buffered saline (PBS; pH 7.4).

Binding of recombinant FITC-labeled A44 to HEP-2 cells. To quantify binding of FITC-labeled r-A44, HEP-2 cells were seeded to 96-well plates (5 × 10³ cells per well) and cultivated overnight in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. After the addition of fresh medium, FITC-labeled A44 was added at concentrations of 0 to 50 μg/well, followed by incubation for 3 to 48 h at 37°C with 5% CO₂. Cells were washed three times with PBS and lysed with 100 μl distilled water. Lysates were transferred to 96-well UV-compatible plates, and fluorescence was measured at 490/520 nm.

* Corresponding author. Mailing address: Department of Molecular Genetics, The Forsyth Institute, Boston, MA 02115. Phone: (617) 892-8292. Fax: (617) 262-4021. E-mail: hboisvert@forsyth.org.

[▽] Published ahead of print on 14 June 2010.

Fractionation of HEP-2 cells. Western blot assays to detect the association of r-A44 with HEP-2 epithelial cells were described previously (4). Briefly, tissue culture plates (petri dishes) were seeded with approximately 1×10^7 HEP-2 cells and incubated overnight at 37°C with 5% CO₂. After the addition of fresh culture medium, r-A44 (2 µg/ml) was added and the plates were incubated for 3 to 48 h. Supernatants were retained as controls to monitor the presence and/or degradation of peptides. Plates were washed three times with PBS, and cells were scraped from wells and transferred to sample tubes. HEP-2 cytoplasmic, cytoskeletal, and nuclear protein fractions were isolated using a ProteoJET kit (Fermentas, MD). The insoluble pellet remaining at the end of the procedure was designated the cytoskeletal fraction. To confirm the correct compartmental separation of proteins, each fraction was size fractionated by SDS-PAGE, then blotted to nitrocellulose membranes. After being blocked overnight with 5% (wt/vol) milk, blots were probed with rabbit primary antibodies to cytoskeletal protein early endosome antigen 1 (EEA1) and cytoplasmic protein glyceraldehyde 3-phosphatase dehydrogenase (GAPDH). Mouse primary antibodies were used to detect mitochondrial protein cytochrome *c* (CytC; Biolegend, CA). Horseradish peroxidase (HRP)-labeled anti-rabbit and anti-mouse secondary antibodies were obtained from Sigma-Aldrich (MO).

Purification of A44-specific antibodies. A44-specific antibodies were purified using a modified immunoblotting technique (39). Briefly, purified r-A44 was fractionated by SDS-PAGE and blotted to nitrocellulose. The membrane was stained with Ponceau S (0.5% [vol/vol] in acetic acid) for 5 min and washed with distilled water. Strips containing proteins were excised, rinsed with water, and blocked for 2 h at room temperature with 5% (wt/vol) milk in PBS. After being washed three times with PBS, the strips were incubated overnight at 4°C with anti-RgpA polyclonal antibody that recognizes all peptides within the RgpA adhesin domain (6). Unbound antibody was removed by washing the strips three times with PBS, and anti-A44-specific antibody was eluted with 100 mM glycine (pH 5.2). The antibody solution was neutralized with 1/10 volume of 1 M Tris (pH 8.0) and stored at -20°C.

Colorimetric cell viability assay. Viability of HEP-2 cells was measured using 3-(4,5-dimethylthiazol-2H)-2,5-diphenyl tetrazolium bromide (MTT). Purified r-A44 was incubated at different concentrations with HEP-2 epithelial cells (3×10^4 cells/well) in 24-well plates for 1 to 24 h at 37°C and 5% CO₂. For some experiments 2 µM staurosporine was added for an additional 24 h. After incubation, wells were washed three times with PBS, MTT stock solution (5 mg/ml in PBS, 10 µl per 100 µl medium) was added, and the plates were incubated for an additional 1 h in a CO₂ chamber. Following the addition of 100 µl acid-isopropanol (0.04 N HCl in isopropanol) to each well to solubilize reduced tetrazolium crystals, the solution was transferred to a 96-well plate and absorption was measured at 570 nm in an HTS 7000 Plus bioassay reader (Perkin Elmer, MA).

Confocal laser scanning and electron microscopy. All fluorescent images were observed with a TCS SP2 microscope (Leica Microsystems GmbH, Germany). In certain experiments latex beads were coated with r-A44 and incubated with HEP-2 epithelial cells; these interactions were observed by double immunofluorescence, as described previously (4). F-actin in HEP-2 cells was stained with rhodamine-phalloidin (Molecular Probes, CA) diluted 1:50 in PBS and incubated for 20 min at room temperature. In certain assays we used an A44-green fluorescent protein (GFP)-expressing HEP-2 cell line. To construct the pEGFP-A44 plasmid, primers 5' CCGCTCGAGATGACGCGTCAGGCCGAG and 5' CGCGTCGACCTTGCCATTGGCCTTGATCT, containing XhoI and SalI restriction sites (underlined), were used to amplify the A44 coding sequence from *P. gingivalis* 33277 chromosomal DNA. Amplification products were cloned into the pJet vector (Fermentas), and the XhoI and SalI fragment from a correct plasmid was isolated. The insert was purified using the Qiaquick kit (Qiagen, CA), ligated into XhoI/SalI-digested plasmid pEGFP-N3, and transformed into *Escherichia coli* DH5α. Plasmid pEGFP-A44 was purified, and transient transfection was carried out using the FuGENE HD transfection reagent (Roche, IN) according to the manufacturer's protocol.

Immunogold-labeling experiments used either HEP-2 epithelial cells grown on plates and incubated with purified r-A44 (2 µg/ml) for 90 min at 37°C and 5% CO₂ or the A44-GFP-expressing HEP-2 cell line. Cells were fixed with 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2), gradually dehydrated with ethanol, and embedded in LR White (Electron Microscopy Sciences, PA). Sections were transferred to carbon-Formvar grids for immunolabeling with anti-His tag- or anti-A44-specific antibodies. Following treatment with gold-labeled (10 nm) anti-rabbit secondary antibodies, grids were counterstained with lead citrate and uranyl acetate. Sections were viewed with a 1200 EX electron microscope (JEOL, MA) operating at 100 kV.

