Piracy of Prostaglandin E2/EP Receptor–Mediated Signaling by Kaposi's Sarcoma-Associated Herpes Virus (HHV-8) for Latency Gene Expression: Strategy of a Successful Pathogen

Arun George Paul¹, Neelam Sharma-Walia¹, Nagaraj Kerur¹, Carl White², and Bala Chandran¹

Abstract

Kaposi's sarcoma-associated herpes virus (KSHV; HHV-8) is implicated in the pathogenesis of KS, a chronic inflammation-associated malignancy. Cyclooxygenase-2 (COX-2) and its metabolite prostaglandin E2 (PGE2), two pivotal proinflammatory/oncogeneic molecules, are proposed to play roles in the expression of major KSHV latency-associated nuclear antigen-1 (LANA-1). Microsomal PGE2 synthase, PGE2, and its receptors (EP1, EP2, EP3, and EP4) were detected in KS lesions with the distinct staining of EP2/EP4 in KS lesions. In latently infected endothelial TIVE-LTC cells, EP receptor antagonists downregulated LANA-1 expression as well as Ca²⁺, p-Src, p-Pi3K, p-PKCζ/λ, and p-NF-κB, which are also some of the signal molecules proposed to be important in KS pathogenesis. Exogenous PGE2 and EP receptor agonists induced the LANA-1 promoter in 293 cells, and YY1, Sp1, Oct-1, Oct-6, C/EBP, and c-Jun transcription factors seem to be involved in this induction. PGE2/EP receptor-induced LANA-1 promoter activity was downregulated significantly by the inhibition of Ca²⁺, p-Src, p-Pi3K, p-PKCζ/λ, and p-NF-κB. These findings implicate the inflammatory PGE2/EP receptors and the associated signal molecules in herpes virus latency and uncover a novel paradigm that shows the evolution of KSHV genome plasticity to use inflammatory response for its survival advantage of maintaining latent gene expression. These data also suggest that potential use of anti-COX-2 and anti-EP receptor therapy may not only ameliorate the chronic inflammation associated with KS but could also lead to elimination of the KSHV latent infection and the associated KS lesions. Cancer Res; 70(9); OF1–12. ©2010 AACR.

Introduction

Kaposi’s sarcoma-associated herpes virus (KSHV; HHV-8) is etiologically associated with KS, the most common and aggressive AIDS-defining malignancy (1–3). KS is characterized by a proinflammatory microenvironment (1–3). Thus, unraveling the biology of KSHV pathogenesis and, therefore, KS is closely tied to understanding the chronic inflammatory conditions that set the stage for KS. Previous reports had shown that cyclooxygenase-2 (COX-2), a proinflammatory molecule, was highly upregulated in vitro by KSHV infection (4, 5). The tumorigenic properties of COX-2 are attributed to its metabolite prostaglandin E2 (PGE2) that exerts its effect through eicosonoid (EP) receptors (EP1–EP4; refs. 6–12). COX-2 inhibition significantly abrogated expression of the major KSHV latent gene latency-associated nuclear antigen-1 (LANA-1) during de novo KSHV infection of fibroblast (HFF) and endothelial (HMVEC-d) cells, and exogenous PGE2 reversed this downregulation (5). These studies have indicated that COX-2/PGE2–mediated inflammation is crucial for KSHV latency program. Although, the role of COX-2 and PGE2 in herpes viral lytic cycle is shown, their role in viral latency has been observed only in KSHV.

However, the mechanistic aspects of how COX-2/PGE2 mediates KSHV latent gene expression is not known and the role of EP receptors is unexplored in herpes virus biology. Our study shows that Ca²⁺, Src, phosphoinositide 3-kinase (PI3K), PKCζ/λ, and NF-κB signal molecules are regulated by EP receptors in latently infected cells and blocking EP receptors downregulated LANA-1 and COX-2 gene expression. PGE2 stimulated the LANA-1 promoter via a network of Ca²⁺, Src, PI3K, PKCζ/λ, and NF-κB activation. Collectively, these studies show that KSHV uses the host proinflammatory COX-2/PGE2/EP receptor pathway for its
own advantage of establishing and maintaining latent gene expression.

Materials and Methods

Cells and KSHV. TIVE-LTC (long term–infected telomerase immortalized umbilical vein endothelial cells) TIVE cells, a gift from Dr. Rolf Renne (University of Florida), and 293 cells were cultured as described before (13). KSHV was prepared and assessed for its infectivity. Mycoplasma, and lipopolysaccharide, as described before (5).

