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Signaling Cascades Triggered by Bacterial Metabolic End Products during Reactivation of Kaposi's Sarcoma-Associated Herpesvirus▽

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The present studies explore the role of polymicrobial infection in the reactivation of Kaposi's sarcoma-associated herpesvirus (KSHV) and analyze signaling pathways activated upon this induction. We hypothesized that activation of the cellular stress-activated mitogen-activated protein kinase (MAPK) p38 pathway would play a key role in the bacterium-mediated disruption of viral latency similar to that of previously reported results obtained with other inducers of gammaherpesvirus lytic replication. KSHV within infected BCBL-1 cells was induced to replicate following exposure to metabolic end products from gram-negative or -positive bacteria that were then simultaneously exposed to specific inhibitors of signal transduction pathways. We have determined that bacterium-mediated induction of lytic KSHV infection is significantly reduced by the inhibition of the p38 MAPK pathway. In contrast, inhibition of the phosphatidylinositol 3-kinase pathway did not impair induction of lytic replication or p38 phosphorylation. Protein kinase C, though activated, was not the major pathway used for bacterium-induced viral reactivation. Furthermore, hyperacetylation of histones 3 and 4 was detected. Collectively, our results show that metabolic end products from these pathogens induce lytic replication of KSHV in BCBL-1 cells primarily via the activation of a stress-activated MAPK pathway. Importantly, we demonstrate for the first time a mechanism by which polymicrobial bacterial infections result in KSHV reactivation and pathogenesis.

Human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is a herpesvirus that has been recognized as a significant viral pathogen, particularly for immunocompromised persons infected with human immunodeficiency virus type 1 (HIV-1). KSHV belongs to a subfamily of gammaherpesviruses (lymphotropic), together with Epstein-Barr virus (EBV), herpesvirus saimiri, and murine gammaherpesvirus 68. This virus has been consistently associated with Kaposi's sarcoma (7), the most common neoplasm observed in individuals infected with HIV-1. KSHV is also associated with two lymphoproliferative diseases, multicentric Castleman's disease (42) and primary effusion lymphoma (PEL) or body cavity-based lymphoma (4). We and others have provided compelling evidence that oral infection of KSHV does occur in patents with Kaposi's sarcoma (15, 47) and among healthy populations (15).

A hallmark of all herpesviruses, including KSHV, is their ability to establish life-long latent infections in their natural host cells (40). In latent infection, the viral genome persists extrachromosomally as a circular episome, viral gene expression is severely attenuated, and viral progeny are not produced (12). During reactivation, KSHV-infected cells express a variety of lytic cycle genes, linear forms of the genome are produced for packaging, and viral progeny are produced, which ultimately results in host cell lysis (33, 37, 38, 49,). The switch from latency to lytic viral gene expression of KSHV is crucial for virus spread between cells and hosts and is also likely to play an important role in the tumorigenesis induced by KSHV (21, 33). Previous studies have demonstrated that KSHV viral replication in PEL and Kaposi's sarcoma tumor cells is tightly regulated, with viral genomes persisting predominantly in a latent state (14, 41). Although the exact mechanism by which latent virus becomes reactivated and begins lytic replication is not entirely known, treatment of PEL cells with chemical agents, such as the phorbol ester phorbol-12-tetradecanoate-13-acetate (TPA) (38), the short-chain fatty acid *n*-butyrate (33), the calcium ionophore ionomycin (5), and the DNA methyltransferase inhibitor 5-azacytidine (8), induced KSHV replication. Other studies have also shown that inflammatory cytokines (5), coinfection with HIV-1 (45), and hypoxic conditions (11) can also directly induce lytic viral reactivation. Certain physiological conditions reactivate the latent virus periodically in most asymptomatic carriers, potentially leading to the onset of disease. Such conditions include immunodeficiency either due to AIDS or as a result of organ transplantation (16, 30).

There is a paucity of knowledge with regard to the influence of local physiologic factors on reactivation of this and other herpesviruses. Recent experiments from our laboratory have shown that spent media from selected gram-negative anaerobic bacteria (*Fusobacterium nucleatum* and *Porphyromonas gingivalis*), which contain lipopolysaccharides and high levels of short-chain fatty acids, such as *n*-butyric, propionic, and isovaleric acids, can also induce the switch between latency and lytic replication of KSHV (the present study).

Contreras et al. (9) detected a positive relationship between subgingival coinfections of herpesviruses and periodontal pathogens, including *P. gingivalis*, *F. nucleatum*, and others;

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while studies by Mardirossian et al. (31) have found that human herpesviruses, including KHSV, occur at a higher frequency in subgingival specimens from periodontitis. In light of the fact that we have shown that metabolic end products from selected gram-negative anaerobic periodontal pathogens can induce KSHV lytic reactivation and a positive relationship exists between herpesviruses and periodontal pathogens, it is conceivable that bacterial infection may pave the way for increased viral replication and enhanced pathogenesis of both organisms. Understanding the molecular pathogenic mechanisms that exist between viruses and bacteria that permit their survival, propagation, and subsequent involvement in disease development is critical for the development of more effective diagnostic, preventative, and treatment strategies.

The current study was designed to begin to decipher these mechanisms by focusing on the signal transduction pathways that might allow bacterial metabolic end products from some of the most common periodontal pathogens to modulate/regulate key molecular events in KSHV reactivation. Specific inhibitors for candidate signaling pathways were used to examine the relevance of each pathway to mediate the induction of lytic replication following exposure of a PEL cell line latently infected with KSHV to the metabolic end products of common periodontal pathogens. Results show that metabolic end products from these pathogens induce lytic replication of KSHV in BCBL-1 cells primarily via activation of the stress-activated mitogen-activated protein kinase (MAPK) pathway. Protein kinase C (PKC), though activated, is not the major pathway used for bacterium-induced viral reactivation.

MATERIALS AND METHODS

Cell lines and cell culture. BCBL-1 is a latently infected KSHV body cavity-based, lymphoma-derived cell line (a gift from Blossom Domania, University of North Carolina, Chapel Hill, NC). DG75 is a KSHV-negative B lymphoblastoid cell line (obtained from ATCC). Cell lines were maintained in 5% CO_2 at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and containing penicillin, streptomycin, sodium pyruvate, and L-glutamine (GIBCO-BRL, Gaithersburg, MD). A293 human embryonic kidney cells (obtained from ATCC) were maintained in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% FBS containing penicillin and streptomycin. *P. gingivalis* strain A7436, *F. nucleatum* strain 1594, *Prevotella intermedia* CB21, *Streptococcus mutans* 10449, and *Staphylococcus aureus* ATCC 25923 were kind gifts from Roland Arnold (University of North Carolina, Chapel Hill, NC). *P. gingivalis*, *F. nucleatum*, *S. mutans*, and *P. intermedia* were grown anaerobically in Wilkins-Chalgren (WC) anaerobic broth (Oxoid) in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2 at 35°C (in a Coy anaerobic chamber). *S. aureus* was grown aerobically in WC broth at 37°C. Spent medium was collected from cells that were harvested from overnight culture at a late exponential stage of growth.