Real-time qRT-PCR. We used quantitative reverse transcription-PCR (qRT-PCR) to analyze changes in the expression of host cell proteins Bcl-2, Bcl-XL, Bax, Bak, tumor necrosis factor alpha (TNF-α), NF-κB, RhoA, Mcl-1, Stat3, and

TABLE 1. Primers used for qRT-PCR

Gene	Primer	
	Direction	Sequence
β-globin gene	Forward	TCAGGATCCACGTGCTTGTCA
	Reverse	TACCTTGGACCCAGAGGTTCTTTGA
<i>bcl-2</i>	Forward	GGTGGAGGAGCTCTTCAGG
	Reverse	ATAGTTCCACAAAGGCATCC
<i>bcl-XL</i>	Forward	CTGAATCGGAGATGGAGACC
	Reverse	TGGGATGTCAGGTCACTGAA
<i>bax</i>	Forward	TCTGACGGCAACTTCAACTG
	Reverse	CGTCCCAAAGTAGGAGAGGA
<i>bak</i>	Forward	TCTGGCCCTACACGTCTACC
	Reverse	ACAAACTGGCCCAACAGAAC
NF-κB gene	Forward	GCACGACAACATCTCATTGG
	Reverse	TCCCAAGAGTCATCCAGGTC
TNF-α gene	Forward	CGTCTCTACCAGACCAAGG
	Reverse	CCAAAGTAGACCTGCCCAGA
<i>rhoA</i>	Forward	CCGGCGCGAAGAGGCTGGA
	Reverse	GCACATACACCTCTGGGAAC
<i>mcl-1</i>	Forward	TAAGGACAAAACGGGACTGG
	Reverse	ACCAGCTCCTACTCCAGCAA
<i>stat3</i>	Forward	TGGCACTTGTAATGGCGTCTTC
	Reverse	CAGCAGGGAGGAGTCACCA
cIAP gene	Forward	TGTTCCAGTTCAGCCTGAGCAG
	Reverse	CACCTCAAGCCACCATCACAA

cIAP in the presence of peptide A44 or live *P. gingivalis* cells. Expression of β-globin was used as the host cell gene internal control. Primer sequences used for qRT-PCR can be found in Table 1. To isolate RNA, HEP-2 cells were seeded on tissue culture plates (1×10^7 cells/plate) and cultivated overnight at 37°C and 5% CO₂. After addition of fresh medium, cells were incubated with either r-A44 (2 µg/ml) or *P. gingivalis* (multiplicity of infection [MOI], 1:100) for 1 to 24 h or 1 to 6 h, respectively, as above. RNA was isolated using the Epicenter Masterpure RNA isolation kit according to the manufacturer's protocol. Total RNA (2 µg) was transcribed to cDNA with oligo(dT) primers using the RevertAid cDNA synthesis kit (Fermentas) according to the manufacturer's protocol. cDNA (1 µl) was used in a 20-µl reaction mixture with 1 µl each of forward and reverse primers and 10 µl 2× SYBR green PCR mix (Bio-Rad, CA). Amplifications were carried out in an iCycler (Bio-Rad) under the following conditions: predenaturation at 95°C for 2 min 30 s followed by 50 cycles each of denaturation at 95°C for 15 s, annealing at 55°C for 15 s, and elongation at 72°C for 15 s. Expression ratios were calculated according to the method of Pfaffl (28) for relative quantification using β-globin values as internal controls.

Detection of activated caspase by Western blotting. HEP-2 cells were seeded in tissue culture plates (1×10^7 cells per plate) and incubated for 16 h with peptide A44 (2 µg/ml) at 37°C and 5% CO₂; control plates were incubated with DMEM alone. Apoptosis was induced with staurosporine (2 µM) for 6 to 24 h, and then cells were washed and harvested. In samples that were incubated with medium alone, cells were collected from the supernatant after staurosporine induction for 12, 16, and 24 h since they detached from the plates. For incubations with peptide A44 and with staurosporine for 24 h, there were also detached cells in the supernatant, and these were collected and combined with attached cells from the same plate. Cell lysates were size separated by SDS-PAGE and transferred to nitrocellulose. Blots were blocked overnight with 5% milk in PBS and probed with anti-caspase-9 antibody (Cell Signaling Technologies, MA). The antibody recognizes procaspase and the activated cleaved caspase form. HRP-labeled anti-rabbit antibody and an ECL kit (Pierce, IL) were used to detect binding of primary antibody.

FIG. 1. Adhesin peptide A44 associates with HEp-2 cells in a time- and dose-dependent manner. (A) Different concentrations of FITC-labeled A44 were incubated with host cells for 1 to 48 h. After washing, A44 fluorescence that remained associated with cells was measured at 490/520 nm. (B to E) Double-immunofluorescence imaging of A44-coated latex beads incubated for 1 to 24 h with host cells. Externally adherent beads are blue, internalized beads are green, and F-actin is shown in red. Internalized beads associated with the actin cytoskeleton appear yellow, and white beads are in the process of internalization involving rearrangement of the actin cytoskeleton.

RESULTS

Gingipain adhesin peptide A44 is not degraded in host cells.