Antibodies and chemicals. The rabbit polyclonal antibody detecting phosphorylated kinase p38 and the respective inactive nonphosphorylated form were purchased from Cell Signaling Technology, Beverly, MA. The rabbit polyclonal antibody against KSHV RTA was a gift from Ren Sun (University of California, Los Angeles, CA). The goat polyclonal antibody against β-actin was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. The KSHV virus-specific monoclonal antibodies for K8.1 and LANA and polyclonal antibody for viral interleukin 6 (vIL-6) were obtained from Advanced Biotechnologies Inc., Columbia, MD. Acetyl-histone 3 and 4 (H3 and H4, respectively) antibodies were obtained from Upstate Biotechnology Inc., Charlottesville, VA. Sodium butyrate (*n*-butyrate) and TPA were purchased from Sigma, St. Louis, MO. The phosphatidylinositol 3-kinase (PI3 kinase) inhibitor LY294002 [2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride] was purchased from Cell Signaling Technology, Beverly, MA. The PKC inhibitor GF109203X (bisindolylmaleimide I [GFX]) was purchased from EMD Biosciences, Inc., San Diego, CA. The p38 kinase inhibitor SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole] was purchased from Sigma, St. Louis, MO, and the inhibitor PD169316 [4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole] was purchased from Calbiochem, San Diego, CA. RQ1 DNase was obtained from Promega, Madison, WI. All reagents were prepared as recommended by their suppliers. Dimethyl sulfoxide (Me_2SO) was used as a vehicle for TPA and each of the inhibitors.

Induction and treatment. Cells were maintained at densities between 2.5×10^5 and 3.0×10^5 cells/ml. Ten milliliters of fresh growth medium was added to the cells prior to drug treatment or exposure to spent medium. The cells were preincubated in culture medium supplemented with kinase inhibitors (SB202190, PD169316, GFX or LY2940020) at 10 μM, 2 μM, 5 μM, and 10 μM, respectively, for 1 h at 37°C. Effects of all inhibitors used were not due to an increase in cellular toxicity (data not shown). These cells were subsequently induced into the lytic cycle by exposure to 0.3 mM sodium butyrate (NaB) or 20 ng TPA per ml. Spent media from the anaerobic gram-negative bacteria *P. gingivalis* and *F. nucleatum* or gram-positive bacteria *S. aureus* and *S. mutans* grown to late log phase were centrifuged at 10,000 rpm for 20 min to remove the bacteria and supernatants and filtered through a 0.45-micrometer-pore-size filter. The supernatants were added at a 1:50 dilution in place of the pharmacological chemical inducer (*n*-butyrate or TPA) to determine their induction capabilities. Aliquots were collected for protein and viral DNA isolation from cells harvested at 24 to 48 h or when indicated at different time intervals postinduction. Successful induction was determined by detection of viral lytic gene expression and virion production.

Extracellular KSHV virion DNA isolation. BCBL-1 cells were removed from the medium by centrifugation at $1,500 \times g$ for 5 min. The supernatant was collected and centrifuged again for 30 min at 3,000 rpm. The cell-free culture medium was filtered through a 0.45-micrometer-pore-size filter distributed into Beckman Ultra Clear tubes, and viral particles were pelleted by ultracentrifugation at 22,000 rpm for 2 h at 4°C. The supernatant was removed, and viral pellets were resuspended in buffer containing 40 mM Tris-HCl (pH 7), 10 mM NaCl, 6 mM $MgCl_2$, and 10 mM $CaCl_2$. RQ1 DNase was added, followed by incubation at 37°C for 1 h to eliminate free DNA. DNase was inactivated by adding EDTA to 20 mM and heating to 80°C for 5 min. Viral DNA was isolated using a High Pure viral nucleic acid kit (Roche, Indianapolis, IN) and used for real-time PCR.

Conditions of infection. A293 cell monolayers were trypsinized, washed, resuspended in fresh culture medium, mixed with the resuspended BCBL virus or DG75 culture medium (mock), and plated in eight-well chamber slides (Falcon) at a concentration of 2.5×10^4 cells/well. At 48 h postinfection, the monolayers were further processed for immunofluorescent antibody assay (IFA) as described below.

Real-time PCR. Viral DNAs were employed for real-time PCR amplifications to detect the KSHV K8.1 region of the genome. Briefly, 100 ng of DNA, SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), and an ABI Prism 7000 sequence detection system were used for amplification. Primer sequences (Lineberger Cancer Center Nucleic Acid Core Facility, University of North Carolina, Chapel Hill, NC) previously described by Fakhari and Dittmer (17) were used to detect the K8.1 viral DNA. DNA from uninfected DG75 cells and reactions run in the absence of added DNA served as negative controls. Analyses of the data were performed with associated Prism 7000 software (Applied Biosystems). The uninduced K8.1 level was arbitrarily set at 1.

Protein preparation and electrophoresis. For extraction of total cellular protein, cells were collected by centrifugation, washed once in phosphate-buffered saline, resuspended in 200 μl ice cold extraction (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% Na deoxycholate, 25 μg/ml leupeptin, and 50 μl protease inhibitor cocktail [Sigma]), passed through a 25-gauge needle, and centrifuged. For nuclear protein extraction, cell pellets were washed as described above and resuspended in 500 μl ice cold extraction buffer A (10 mM HEPES [pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 1.0 mM dithiothreitol) and centrifuged. Cells were then resuspended in 200 μl buffer A containing 0.1% NP-40 and 1× complete proteinase inhibitor, incubated on ice for 10 min, and centrifuged to pellet nuclei. Nuclear pellets were washed in buffer A, centrifuged, and resuspended in 50 μl of extraction buffer C (10 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1.0 mM dithiothreitol, 25% glycerol), incubated on ice for 30 min with occasional vortexing, and centrifuged. Cell lysates were resuspended in SDS sample buffer, and 50 to 150 μg of proteins was loaded in lanes for SDS-polyacrylamide gel electrophoresis.

Immunoblot analysis. Immunoblot analysis was performed for the detection of activated forms of proteins by immunoblotting with antiphospho antibodies (phosphorylated p38). Blots were also reacted with antibodies to detect β-actin to confirm equal protein loading. Virus-specific antibodies were used to detect the expression of viral proteins RTA, vIL-6, LANA, or K8.1 and cellular acetylated histone 3 or 4. The proteins were transferred overnight onto polyvinylidene difluoride membranes (Millipore), and immunocytochemistry protocols for each

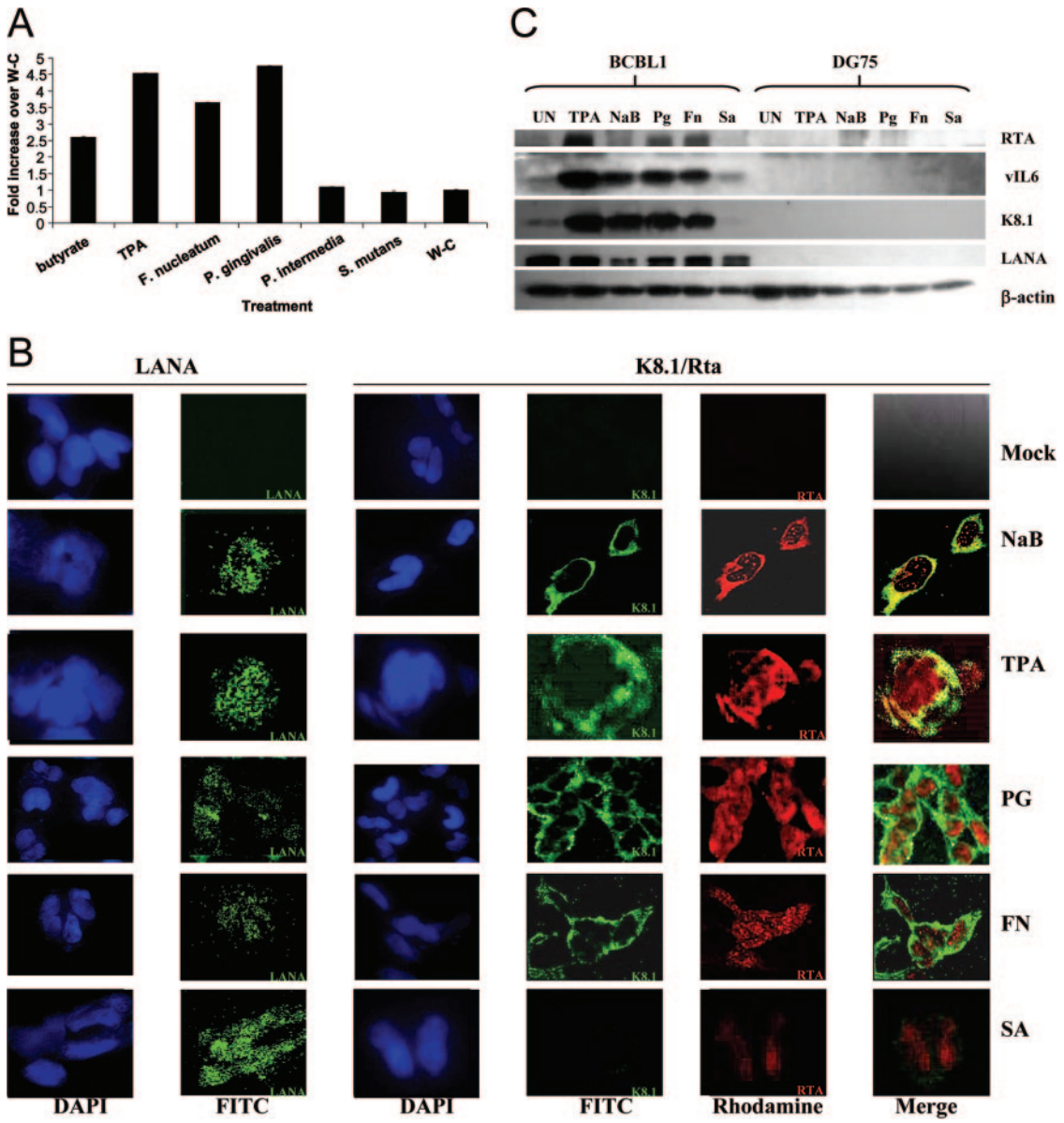

FIG. 1. (A) Increased virion release induced by spent media from periodontopathic bacteria. BCBL-1 cells were treated with or without spent media from *P. gingivalis*, *F. nucleatum*, *P. intermedia*, and *S. mutans* and NaB or TPA (positive controls). The level of viral DNA was quantified using real-time PCR to amplify the K8.1 region of the viral genome 24 h after treatment. (B) Productive infection of A293 cells by KSHV virions isolated following induction by spent medium from selected periodontopathic bacteria. A293 cell monolayers were fixed and incubated with antibodies against glycoprotein K8.1 or RTA at 48 h postinfection with virus isolated from TPA or *n*-butyrate (positive controls) or spent media from gram-negative or -positive bacteria. Virus isolated from WC (uninduced) medium was used as a negative control. Secondary monoclonal or polyclonal antibodies conjugated to Alex Flour 488 (green) or Alex Fluor 546 (red), respectively, were used to detect the primary antibody bound to KSHV antigens, and DAPI stain was used to detect the cell nuclei. The fluorescein isothiocyanate (FITC) column shows the Alex Flour 488 channel (K8.1 or LANA), the rhodamine column shows the Alex Fluor 546 channel (RTA), and the merge column shows the Alex Flour 488 and Alex Fluor 546 overlay (K8.1/RTA). (C) Effect of spent media from periodontopathic bacteria on the induction of immediate early, early, and late lytic KSHV gene expression. BCBL-1 cells were treated with or without TPA or sodium butyrate (NaB) (positive controls) or spent media from *P. gingivalis*, *F. nucleatum*, or *S. aureus*. Whole cellular protein was extracted and run on a 10% SDS-polyacrylamide gel. The expression of immediate early (RTA), early lytic (vIL-6), late lytic (K8.1), and latent (LANA) as well as the cellular β-actin protein was examined by immunoblot analysis using anti-RTA, anti-vIL-6, anti-K8.1, anti-LANA, and anti-β-actin antibodies, respectively. (D) Expression of early and late lytic KSHV genes following induction by active spent media. Immunofluorescence confocal microscopic detection of KSHV antigens. KSHV latently infected BCBL-1 cell monolayers were fixed and incubated with antibodies against glycoprotein K8.1, RTA, and LANA at 24 h postinduction with TPA, *n*-butyrate (positive controls), or spent media from gram-negative or -positive bacteria. Uninfected DG75 cells were used as negative controls. Secondary monoclonal or polyclonal antibodies conjugated to Alex Flour 488 (green) or Alex Fluor 546 (red), respectively, were used to detect the primary antibody bound to KSHV antigens, and DAPI stain was used to detect the cell nuclei. The FITC columns show the Alex Flour 488 channel (K8.1 or LANA), the rhodamine column shows the Alex Fluor 546 channel (RTA), and the merge column shows the Alex Flour 488 and Alex Fluor 546 overlay (K8.1/RTA).

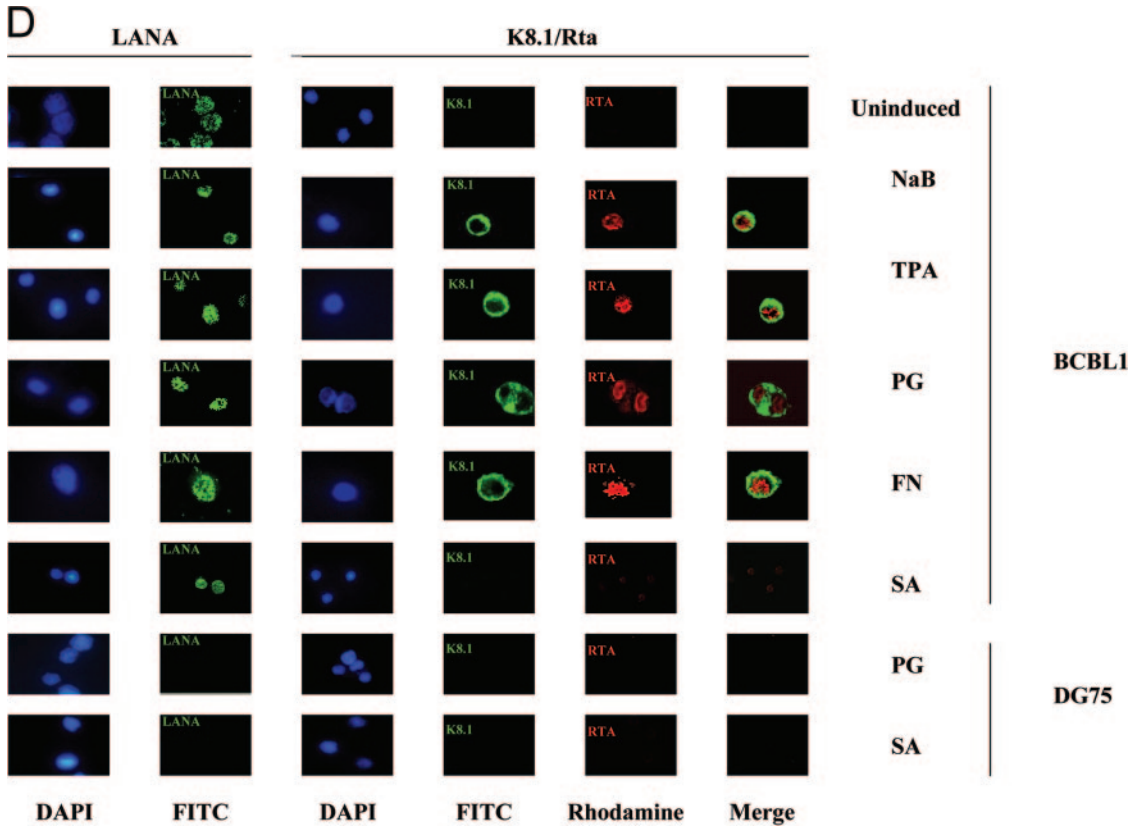

FIG. 1—*Continued*.

specific antibody were followed as instructed by the manufacturer. Immunoreactive bands were visualized with SuperSignal West Pico chemiluminescence substrate (Pierce) according to the manufacturer's instructions. The relative level of expression of each cellular or viral protein was determined by quantitative densitometry. The increase (*n*-fold) over that expressed in the uninduced state was calculated.