We examined adherence of A44 to epithelial monolayers as a first step toward determining the fate of A44 within host cells. In these experiments, increasing amounts of FITC-labeled peptide were incubated with HEp-2 epithelial monolayers for 3 to 48 h. Peptide adherence (detected by fluorescence) was dose dependent, with the most rapid accumulation occurring during the first 24 h of incubation (Fig. 1A). We assumed that most of the accrual was due to aggregation of peptide externally on the surfaces of HEp-2 cells; therefore, we also carried out experiments to assess A44 accumulation within cells over time. Peptide-coated latex beads (10^8 /ml) maximally coated with $50 \mu\text{g}$ A44 were incubated with 10^6 HEp-2 cells, and the intracellular accumulation of beads was observed by confocal microscopy after 1, 3, 6, and 24 h of incubation. Cells were depicted in red by staining actin with rhodamine-phalloidin (Fig. 1B), and beads (green) were already seen inside cells after 1 h of incubation; internalized beads associated with actin are yellow, and external beads are blue. Numbers of internalized beads increased by 3 and 6 h, and by 24 h some cells appeared packed with beads.

Next, we investigated the cellular location of internalized A44. In a preliminary experiment, HEp-2 cell lysates were fractionated using a commercial kit and partitioning of proteins into the correct cell compartments was verified by probing blots of the fractions for compartment-specific proteins. Figure 2A shows a Coomassie-stained protein gel of HEp-2 cell supernatant, cytoskeletal, cytoplasmic, and nucleic protein fractions. We used an antibody against early endosomal antigen 1 (EEA1) to validate the cytoskeletal protein fraction and anti-GAPDH antibody for the cytoplasmic fraction. Both an-

tibodies detected their target within the predicted fractions, showing that the separation of cytoplasmic and cytoskeletal proteins from the other fractions was correct and that no cross-contamination occurred. We also confirmed the presence of mitochondrial contents within the nuclear protein preparation by using antibodies against cytochrome *c*, a small haem protein found exclusively in the inner membranes of intact mitochondria (Fig. 2A). This result suggests that in this procedure the “nuclear” fraction contains both mitochondrial and nuclear proteins.

To determine whether A44 was degraded inside cells, the recombinant peptide ($2 \mu\text{g}/\text{ml}$) was incubated with HEp-2 cells for up to 48 h and then host cells were lysed and separated as described above into cytoskeletal, cytoplasmic, and nuclear/mitochondrial protein fractions. Proteins in the culture supernatants and cell fractions were resolved by SDS-PAGE, blotted to membranes, and probed with anti-His tag antibodies to detect A44. Peptide was detected in all supernatants and cytoskeletal fractions and by 3 h was also associated with the nuclear/mitochondrial fraction. Interestingly, intact A44 was present in the cytoskeletal and nuclear/mitochondrial fractions even after 24 h of incubation; however, after 48 h the peptide was no longer detected in the latter fraction, suggesting that degradation may have occurred in this compartment (Fig. 2B).

Detection of peptide A44 within host mitochondria. Previously, Scragg et al. (34) demonstrated that the complete adhesin domain of RgpA translocated to the perinuclear region of KB eukaryotic cells. To identify the subcellular localization of peptide A44 in the present study, the DNA sequence was cloned into plasmid pEGFP-N3 and introduced into HEp-2 epithelial cells. Immunofluorescent imaging was used to determine the distribution of A44-GFP signals (green); nuclei were

FIG. 2. Translocation of peptide A44. (A) Coomassie-stained gel and Western blots of HEP-2 protein fractions: supernatant (SN), cytoskeletal (Sk), cytoplasmic (Cp), and nuclear (Nuc). Correct separation of fractions is shown using antibodies against cytoskeletal protein early endosome antigen 1 (EEA1), cytoplasmic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and mitochondrial marker cytochrome *c* (CytC). α , anti. (B) Detection of peptide A44 in HEP-2 fractions on prolonged incubation. Peptide A44 was detected in host cell fractions with anti-His tag antibodies.

stained blue after treatment with the TO-PRO-3 DNA stain, and rhodamine-phalloidin (red) was used to reveal host actin and cell structure. Figure 3A and B show several cells expressing A44-GFP in close proximity to TO-PRO-3-stained nuclei; however, the blue DNA stain appeared to colocalize with A44-GFP in the perinuclear region. Expression of GFP can be toxic to cells, and in these experiments relatively few A44-GFP-expressing cells were observed within fields of view. Therefore,

FIG. 3. Confocal microscopy reveals that peptide A44 translocates to mitochondria. (A, B) A44-GFP expressed in host cells colocalizes with nucleic acid stain TO-PRO-3 outside host nuclei. (C) MitoTracker (red) was used to label mitochondria. The merged image shows FITC-labeled A44 (green) translocated to host mitochondria (colocalization is shown in yellow).

FIG. 4. Localization of A44 within mitochondria. Shown is transmission electron microscopy of sections of host cells either expressing A44-GFP (A) or incubated with exogenous peptide A44 (B). A44 was detected with A44-specific (A) or anti-His tag (B) antibodies. Secondary antibodies were 10-nm-gold-labeled anti-rabbit antibodies. Arrows indicate internalized, gold-labeled A44. Scale bars, 100 nm.

the experiments were repeated with FITC-labeled A44 (green) and MitoTracker (red) to stain membranes of mitochondria, the only other DNA-containing organelles in eukaryotic cells. As shown in Fig. 3C, FITC-labeled A44 colocalized with mitochondria in the perinuclear region, implying that A44 trafficked to mitochondria and not cell nuclei.

As an additional approach, we used immunogold labeling and transmission electron microscopy to confirm the translocation of peptide A44 to mitochondria. First, we treated preparations of the A44-GFP-expressing HEp-2 cell line with A44-specific antibodies, followed by 10-nm-gold-labeled anti-rabbit secondary antibodies. As shown in Fig. 4A, gold particles can be seen within mitochondrial structures, confirming the trafficking of A44 to and localization within mitochondria. In a second set of experiments to determine whether exogenously added A44 also translocates to host mitochondria, we added A44 (2 μ g/ml) to HEp-2 epithelial cells, then used the same immunogold-labeling technique and transmission electron microscopy to visualize the peptide (Fig. 4B). There were no differences in localization of endogenous transiently expressed A44 and exogenously added peptide, as both migrated to host mitochondria. However, as measured previously, the amount of A44 detected within cells was much larger when the peptide was added exogenously. These data suggest that even though

A44 is intimately associated with mitochondria there are no toxic effects on cell viability.

Peptide A44 blocks apoptosis in host epithelial cells. To investigate whether uptake of A44 into mitochondria affected host cell viability, we used a quantitative colorimetric assay with MTT as an indicator of mitochondrial dehydrogenase (oxidoreductase) activities (23). Since the tetrazolium salt is cleaved only by hydrogenases produced from active mitochondria, the formation of a colored product (reduced tetrazolium) is an indicator of living cells. Monolayers of epithelial cells were incubated with A44 (2 μ g/ml) for up to 24 h, and there was no reduction in indicator color compared to the negative control (monolayers to which no peptide was added) (Fig. 5, -STS group), indicating that A44 does not damage host mitochondria and affect viability.

Next, we investigated whether A44 could protect host cells from staurosporine-induced apoptosis. As above, cells were preincubated with A44 (2 μ g/ml) for 1 to 24 h, followed by the addition of fresh medium containing 2 μ M staurosporine to induce apoptosis, and incubation continued for a further 24 h. As shown in Fig. 5 (+STS group), cells that were not pretreated with A44 before the staurosporine challenge showed the lowest viability (10 to 15%), and a similar result was obtained when cells had only a short (1-h) pretreatment with

FIG. 5. Peptide A44 prevents staurosporine-induced apoptosis in host cells. Apoptosis was induced with 2 μ M staurosporine for 24 h in cells preincubated with peptide A44. Percentages of live cells were extrapolated from amounts of MTT indicator taken up by cells. Student's *t* test from at least three different experiments revealed statistical significance of viable cells (*, $P < 0.05$).

FIG. 6. Expression of apoptosis-related genes in host cells after incubation with A44 or live *P. gingivalis*. (A) After 1 to 24 h of incubation with peptide A44, relative gene expression was measured by quantitative RT-PCR. (B) Relative gene expression in cells incubated for 1, 3, and 6 h with *P. gingivalis* (Pg) 33277 was measured by qRT-PCR. Using Student's *t* test statistical significance in expression levels from at least three separate experiments was obtained (*, $P < 0.05$; **, $P < 0.1$).

peptide A44 (15 to 20% live cells). As shown in Fig. 5, longer pretreatments of host cells with A44 afforded greater protection against staurosporine-induced cell death; for example, host cell cultures pretreated for 3 and 6 h showed higher percentages of live cells, 43% and 57%, respectively. Preincubation with peptide for 16 h gave the most protection, with all cells retaining viability. Lastly, the antiapoptotic effect of A44 decreased over time since a 24-h pretreatment yielded approximately 59% cell viability.

A44 induces the expression of antiapoptotic factors. Apoptosis is controlled by the balance of pro- and antiapoptotic factors at the mitochondrial membrane, and the expression of these factors is regulated by several signal transduction systems. Therefore, we investigated whether peptide A44 and live *P. gingivalis* affect the expression of these factors in HEp-2 epithelial cells. Following treatment of monolayers with either purified peptide A44 (2 μ g/ml) for up to 24 h or *P. gingivalis* for up to 6 h, HEp-2 cellular RNA (2 μ g) was transcribed into cDNA using oligo(dT) primers, and the expression of several pro- and antiapoptotic factors was quantified by qRT-PCR. We used the β -globin gene as the internal control host gene to calculate the relative expression levels of 4 antiapoptotic genes, *bcl-2*, *bcl-XL*, *mcl-1*, and the gene encoding NF- κ B, which activates inhibitor of apoptosis proteins (IAP). In addition, we measured the expression of 3 proapoptotic genes, *bax*, *bak*, and the TNF- α gene, and of prosurvival factors *rhoA*, *stat3*, and *cIAP1*. As depicted in Fig. 6A, *bcl-2* expression is over 2-fold higher in cells incubated with peptide A44 for 1, 3, or 6 h than

in cells without A44. After 6 h a 2-fold induction for *bcl-XL* was also detected. We detected up to 3-fold-higher expression levels of *bcl-2* after 16 h of incubation compared to those for cells without A44. These results suggest that the induction of antiapoptotic factors was an early effect of A44 treatment. When HEp-2 epithelial cells were incubated with A44 for 24 h, quantitative analysis did not show induced gene expression, and we concluded that the effect of A44 on antiapoptotic factors Bcl-2 and Bcl-XL was transient and limited to the early stages of infection.

Expression of the same genes was quantified after 1, 3, and 6 h of incubation with *P. gingivalis* cells (Fig. 6B). Incubation for 1 h did not change the expression profile in cells; however, a significant increase (2-fold) in *bcl-2* expression was observed after 3 and 6 h of incubation with bacteria. *bcl-XL* expression was also upregulated approximately 2-fold after 6 h. We also detected a 2-fold induction of *mcl-1* expression and up to 3-fold increases in NF- κ B and TNF- α RNA transcripts after 6 h of incubation. After longer incubations HEp-2 cells detached from plates, indicative of loss of viability due to apoptosis. Thus, as with A44, the early events in *P. gingivalis* infection appear to be antiapoptotic and temporary.