IFA. The detection of KSHV antigens in infected cells was performed as previously described (15). Briefly, cells were fixed in 1:1 methanol-acetone, washed in PBS, and blocked overnight at 4°C with 20% normal goat serum in PBS. Cells were incubated overnight at 4°C with primary antibodies diluted in 20% normal goat serum, as follows: a mouse monoclonal antibody against the lytic infection-associated viral glycoprotein K8.1, a rabbit polyclonal antibody against the viral RTA, a rat monoclonal antibody against KSHV LANA, and rabbit polyclonal antibodies against histone 3 or 4 (Upstate Biotechnology, Inc., Charlottesville, VA.). Last, cells were washed, stained with goat anti-mouse secondary antibody conjugated to Alex Fluor 488 (K8.1), goat anti-rat secondary antibody (LANA) conjugated to Alex Fluor 488 or goat anti-rabbit secondary antibody (gB) (acetylated histone 3 or 4) conjugated to Alex Fluor 546 (Molecular Probes, Inc.) for 1 h at an ambient temperature. Slides were washed with PBS, then mounted and coverslipped using Vectoshield containing 4′,6′-diamidino-2-phenylindole (DAPI), and analyzed by confocal microscopy using a Zeiss LSM Pascal microscope.

RESULTS

Anaerobic bacterium spent media induce the KSHV lytic cycle. Because of the increase in viral production in the setting of periodontal disease, we examined the potential for metabolic end products in the bacterial spent media to induce virus production and secretion. In order to quantify the level of viral DNA subsequent to bacterial induction, real-time PCR was employed to amplify the K8.1 region of the viral genome from the culture medium of uninduced control BCBL-1 cells and BCBL-1 cells incubated with the pharmacologic inducer *n*-butyrate or TPA or spent media from *P. gingivalis*, *F. nucleatum*, *P. intermedia*, and *S. mutans*. In replicate experiments, we detected a 4.5- to 6-fold increased production over that of uninduced cells in the amplification of the DNase-resistant K8.1 region of KSHV virion DNA release from cells treated with spent media from *P. gingivalis* and *F. nucleatum* but not medium from *P. intermedia* or *S. mutans* (Fig. 1A). Significant amplification was also seen in cells treated with the pharmacological inducers *n*-butyrate and TPA (positive controls). Each of these experiments was performed in triplicate. Virion production was confirmed by the infection of A293T cells with supernatant-derived virus from BCBL-1 cells treated with the metabolic end products of *P. gingivalis* and *F. nucleatum*. These infections were primarily lytic in nature, simultaneously expressing RTA and K8.1, similar to that of NaB- and TPA-treated cells. LANA was also detected in infected cells but was not detected in mock infection (Fig. 1B). We have also determined by immunoblotting that spent media from *P. gingivalis* and *F. nucleatum* induced expression of the immediate early protein ORF50, the early protein vIL-6, and the late lytic protein K8.1 to levels comparable to that of the known pharmacologic inducers TPA and *n*-butyrate (Fig. 1C.). LANA protein levels remained constant upon induction and were comparable to LANA levels in uninduced and gram-positive medium-treated cells (Fig. 1C). We have also determined, using real-time PCR, that these anaerobic spent media are also

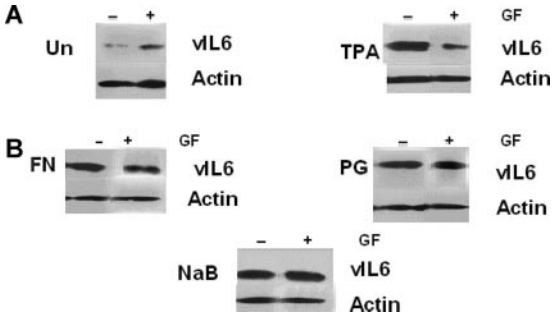

FIG. 2. Effect of PKC inhibition on the induction of early lytic KSHV gene expression. BCBL-1 cells were treated with or without (Un) TPA (positive control) (A) or spent medium from *P. gingivalis* (PG), *F. nucleatum* (FN), or NaB (negative control) (B) and the PKC inhibitor (GFX). The expression of early lytic (vIL-6) as well as the cellular β-actin protein was examined by immunoblot analysis.

capable of EBV lytic induction (data not shown). These results were confirmed by simultaneous immunofluorescent detection of RTA and K8.1 by confocal analysis upon incubation of BCBL-1 cells with spent media from the gram-negative anaerobes *P. gingivalis* and *F. nucleatum* or with the pharmacologic inducer *n*-butyrate or TPA. Spent medium from the gram-positive organism *S. aureus* did not result in expression of K8.1 (Fig. 1D, lanes 3 to 6), although minimal expression of RTA was observed. Accordingly, nuclear expression of LANA was detected irrespective of treatment (Fig. 1D, lanes 1 and 2). No signal was detected from DG75 negative control cells for all treatments. Together, these results suggest that metabolic end products in the spent media from the gram-negative anaerobic bacteria *P. gingivalis* and *F. nucleatum*, but not *P. intermedia*, *S. mutans* (Fig. 1A), or *S. aureus* (Fig. 1B to D), are capable of inducing KSHV viral reactivation, inducing the KSHV temporal cascade and production of infectious virus.

PKC inhibition blocks KSHV induction by TPA, but not by *n*-butyrate or spent media of *P. gingivalis* and *F. nucleatum*. To establish the role of PKC in KSHV lytic reactivation, we investigated the effect of a PKC inhibitor in PEL-derived KSHV-infected BCBL-1 cells by using Western blotting with an antibody specific for vIL-6. Cell cultures were treated with the PKC inhibitor GFX 1 h prior to induction with TPA, *n*-butyrate, or bacterial spent media. The cells were harvested 24 h postinduction. As demonstrated by virion detection, we consistently obtained KSHV lytic reactivation in cultures treated with TPA, *n*-butyrate, *F. nucleatum*, and *P. gingivalis* but not in uninduced cultures in the absence of GFX. Reactivation was confirmed by measuring the level of early lytic protein vIL-6 expression by Western blotting analysis (Fig. 2A and B). When 5 μM GFX, which inhibits the PKC α, β, γ, δ, and ε isoforms (24), was added, virus reactivation was largely inhibited in the TPA-treated culture (Fig. 2A) but not in the cultures treated with *n*-butyrate, *F. nucleatum* or *P. gingivalis* (Fig. 2B). Results are representative of at least two experiments. These results suggest that KSHV reactivation by TPA depends to a large extent on the activity of one or more isoforms of PKC, similar to that observed by Deutsch et. al. (13). On the other hand, the PKC pathway does not appear to be important for *n*-butyrate or bacterial metabolic end-product-mediated viral reactivation.