Peptide A44 delays activation of caspase-9. Overexpression of antiapoptotic *bcl-2* family members is known to preserve mitochondrial membrane potential integrity and therefore prevent leakage of cytochrome *c* into the cytoplasm. Release of cytoplasmic cytochrome *c* activates caspase-9, which in turn activates caspase-3, an executioner caspase that initiates irre-

FIG. 7. Western blot analysis of activated caspase-9 in cell extracts. HEp-2 cells preincubated without (–) and with (+) peptide A44 for 16 h were incubated for 6, 8, 12, 16, and 24 h with 2 μ M staurosporine to activate caspase-9.

versible cell death. To address the question of how the protective, antiapoptotic effect of A44 functioned at the protein level, we investigated whether expression of *bcl-2* and *bcl-XL* induced by A44 prevented the staurosporine response of caspase-9 activation. To afford maximum protection, peptide A44 was incubated with host cells for 16 h; cells without peptide were used as negative controls. Incubations with and without staurosporine were for 6, 8, 12, 16, and 24 h. All cells from the same plate were collected, including detached cells in supernatants. Western blots of cell lysates probed with caspase-9 antibodies revealed activation of caspase-9 in cultures after 6 h of incubation with staurosporine in the absence of peptide A44, as shown in Fig. 7 by the appearance of a smaller band, corresponding to activated cleaved caspase-9. However, lysates of cells preincubated with peptide A44 before staurosporine treatment for up to 12 h contained only a strong pro-caspase-9 band. Longer incubation with staurosporine led to the appearance of cleaved caspase-9 in lysates even after A44 treatment, suggesting that the protective properties of the peptide, possibly due to upregulation of *bcl-2* and *bcl-XL*, are temporary.

DISCUSSION

Recently, we showed that RgpA adhesin peptide A44 and *P. gingivalis* use a clathrin-dependent pathway to enter host epithelial cells (4). In the present study we investigated the intracellular location of A44 following internalization. Increasing amounts of labeled peptide and A44-coated beads accumulated on and within HEp-2 cells over time (Fig. 1A to D). Cell fractionation and Western blot assays revealed that A44 associated with the host nuclear fraction, which we showed also contained mitochondrial components (Fig. 2A). Even after 48 h of incubation the intact peptide was associated with the cytoskeletal fraction but was no longer detected in the nuclear/mitochondrial fraction, possibly because of degradation by cellular proteases (Fig. 2B). Previously, Scragg et al. showed that the complete adhesin domain of RgpA translocated to the perinuclear region in host cells (34). In the present study we observed that TO-PRO-3 nuclear stain (blue) colocalized with GFP-labeled A44 (green) outside the nucleus (Fig. 3A and B), suggesting that DNA-containing mitochondria, not nuclei, were targets of peptide A44. We tested this model using MitoTracker (red) to stain mitochondria and exogenously added FITC-labeled peptide and observed colocalization of A44 with host mitochondria by confocal microscopy (Fig. 3C). This was confirmed by transmission electron microscopy of A44-GFP-expressing HEp-2 cells that were immunogold labeled with A44-specific antibodies (Fig. 4).

A number of pathogens target mitochondria, which play a central role in apoptosis. During programmed cell death, de-

polarized mitochondria swell and release proapoptotic factors that activate caspases. These enzymes cleave cellular proteins, resulting in mitochondrial membrane changes that lead to apoptosis. Bacterial pathogens manipulate different pathways to either induce or inhibit apoptosis (3). An advantage of apoptosis over necrosis is the reduced migration of inflammatory cells to the infection site. On the other hand, apoptosis breaks down epithelial/endothelial barriers, and blocking this process enables bacteria to colonize a site, albeit transiently. Mechanistically, caspases are activated through the binding of ligands to death receptors and the release of proapoptotic factors from mitochondria (40) or through stress-induced release of Ca^{2+} from the endoplasmic reticulum (ER) (5, 33). Apoptosis can also be mediated by noncaspase proteases or by nonmitochondrial proteins known as apoptosis-inducing factors (AIF) (16). To determine whether peptide A44 induced apoptosis, purified peptide was incubated with HEp-2 cells and cell viability was quantified using reduction of MTT as an indicator of mitochondrial activity (23). As shown in Fig. 5A, incubation with A44 for up to 24 h did not affect host cell viability, and we concluded that the peptide does not induce apoptosis during the early stages of internalization.

Bacteria also manipulate host cells to block apoptosis, and pretreatment with A44 protected HEp-2 cells from staurosporine-induced apoptosis for up to 24 h (Fig. 5). Three different antiapoptotic mechanisms used by bacteria have been described so far (reviewed in reference 13). For example, *Chlamydia* spp. prevent the release of cytochrome *c* from mitochondria by blocking proapoptotic factors (12, 43, 49). A second strategy is activation of cell survival pathways, as exemplified by upregulation of antiapoptotic factors *bcl-2* and *mcl-1* in macrophages by *Staphylococcus aureus* (20). Lastly, *Shigella flexneri* (8) and *Legionella* (1) interact directly with caspases and block caspase-3 activation. Anti- and proapoptotic factors also interact with each other, and the balance of these activities at the mitochondrial membrane determines life or death for the cell (15). Members of the antiapoptotic Bcl-2 family, e.g., Bcl-2 itself, Bcl-XL, and Mcl-1, preserve the integrity of the mitochondrial membrane by binding to porin channels (38). Two additional classes of proapoptotic proteins are the Bax subfamily (i.e., Bax and Bak) and BH3-only proteins (i.e., Bim). The latter act directly with and antagonistically to antiapoptotic proteins to inhibit their activities; subsequently, Bax and Bak form homo- and heterodimers within the mitochondrial membrane, leading to release of cytochrome *c* (9, 31). We examined expression of specific pro- and antiapoptotic factors after incubation of HEp-2 cells with peptide A44 and quantified expression of antiapoptotic proteins Bcl-2, Bcl-XL, and Mcl-1, of proapoptotic proteins Bax, Bak, and TNF- α , and of

prosurvival factors NF- κ B, Stat3, RhoA, and cIAP-1. Expression of Bcl-2 was upregulated in cells incubated with A44 for up to 16 h (Fig. 6). Upregulation of antiapoptotic proteins such as Bcl-2 prevents release of cytochrome *c* and has also been shown to abrogate the function of proapoptotic proteins and protect against cell death (18). We also detected a transient 2-fold induction of *bcl-XL* after 6 h of incubation; however, by 24 h expression of *bcl-XL* decreased to control levels, as measured in cells without the peptide. The half-life of *bcl-2* RNA is relatively short compared to the long half-life (up to 10 h) of the Bcl-2 protein, and differences in mRNA levels detected in our qRT-PCR experiments and the actual number of live cells in MTT assays could be due to the different turnover rates for RNA and protein (30). Timelines for and the progression of various apoptotic events vary greatly in cells, and the time required for an activity to peak in one cell line might differ from that required in another. Several studies showed that overexpression of Bcl-2 was not effective in protecting cells from staurosporine-induced apoptosis; however, overexpressed Bcl-XL was able to protect neuroblastoma cells (47). In our experiments A44 delayed staurosporine-induced apoptosis for up to 24 h, at which point there were still more live cells (35 to 40%) than in controls without A44 treatment.