Inhibitors of p38, but not PI3 kinase pathway, inhibit KSHV lytic reactivation. Previous reports suggested that the PI3 kinase pathway is required for KSHV entry into target cells, while the stress-activated p38 MAPK pathway is minimally involved (34, 48). To determine whether the PI3 kinase and p38 kinase pathways were critical during bacterium-mediated viral reactivation from latency, BCBL-1 cells were preincubated for 1 h with the PI3 kinase inhibitor LY294002 prior to induction with spent medium from *P. gingivalis* or *S. aureus*. Treatment with spent medium from *S. aureus* did not result in activation, while treatment with spent medium from *P. gingivalis* did induce replication (Fig. 3, lanes 1 and 2). β-Actin was used as a loading control. Viral reactivation was measured by detection of early lytic anti-vIL-6 by Western blotting analysis. Results are representative of at least two experiments. Two distinct exposures are shown, each reflecting a similar trend. vIL-6 expression increased and remained largely unchanged in the presence of the PI3 kinase inhibitor (Fig. 3, lane 2). In contrast, the p38 inhibitors SB202190 and PD169316 abolished vIL-6 expression (Fig. 3, lanes 3 and 4). These results clearly demonstrate that the PI3 kinase pathway is not important for induction mediated by metabolic end products of gram-negative anaerobic bacteria. The p38 pathway, however, appears to be critical for expression of the early viral lytic gene and might therefore function as a potential upstream mediator of vIL-6 expression. Spent medium from *S. aureus* failed to increase vIL-6 expression. Accordingly, inhibition of the catalytic activity of the phosphorylated form of p38 kinase precluded viral reactivation, as shown by the decrease in detection of K8.1 expression upon treatment with the p38 inhibitor SB202190. This reduction was most dramatic at 24 h posttreatment (Fig. 3B).

Phosphorylation-specific antibodies were used to examine the phosphorylation state of p38 MAPK upon treatment with bacterial spent media. Figure 3 shows a significant increase in the phosphorylation state upon induction with *P. gingivalis.* Phosphorylation of p38 MAPK was also observed in the presence of the PI3 kinase and p38 inhibitors; however, a significant decrease in the expression of vIL-6 and K8.1 was observed only in the presence of the p38 kinase inhibitors (Fig. 3A, lanes 3 and 4, and Fig. 3B, lanes 3 and 5, respectively). Hence, p38 appears to be phosphorylated, resulting in viral reactivation independent of PI3 kinase activation (Fig. 3A, lane 2). In contrast, inhibition of the catalytic activity of the activated (phosphorylated) form of p38 kinase precluded viral reactivation, as shown by a decrease in the expression of early lytic vIL-6 and late lytic K8.1 (Fig. 3A, lanes 3 and 4, and Fig. 3B, lanes 3 and 5). This is consistent with the known function of the inhibitors SB202190 and PD169316 to allow phosphorylation (activation) but inhibit the catalytic activity of p38 MAPK. Total p38 was consistently detected regardless of treatment.

Spent media from gram-negative anaerobic bacteria inhibit histone deacetylase activity, resulting in hyperacetylation of histones. Several studies have shown that butyrate inhibits histone deacetylase (HDAC) activity and promotes hyperacetylation of histones, leading to the regulation of gene expression (1, 3, 10, 43, 46). Furthermore, activation of both the extracellular signal-regulated kinase (ERK) and the p38 MAPK signaling pathways has been tied to a global increase in H3 acetylation and phosphorylation (28). Inhibition of

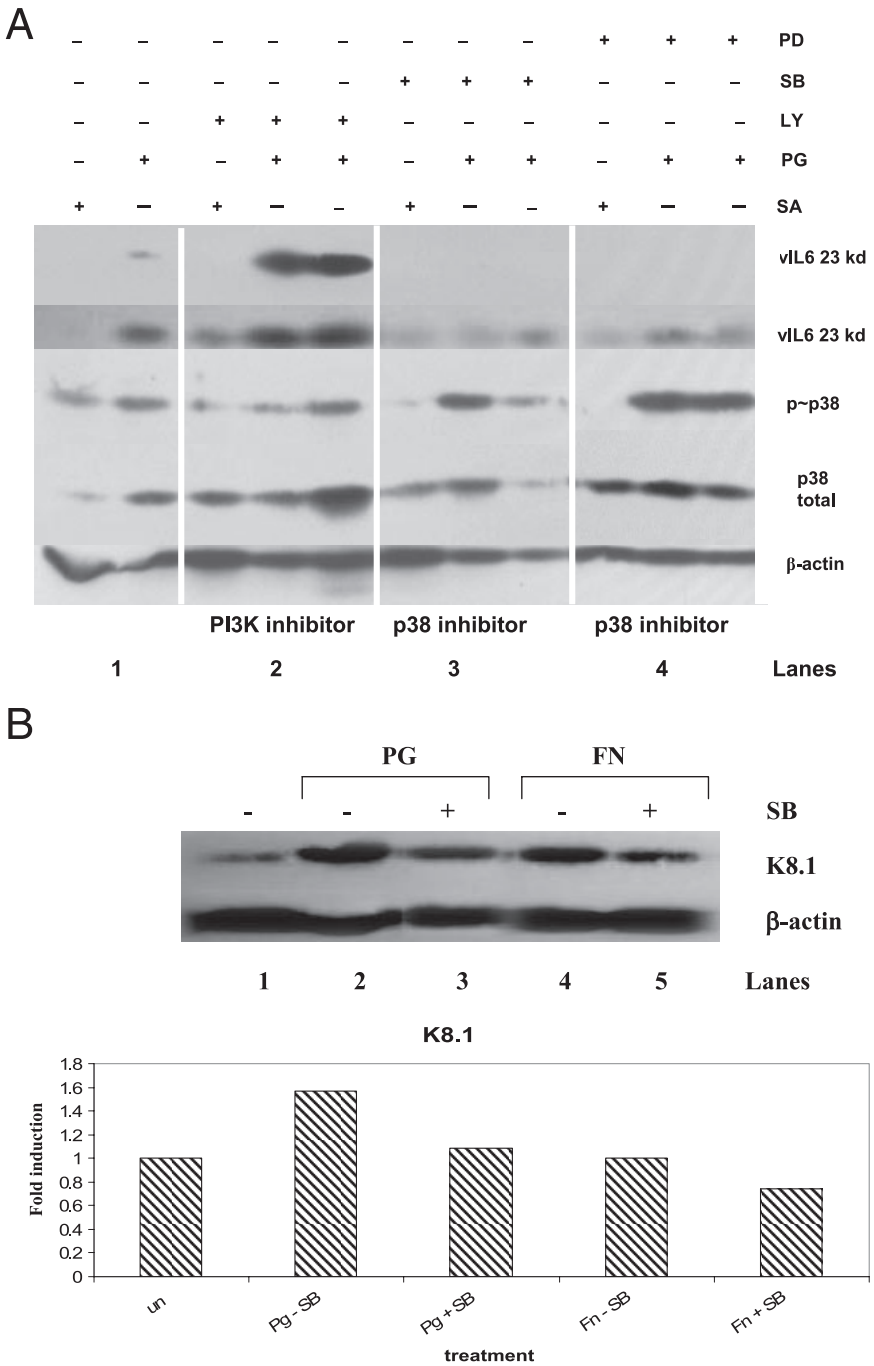

FIG. 3. (A) Effect of p38 and PI3K inhibition on the induction of early lytic KSHV gene expression. BCBL-1 cells were pretreated with the PI3 kinase (LY294002) or p38 (SB202190 and PD169316) inhibitors for 1 h at 37°C and then treated with spent medium from *S. aureus* or *P. gingivalis* for 24 or 48 h. The expression of early lytic (vIL-6), phospho-p38 (p~p38), total p38, and cellular β-actin proteins was examined by immunoblot analysis. PD, PD169316; SB, SB202190; LY, LY294002; PG, *P. gingivalis*; SA, *S. aureus*. (B) Effect of p38 inhibition on the induction of late lytic KSHV gene expression. BCBL-1 cells were pretreated with the p38 inhibitor (SB202190) for 1 h at 37°C and induced with spent media from *P. gingivalis* (PG) and *F. nucleatum* (FN). The expression of late lytic (K8.1) and cellular β-actin proteins was examined by immunoblot analysis using anti-K8.1 and anti-β-actin antibodies.

deacetylation leads to hyperacetylation of histones, and *n*-butyrate has been shown to increase the level of acetylated histones (39). To directly test the effects of the spent media on histone 3 and histone 4 acetylation levels, whole-cell protein extracts from BCBL-1 cells cultured in the presence of spent medium from *P. gingivalis* were examined by Western blotting analysis using antibodies specific for acetylated histone 3 or histone 4. Cells were cultured in the presence of *n*-butyrate, a known promoter of histone 3 hyperacetylation (positive control), TPA, a phorbol ester known to not have HDAC inhibi-

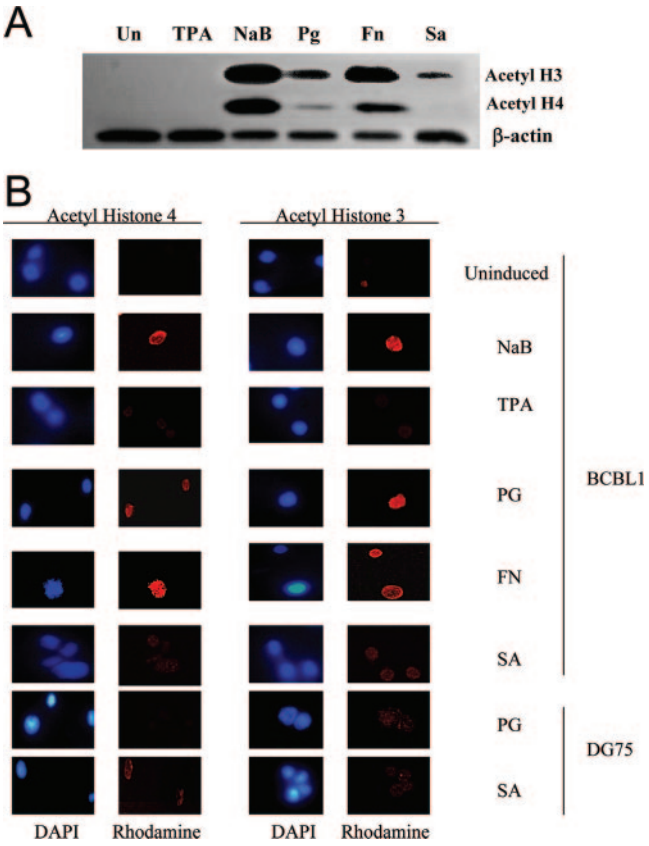

FIG. 4. (A) Hyperacetylation of histones 3 and 4 following induction by bacterial spent media. Nuclear protein was extracted from BCBL-1 cells left untreated (Un) or cultured in the presence of spent medium from *P. gingivalis* (PG) or NaB (positive control) for 24 h and run on an 18% SDS-polyacrylamide gel. Equal amounts of lysate were immunoblotted with anti-acetyl H3 or H4 antibodies. Fn, *F. nucleatum*; Sa, *S. aureus*. (B) Immunofluorescence detection of hyperacetylated histones 3 and 4 following induction by bacterial spent medium. Immunofluorescence confocal microscopy was used to detect acetylated histones 3 and 4 in latently infected KSHV BCBL-1 and uninfected DG75 cells 24 h postinduction with spent medium from gram-negative or -positive bacteria or TPA and *n*-butyrate. Uninduced BCBL-1 cells served as negative controls. Secondary polyclonal antibodies conjugated to Alex Fluor 546 (red) were used to detect the primary antibody bound to the acetylated histones, and DAPI stain was used to detect the cell nuclei. The rhodamine column shows the Alex Fluor 546 channel (acetyl-histone 3 or 4). PG, *P. gingivalis*; FN, *F. nucleatum*; SA, *S. aureus*.

tion activity (negative control), or spent medium from *P. gingivalis*, *F. nucleatum*, or *S. aureus* for 24 h. Equal amounts of lysate were immunoblotted with antibodies specific for acetyl H3 and acetyl H4 (Fig. 4A). Results are representative of at least two experiments. As expected, acetylation of both histone 3 and histone 4 significantly increased when the cells were cultured in the presence of the *P. gingivalis* and *F. nucleatum* spent media and *n*-butyrate (Fig. 4A, lanes 2 and 4). No effect was observed for the uninduced cells or for cells induced with TPA (Fig. 4A, lanes 1 and 3). A much milder effect was detected upon treatment with *S. aureus.* Furthermore, we have demonstrated H3 and H4 hyperacetylation in *P. gingivalis*-, *F. nucleatum*-, and NaB-treated cells by IFA. Acetylated histones 3 and 4 were detected in the nuclei of treated BCBL-1 cells, while those cells treated with TPA, *S. aureus*, or WC broth did not demonstrate expression of hyperacetylated H3 or H4 (Fig. 4B). These results clearly demonstrate the presence of H3 and H4 that is modified by acetylation and is dependent on exposure of the cells to *n*-butyrate, *F. nucleatum*, or *P. gingivalis* metabolic end products. Minimal acetylation was observed for DG75 cells treated with spent medium from *P. gingivalis* or *S. aureus*.

Inhibition of p38 kinase blocks hyperacetylation of histone 3. The determination that p38 kinase phosphorylation is induced by the gram-negative anaerobic bacterial spent media led us to examine the role of p38 kinase in mediating H3 acetylation. BCBL-1 cells were pretreated with the p38 kinase inhibitor SB202190 for 1 h and then induced with spent medium from *P. gingivalis* or *F. nucleatum*. Protein extracts were examined by Western blotting analysis using antibodies specific for acetylated histone 3 and vIL-6. As shown in Fig. 5, lanes 2 and 4, both the hyperacetylation of H3 and the KSHV reactivation (vIL-6 expression) were induced by treatment with the gram-negative spent media from *P. gingivalis* and *F. nucleatum* and blocked by the inhibition of p38 (Fig. 5, lanes 3 and 5). This result implies that activation of the p38 kinase signaling pathway mediates spent-medium-induced hyperacetylation of H3. This correlates well with vIL-6 expression and suggests that p38 kinase activation and H3 acetylation might be crucial upstream events required for viral reactivation. In addition, the presence of hyperacetylated H3 as early as 1 h postinduction (Fig. 6, lane 2) suggests that p38 is activated, resulting in hyperacetylation of H3 prior to de novo expression of early and late viral gene products. Results are representative of at least two experiments.