When HEP-2 cells were incubated with live *P. gingivalis* for up to 6 h, we detected 2-fold increases in levels of Bcl-2 and Bcl-XL (Fig. 6B). We also found significantly higher levels of the transcript for Mcl-1, which blocks proapoptotic factors and inhibits the oligomerization of Bak and Bax in the mitochondrial membrane. Mcl-1 has a faster turnover rate than Bcl-2 and is modulated and activated by several growth factors, including phosphorylated Akt and phosphatidylinositol 3-kinase (10, 29, 30). In addition, NF- κ B and TNF- α levels were 2.5- and 3.3-fold higher, respectively, than those in uninfected cells. While NF- κ B prevents apoptosis by blocking cytochrome *c* release and activation of caspases-9 and -3, TNF- α induces apoptosis by activating caspase-8 in the extrinsic apoptosis pathway. In our assays, cells incubated with *P. gingivalis* for more than 6 h began detaching from tissue culture plates (data not shown), indicative of loss of viability. Thus, it is possible that, upon longer incubation, *P. gingivalis* activation of death receptors via TNF- α cannot be offset by simultaneously activated NF- κ B, Bcl-2, Bcl-XL, and Mcl-1.

There are contradictory reports on whether *P. gingivalis* has pro- or antiapoptotic effects on host cells. In one study, the analysis of the transcriptional profile of gingival epithelial cells exposed for 2 h to *P. gingivalis* showed an increase in *bcl-2* and *bif-1*, which protected host cells from camptothecin-induced apoptosis (17). In an earlier study it was also shown that *P. gingivalis* 33277 rescued primary gingival epithelial cells from camptothecin-induced apoptosis since early upregulation of *bax* expression preceded upregulation of *bcl-2* at 24 h, leading to the conclusion that the first cellular response of apoptosis was subsequently delayed by *P. gingivalis* (25). In another study with *P. gingivalis* 33277, staurosporine-induced apoptosis was blocked but restored in the presence of a phosphatidylinositol 3-kinase inhibitor, implicating a pathway in which phosphorylated Akt and activated NF- κ B prevented depolymerization of the mitochondrial membrane and cytochrome *c* release induced by staurosporine (45). This is consistent with our observation of higher NF- κ B levels in cells exposed to the same *P. gingivalis* strain. Another antiapoptotic path-

way was detected when gingival epithelial cells were infected with *P. gingivalis* 33277 whereby, in addition to Stat3, Jak, an upstream kinase, was also upregulated (21). In the present study we did not detect differences in *stat3* levels, possibly due to the different host cell types used; however, Stat3-induced survival responses such as upregulated *bcl-2*, *bcl-XL*, and *mcl-1* were observed, and the overall antiapoptotic effect was the same. Recently, it was suggested that gingipains of *P. gingivalis* strain W50 mediate apoptosis in a dose-dependent manner (26). Strain W83 induced apoptosis by gingipain-mediated cleavage of N- and VE-cadherins, resulting in loss of epithelial and endothelial cell adhesion by caspase-dependent and -independent mechanisms (7, 36, 37). Indeed, several other studies demonstrated that on prolonged incubation proteases destroy cells and tissue and induce apoptosis, implying that gingipain activity is decreased when *P. gingivalis* is internalized by host cells, allowing survival (48). We also observed loss of cell viability after infection with *P. gingivalis* 33277, but only after prolonged incubation of more than 6 h. In the studies cited above, concentrations of *P. gingivalis* and/or gingipains were much higher than those used in the present study; however, we also found that incubations with similarly high concentrations of bacteria or proteases led to host cell death (H. Boisvert, unpublished data).

Staurosporine-induced apoptosis results in the translocation of Bax from the cytosol to mitochondria and loss of membrane potential due to opening of mitochondrial pores. The subsequent releases of cytochrome *c* and apoptosis-inducing factors initiate the apoptotic cascade through activation of caspase-9 (19, 27, 42). Our hypothesis that A44 delays apoptosis was tested with staurosporine-treated cells, and we demonstrated that 16 h of preincubation with peptide A44 delayed activation of caspase-9 for up to 12 h compared to activation in cells without peptide A44. However, A44 could not prevent cleavage and activation of caspase-9 after longer staurosporine exposure.

In summary, our previous work demonstrated, for the first time, that host cells internalized RgpA peptide A44 and live *P. gingivalis* via a clathrin-dependent mechanism. In the present study we continued this mechanistic analysis and identified events following A44 entry into cells. Our data showed that peptide A44 entered host cells efficiently and was not degraded. We discovered that the peptide trafficked to mitochondria, where it has the potential to direct host cells toward survival or death by manipulating apoptosis. By quantifying of transcription of pro- and antiapoptotic factors produced by cells in response to A44, a model emerged whereby the peptide induced expression of antiapoptotic factors, confirmed by the observation that A44 protected against staurosporine-induced apoptosis. On the other hand, the initial antiapoptotic effect was short-lived in cells infected with live *P. gingivalis*, as indicated by observations of cell rounding and detachment after 6 h of incubation. This result could be anticipated since whole bacteria present to the cell a more complex mixture of surface proteins that potentially promote a more effective apoptotic response.