DISCUSSION

The data presented in this study support our hypothesis that metabolic end products of the gram-negative anaerobic periodontopathogens *F. nucleatum* and *P. gingivalis* can efficiently reactivate KSHV lytic replication using stress-activated pathways, resulting in an increase in the release of virions, similar to that of physiological concentrations (0.3 mM) of the pharmacologic inducer *n*-butyrate. In contrast, *S. aureus* and *S. mutans*, gram-positive organisms with distinctly different end products, did not result in activation of these pathways or in viral reactivation. This hypothesis was plausible since both of these organisms produce high levels of butyric acid (26), a major by-product of anaerobic metabolism, which can be detected in gingival fluid from periodontal pockets (2). Niederman et al. (35) report severely diseased subjects exhibited >10-fold increases in the concentrations of this short-chain fatty acid compared to that of mildly diseased subjects (where the mean severe butyric acid concentration was 2.6 ± 0.4 mM, and the mean mild concentration was 0.2 ± 0.04 mM). Therefore, it is possible that this short-chain fatty acid and others (e.g., propionic acid), in addition to yet-unidentified factors within the crude media, might play a contributory role in the initiation and/or perpetuation of KSHV reactivation and pathogenesis. Furthermore, *P. intermedia*, a gram-negative anaerobe which does not secrete these short-chain fatty acids and has a lipopolysaccharide (LPS) that is structurally similar to that of *P. gingivalis*, does not result in significant reactivation

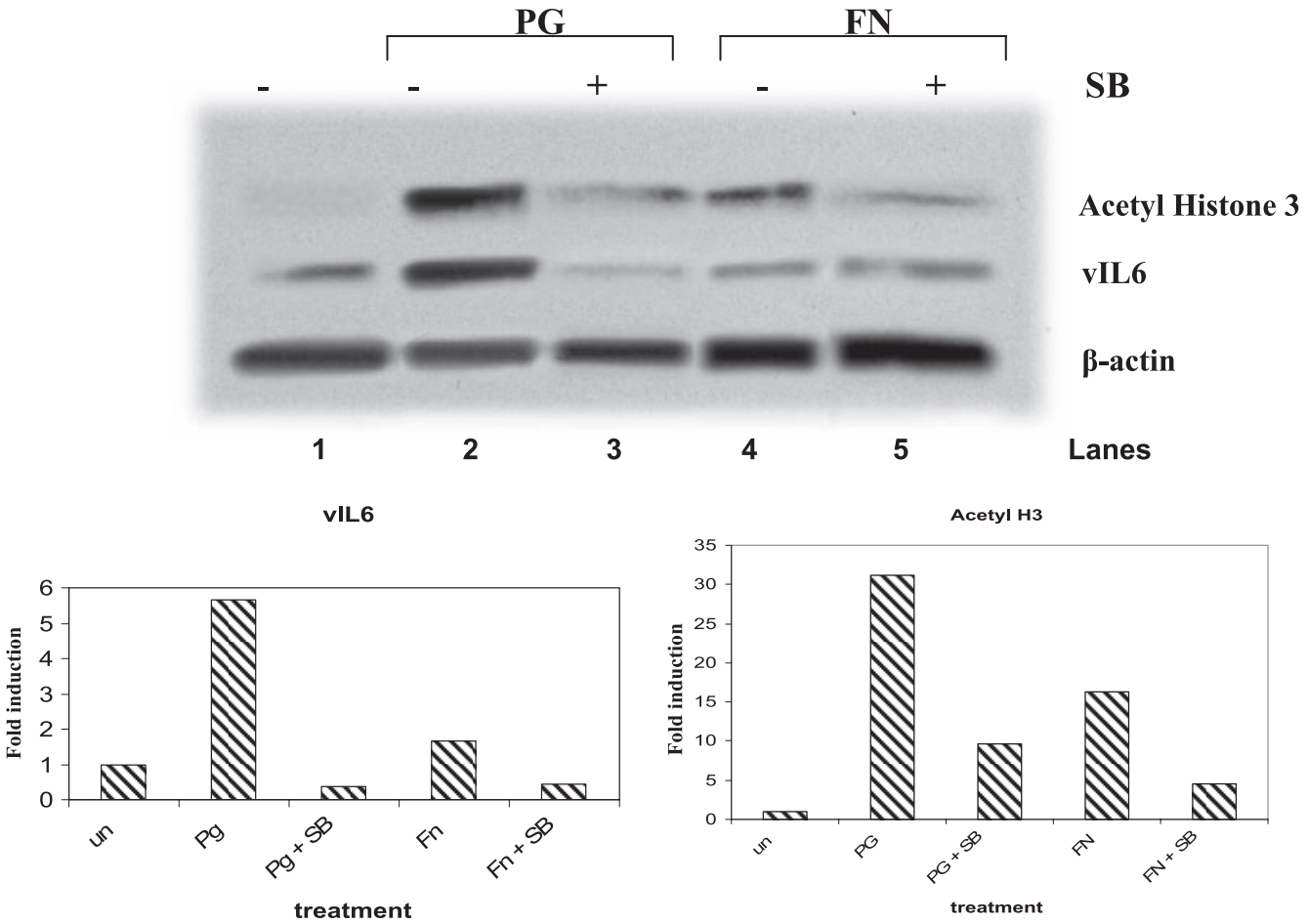

FIG. 5. Effect of p38 inhibition on the hyperacetylation of H3 and induction of late KSHV gene expression. Whole-cell protein was extracted from BCBL-1 cells pretreated with the p38 inhibitor (SB202190) for 1 h at 37°C and induced with spent media from *P. gingivalis* (PG) and *F. nucleatum* (FN). Extracts were run on an 18% SDS-polyacrylamide gel. The expression of early lytic vIL-6, the acetylation status of H3, and the cellular β-actin protein were examined by immunoblot analysis using anti-vIL-6, anti-acetyl H3, and anti-β-actin antibodies, respectively. SB, SB202190.

and productive infection. (Fig. 1A). It may therefore be reasonable to hypothesize that LPS may not be the primary factor responsible for viral reactivation in our system. Despite the impressive array of evidence implicating PKC in the switch between latency and reactivation of the gammaherpesviruses EBV and KSHV (13, 18, 51), our studies have shown that although PKC may mediate KSHV lytic reactivation after TPA stimulation (Fig. 2A), bacterium-induced viral reactivation, like that by *n*-butyrate, proceeds by a PKC-independent mechanism as determined by GFX failure to block lytic-cycle induction. (Fig. 2B). Similar results by Gradoville et al. (20) have been demonstrated for certain latently infected EBV cell lines. They have shown that activation of PKC is not an obligatory event for the switch between latency and lytic-cycle gene expression of EBV in HH514-16, FF41, and W91 cells. In addition, lytic-cycle induction by HDAC inhibitors (e.g., *n*-butyrate and trichostatin A) proceeded by a PKC-independent mechanism in HH514-16 and FF41 cells (20), similar to that shown for BCBL-1 cells infected with KSHV in our studies. It therefore appears that although the TPA-induced PKC signaling pathway might mediate viral reactivation, a separate signaling pathway independent of PKC activation is primarily used for bacterium-induced reactivation and histone deacetylase inhibitors. Finally, Yu et al. (49) have demonstrated that *n*-butyrate is much more effective than the phorbol ester TPA in inducing high levels of KSHV class II and III virus transcription and viral DNA replication. This would suggest that although the gram-negative anaerobes also produce LPS, which acts as a PKC activator, activation of an alternate pathway induced by

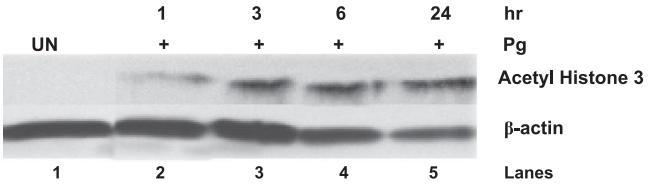

FIG. 6. Effect of *P. gingivalis* (Pg) on H3 acetylation. BCBL-1 cells were induced with spent medium from *P. gingivalis*. Cellular lysate was collected at the times indicated, and the acetylation status of H3, as well as the cellular β-actin protein, was examined by immunoblot analysis using anti-acetyl H3 and anti-β-actin antibodies. UN, untreated.