ACKNOWLEDGMENTS

We thank Ziedonis Skobe and Justine Dobeck for advice and help with electron and confocal microscopy.

This work was supported by U.S. National Institutes of Health grants R01-DE015931 and T32-DE007327-08.

REFERENCES

1. Abu-Zant, A., M. Santic, M. Molmeret, S. Jones, J. Helbig, and Y. Abu Kwaik. 2005. Incomplete activation of macrophage apoptosis during intracellular replication of *Legionella pneumophila*. *Infect. Immun.* **73**:5339–5349.
2. Blanke, S. R. 2005. Micro-managing the executioner: pathogen targeting of mitochondria. *Trends Microbiol.* **13**:64–71.
3. Bohme, L., and T. Rudel. 2009. Host cell death machinery as a target for bacterial pathogens. *Microbes Infect.* **11**:1063–1070.
4. Boisvert, H., and M. J. Duncan. 2008. Clathrin-dependent entry of a gingipain adhesin peptide and *Porphyromonas gingivalis* into host cells. *Cell. Microbiol.* **10**:2538–2552.
5. Breckenridge, D. G., M. Germain, J. P. Mathai, M. Nguyen, and G. C. Shore. 2003. Regulation of apoptosis by endoplasmic reticulum pathways. *Oncogene* **22**:8608–8618.
6. Chen, T., and M. J. Duncan. 2004. Gingipain adhesin domains mediate *Porphyromonas gingivalis* adherence to epithelial cells. *Microb. Pathog.* **36**:205–209.
7. Chen, Z., C. A. Casiano, and H. M. Fletcher. 2001. Protease-active extracellular protein preparations from *Porphyromonas gingivalis* W83 induce N-cadherin proteolysis, loss of cell adhesion, and apoptosis in human epithelial cells. *J. Periodontol.* **72**:641–650.
8. Clark, C. S., and A. T. Maurelli. 2007. *Shigella flexneri* inhibits staurosporine-induced apoptosis in epithelial cells. *Infect. Immun.* **75**:2531–2539.
9. Cory, S., and J. M. Adams. 2002. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat. Rev. Cancer* **2**:647–656.
10. Cuconati, A., C. Mukherjee, D. Perez, and E. White. 2003. DNA damage response and MCL-1 destruction initiate apoptosis in adenovirus-infected cells. *Genes Dev.* **17**:2922–2932.
11. Dimmer, K. S., and D. Rapaport. 2008. Proteomic view of mitochondrial function. *Genome Biol.* **9**:209.
12. Dong, F., M. Pirbhaj, Y. Xiao, Y. Zhong, Y. Wu, and G. Zhong. 2005. Degradation of the proapoptotic proteins Bik, Puma, and Bim with Bcl-2 domain 3 homology in *Chlamydia trachomatis*-infected cells. *Infect. Immun.* **73**:1861–1864.
13. Faherty, C. S., and A. T. Maurelli. 2008. Staying alive: bacterial inhibition of apoptosis during infection. *Trends Microbiol.* **16**:173–180.
14. Grassme, H., V. Jendrosseck, and E. Gulbins. 2001. Molecular mechanisms of bacteria induced apoptosis. *Apoptosis* **6**:441–445.
15. Green, D. R., and J. C. Reed. 1998. Mitochondria and apoptosis. *Science* **281**:1309–1312.
16. Hail, N., Jr., B. Z. Carter, M. Konopleva, and M. Andreeff. 2006. Apoptosis effector mechanisms: a requiem performed in different keys. *Apoptosis* **11**:889–904.
17. Handfield, M., J. J. Mans, G. Zheng, M. C. Lopez, S. Mao, A. Progsulke-Fox, G. Narasimhan, H. V. Baker, and R. J. Lamont. 2005. Distinct transcriptional profiles characterize oral epithelium-microbiota interactions. *Cell. Microbiol.* **7**:811–823.
18. Kempf, V. A., A. Schairer, D. Neumann, G. A. Grassl, K. Lauber, M. Lebiedziewski, M. Schaller, P. Kyme, S. Wesselborg, and I. B. Autenrieth. 2005. *Bartonella henselae* inhibits apoptosis in Mono Mac 6 cells. *Cell. Microbiol.* **7**:91–104.
19. Kluck, R. M., E. Bossy-Wetzel, D. R. Green, and D. D. Newmeyer. 1997. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**:1132–1136.
20. Koziel, J., A. Maciag-Gudowska, T. Mikolajczyk, M. Bzowska, D. E. Sturdevant, A. R. Whitney, L. N. Shaw, F. R. DeLeo, and J. Potempa. 2009. Phagocytosis of *Staphylococcus aureus* by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors. *PLoS One* **4**:e5210.
21. Mao, S., Y. Park, Y. Hasegawa, G. D. Tribble, C. E. James, M. Handfield, M. F. Stavropoulos, O. Yilmaz, and R. J. Lamont. 2007. Intrinsic apoptotic pathways of gingival epithelial cells modulated by *Porphyromonas gingivalis*. *Cell. Microbiol.* **9**:1997–2007.
22. Reference deleted.
23. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55–63.
24. Nakagawa, I., A. Amano, M. Kuboniwa, T. Nakamura, S. Kawabata, and S. Hamada. 2002. Functional differences among FimA variants of *Porphyromonas gingivalis* and their effects on adhesion to and invasion of human epithelial cells. *Infect. Immun.* **70**:277–285.
25. Nakhjiri, S. F., Y. Park, O. Yilmaz, W. O. Chung, K. Watanabe, A. El-Sabaeny, K. Park, and R. J. Lamont. 2001. Inhibition of epithelial cell apoptosis by *Porphyromonas gingivalis*. *FEMS Microbiol. Lett.* **200**:145–149.
26. O'Brien-Simpson, N. M., R. D. Pathirana, G. D. Walker, and E. C. Reynolds. 2009. *Porphyromonas gingivalis* RgpA-Kgp proteinase-adhesin complexes penetrate gingival tissue and induce proinflammatory cytokines or apoptosis in a concentration-dependent manner. *Infect. Immun.* **77**:1246–1261.
27. Petit, P. X., M. Gubern, P. Diolez, S. A. Susin, N. Zamzami, and G. Kroemer. 1998. Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition. *FEBS Lett.* **426**:111–116.
28. Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**:e45.
29. Rajalingam, K., M. Sharma, C. Lohmann, M. Oswald, O. Thieck, C. J. Froelich, and T. Rudel. 2008. Mcl-1 is a key regulator of apoptosis resistance in *Chlamydia trachomatis*-infected cells. *PLoS One* **3**:e3102.
30. Reed, J. C. 1996. A day in the life of the Bcl-2 protein: does the turnover rate of Bcl-2 serve as a biological clock for cellular lifespan regulation? *Leuk. Res.* **20**:109–111.
31. Roset, R., L. Ortet, and G. Gil-Gomez. 2007. Role of Bcl-2 family members on apoptosis: what we have learned from knock-out mice. *Front. Biosci.* **12**:4722–4730.
32. Scheffler, I. E. 2001. A century of mitochondrial research: achievements and perspectives. *Mitochondrion* **1**:3–31.
33. Scorrano, L., S. A. Oakes, J. T. Opferman, E. H. Cheng, M. D. Sorcinelli, T. Pozzan, and S. J. Korsmeyer. 2003. BAX and BAK regulation of endoplasmic reticulum Ca^{2+} : a control point for apoptosis. *Science* **300**:135–139.
34. Scragg, M. A., A. Alsam, M. Rangarajan, J. M. Slaney, P. Shepherd, D. M. Williams, and M. A. Curtis. 2002. Nuclear targeting of *Porphyromonas gingivalis* W50 protease in epithelial cells. *Infect. Immun.* **70**:5740–5750.
35. Scragg, M. A., S. J. Cannon, M. Rangarajan, D. M. Williams, and M. A. Curtis. 1999. Targeted disruption of fibronectin-integrin interactions in human gingival fibroblasts by the RI protease of *Porphyromonas gingivalis* W50. *Infect. Immun.* **67**:1837–1843.
36. Sheets, S. M., J. Potempa, J. Travis, C. A. Casiano, and H. M. Fletcher. 2005. Gingipains from *Porphyromonas gingivalis* W83 induce cell adhesion molecule cleavage and apoptosis in endothelial cells. *Infect. Immun.* **73**:1543–1552.
37. Sheets, S. M., J. Potempa, J. Travis, H. M. Fletcher, and C. A. Casiano. 2006. Gingipains from *Porphyromonas gingivalis* W83 synergistically disrupt endothelial cell adhesion and can induce caspase-independent apoptosis. *Infect. Immun.* **74**:5667–5678.
38. Shimizu, S., M. Narita, and Y. Tsujimoto. 1999. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* **399**:483–487.
39. Smith, D. E., and P. A. Fisher. 1984. Identification, developmental regulation, and response to heat shock of two antigenically related forms of a major nuclear envelope protein in *Drosophila* embryos: application of an improved method for affinity purification of antibodies using polypeptides immobilized on nitrocellulose blots. *J. Cell Biol.* **99**:20–28.
40. Thornberry, N. A., and Y. Lazebnik. 1998. Caspases: enemies within. *Science* **281**:1312–1316.
41. Weinrauch, Y., and A. Zychlinsky. 1999. The induction of apoptosis by bacterial pathogens. *Annu. Rev. Microbiol.* **53**:155–187.
42. Wolf, C. M., and A. Eastman. 1999. The temporal relationship between protein phosphatase, mitochondrial cytochrome c release, and caspase activation in apoptosis. *Exp. Cell Res.* **247**:505–513.
43. Xiao, Y., Y. Zhong, W. Greene, F. Dong, and G. Zhong. 2004. *Chlamydia trachomatis* infection inhibits both Bax and Bak activation induced by staurosporine. *Infect. Immun.* **72**:5470–5474.
44. Yilmaz, O. 2008. The chronicles of *Porphyromonas gingivalis*: the micro-bium, the human oral epithelium and their interplay. *Microbiology* **154**:2897–2903.
45. Yilmaz, O., T. Jungas, P. Verbeke, and D. M. Ojcius. 2004. Activation of the phosphatidylinositol 3-kinase/Akt pathway contributes to survival of primary epithelial cells infected with the periodontal pathogen *Porphyromonas gingivalis*. *Infect. Immun.* **72**:3743–3751.
46. Yilmaz, O., K. Watanabe, and R. J. Lamont. 2002. Involvement of integrins in fimbriae-mediated binding and invasion by *Porphyromonas gingivalis*. *Cell. Microbiol.* **4**:305–314.
47. Yuste, V. J., I. Sanchez-Lopez, C. Sole, M. Encinas, J. R. Bayasas, J. Boix, and J. X. Comella. 2002. The prevention of the staurosporine-induced apoptosis by Bcl-X(L), but not by Bcl-2 or caspase inhibitors, allows the extensive differentiation of human neuroblastoma cells. *J. Neurochem.* **80**:126–139.
48. Zhang, Y., T. Wang, W. Chen, O. Yilmaz, Y. Park, I. Y. Jung, M. Hackett, and R. J. Lamont. 2005. Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components. *Proteomics* **5**:198–211.
49. Zhong, Y., M. Weininger, M. Pirbhaj, F. Dong, and G. Zhong. 2006. Inhibition of staurosporine-induced activation of the proapoptotic multidomain Bcl-2 proteins Bax and Bak by three invasive chlamydial species. *J. Infect.* **53**:408–414.