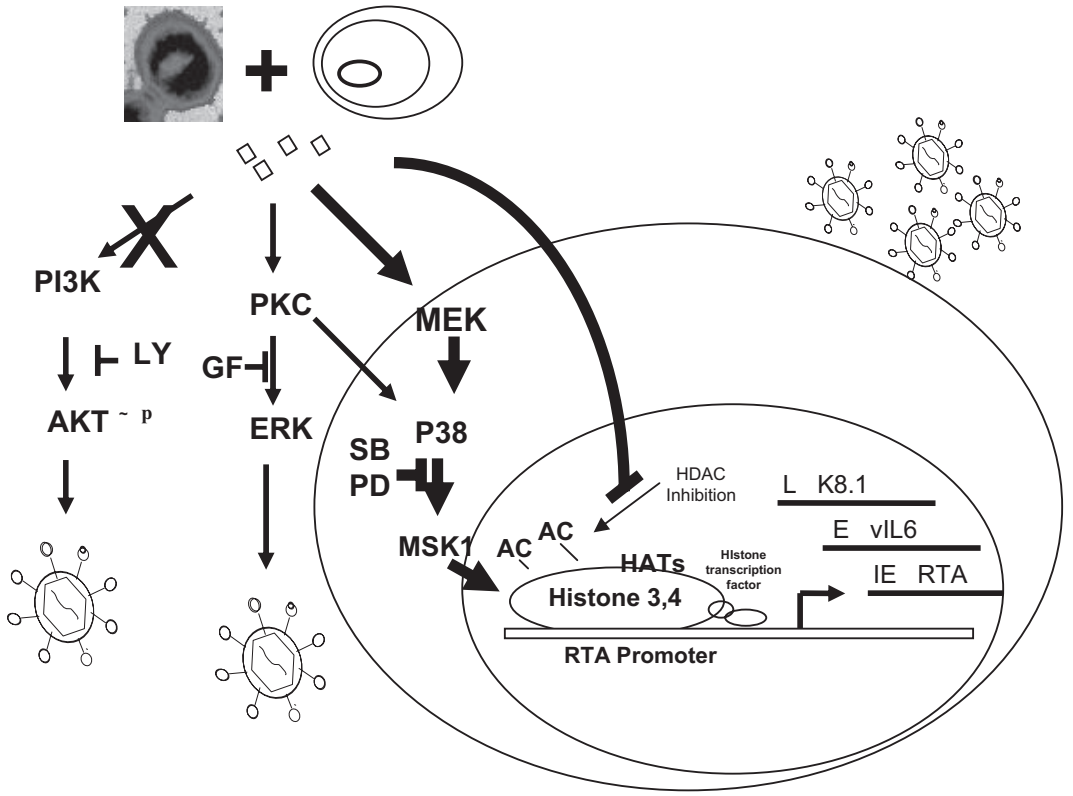

FIG. 7. Model for induction of KSHV reactivation by metabolic end products from gram-negative bacteria, which contain high levels of butyric acid, inhibit cellular HDACs, and activate the p38 kinase pathway. Activation of the p38 pathway may lead to phosphorylation of cellular histones, which results in increased sensitivity to acetylation by histone acetyltransferases. The combined effects of HDAC inhibition and increased acetylation sensitivity result in hyperacetylation of the histones on immediate early viral promoters. The acetylation event neutralizes the positive charge of the histone tail and remodels chromatin structure, making the nucleosome accessible for binding to transcription factors and activation of the temporal cascade of viral gene expression and subsequent viral production. Viral genes are denoted as E (early), IE (immediate early), and L (late). MEK, mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase; SB, SB202190; PD, PD169316.

n-butyrate and the spent media overrides the LPS-induced PKC pathway. One possible explanation for this observation might be that the KSHV K1 protein, the expression of which is significantly induced during the lytic phase of the viral life cycle, suppresses TPA-mediated (and therefore, LPS-mediated) KSHV reactivation (27). Alternatively, Jung et al. (23) have shown that HDAC inhibitors can prevent TPA-induced responses via modulation of MAP kinases; therefore, it is possible that bacterial metabolites/*n*-butyrate could suppress the TPA-induced response in our system by a similar mechanism. Activation of PKC, however, may play a yet-unexplored secondary role.

To explore other candidate signaling pathways potentially activated during viral reactivation by the bacterial spent media, specific inhibitors of the PI3 and p38 kinase pathways were used. Our data showed that lytic reactivation was blocked in the presence of the p38 kinase inhibitors yet persisted in the presence of the PI3 kinase inhibitor (Fig. 3). Interestingly, upon bacterium-induced reactivation, Akt phosphorylation also increased over 48 h, even in the presence of the PI3 kinase inhibitor (data not shown). It is possible that Akt is phosphorylated in a PI3 kinase-independent manner. Activation of Ca^{2+}/calmodulin-dependent protein kinases (36) or an as-yet-unidentified cellular or viral kinase may result in this modification upon bacterium-induced reactivation.

Activation of p38 MAPK signaling pathways results in a global increase in coupled histone H3 phosphorylation (Ser-10) and acetylation (Lys-14) (H3 phosphoacetylation) and H4 acetylation via MSK1 (28). Studies have shown that HDAC inhibitors can induce phosphorylation of H3, thus facilitating acetylation (1, 3, 39, 43, 46), and this phosphorylation can be blocked by p38 inhibition (50). Observations by Lu et al. (29) have demonstrated that the HDAC inhibitor *n*-butyrate induces H3 and H4 hyperacetylation at ORF50, encoding the immediate early protein KSHV RTA, resulting in the initiation of KSHV lytic-cycle replication. Similar studies have shown that HDAC-induced acetylation of histones activates the transcription of the Epstein-Barr virus lytic gene BRLF1 (6).

P. gingivalis and *F. nucleatum*, two common oral pathogens associated with human periodontitis (44) and potent activators of viral reactivation, release the short-chain fatty acid (butyrate) as one of the major by-products into the microenvironment (19), along with other volatile fatty acids (25). Moreover, histone hyperacetylation in T cells has been reported following the exposure of T cells to culture supernatants of *P. gingivalis* (22). In the present work, we provide evidence that culture

supernatants from *P. gingivalis and F. nucleatum* inhibit HDACs in a fashion similar to butyrate and other histone deacetylase inhibitors and that this process is associated with an increase in the levels of histone 3 and 4 acetylation (Fig. 4, 5, and 6). Consistent with the notion that the pathway utilized for TPA-induced viral reactivation is distinct from that used for anaerobic spent media and *n*-butyrate-induced reactivation, TPA induces KSHV reactivation through PKC (Fig. 2A), which did not result in acetylation of either histone. (Fig. 4). These results imply that upon bacterium-mediated viral induction, modification of cellular histones 3 and 4 occurs as a result of HDAC inhibition. Bacterium-induced H3 acetylation is an early event, detectable after 1 h posttreatment with spent media in significant advance of de novo viral protein synthesis (Fig. 6). It would therefore seem reasonable to suggest that acetylation of H3 and/or H4 induced by culture supernatants from *P. gingivalis* and *F. nucleatum* might potentially play a critical role in chromatin remodeling and subsequent regulation of cellular and/or viral gene expression.

Our current study has shown that spent-medium-induced hyperacetylation of H3 and viral reactivation are mediated by p38 kinase. p38 kinase phosphorylation and H3 acetylation are induced by gram-negative anaerobic bacterial spent media and are correlated with viral reactivation. p38 pathway usage was verified by using p38-specific inhibitors prior to induction. In the presence of the inhibitors, H3 acetylation was significantly diminished following induction by the gram-negative anaerobic bacterial spent media as well as viral reactivation.

In summary, we have identified a novel mechanism for KSHV reactivation and important signaling pathways triggered during the reactivation process (Fig. 7). We have shown for the first time that p38 is important to KSHV reactivation and that p38 kinase activation and H3 acetylation might be crucial upstream events for bacterium-induced reactivation of lytic replication. Bacterium-virus interactions in the oral cavity, gut, and genitourinary tract might be key to viral reactivation in these settings. Deciphering the mechanism of bacterium-induced viral reactivation has important implications for developing effective therapeutic measures to control these polymicrobial infections in vivo.

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