Plasmids. LANA-1 promoter sequence (pGL3.6 or p-LANA-1-Luc) and the LANA-1 promoter deletion sequences (pGL3.4, pGL3.3, pGL3.2, and pGL3.1) cloned in pGL3.0 vector (Promega) with the reporter gene Firefly luciferase were gifts from Dr. Yuan Chang, University of Pittsburgh (14).

Reagents. Akt 1/2 inhibitor, TMB-8, PD98059, wortmannin, Ly290042, U0126, and LPA were from Sigma. GFX, GO:6976, PP2, and Bayl17-0855 were from Calbiochem. PGE2, EP1-4 agonists, AHE6809, and GW627368X were from Cayman Chemical. Fura-2AM was from Invitrogen. SC-51322 was from Enzo Life Sciences.

Antibodies. Anti-mouse (COX-1 and COX-2) antibodies as well as anti-rabbit [microsomal PGE2 synthase (mPGES), EP1, EP2, EP3, and EP4] antibodies were from Cayman Chemicals. Anti-mouse (PI3K, α-tubulin, and p-SrC) antibodies were from BD Biosciences, Sigma, and Calbiochem, respectively. Anti-rabbit (p-NF-αB, p-Akt, and p-ERK 1/2) and antirabbit (Akt, Src, NF-αB p65, p-PKCζ/λ, and p-PI3K) antibodies were from Cell Signaling Technology, Inc. Anti-rabbit PGE2 was from Abcam. Anti-rabbit [PKCζ and extracellular signal–regulated kinase 2 (ERK2)] antibodies were from Santa Cruz Biotechnology, Inc. Production and characterization of antibodies against full-length KSHV LANA-1 protein have been described before (15).

Transfection and luciferase reporter assay. Transfections on 293 cells were conducted as described before (5). The luciferase assays were conducted as per the manufacturer’s guidelines (Promega). The relative LANA-1 promoter activity or relative luciferase units (RLU) were normalized to Renilla luciferase protein levels.

Fluorescence-activated cell sorting. Samples for fluorescence-activated cell sorting (FACS) analysis were prepared as per manufacturer’s guidelines (BD Biosciences). The data were collected using FACSCalibur flow cytometer (Becton Dickinson) and analyzed with CellQuest Pro software (Becton Dickinson) at the RFUMS flow cytometry core facility.

Western blotting and measurement of PGE2. Total cell lysates prepared from cells after respective treatments were used for Western blotting and quantified as described before (5). α-Tubulin was used as the loading control for all the blots. Secreted amounts of PGE2 were measured using a PGE2 enzyme-linked immunosorbent assay (ELISA) kit as per the manufacturer’s guidelines (R&D Systems).

Real-time reverse transcription–PCR. LANA-1, COX-2, and COX-1 transcripts were detected by real-time reverse transcription–PCR (RT-PCR) as described before (5).

Confocal microscopy and immunohistochemistry. Confluent TIVE and TIVE-LTC cells were used for confocal microscopy using EP1-4 antibodies as before (13). Tissue sections from three healthy subjects and three KS+ patients were obtained from AIDS and Cancer Specimen Resource, National Cancer Institute. Immunohistochemistry in Fig. 1 was done by similar method as described before (13).

Measurement of Ca²⁺. 293 or TIVE-LTC cells plated onto coverslips and placed in six-well plates were mounted on the stage of an inverted microscope (Olympus IX71) and incubated with 5 μmol/L of Ca²⁺ indicator fura-2AM (Invitrogen) for 30 minutes at 37°C in HBSS (Sigma; pH 7.4). Cells were then continuously perfused with Ca²⁺–free HBSS (pH 7.4). When cytoplasmic Ca²⁺ levels increase after respective treatments, if any, Ca²⁺ will bind to fura-2AM in the cytoplasm, inducing a fluorescence signal that can be used as a measure of the level of cytoplasmic calcium. The calcium-induced fluorescence signal of fura-2AM was measured by alternatively exciting at 340 and 380 nm and collecting the emitted fluorescence at 510 nm using a CCD-based imaging system running SimplePCI software (Hamamatsu Corporation). Changes in cytoplasmic Ca²⁺ ([Ca²⁺]ᵢ) are reported as the ratio of 340/380 emissions. Data analysis was performed using Origin Pro software (Origin Lab Corporation). The Ca²⁺ studies were conducted at RFUMS Ca²⁺ imaging facility.

Statistical analysis. In Fig. 2, the statistical significance (t test) was conducted with respect to untreated or uninfected cells. In Fig. 3, the statistical significance (t test) was conducted with respect to supratumoral treatment. In Fig. 4, the statistical significance (t test) was conducted similar to Fig. 2. In Fig. 5, the statistical significance (t test) was calculated with respect to PGE2 alone treatment. In Fig. 6, the statistical significance (t test) was conducted similar to Fig. 2 (*, P < 0.01; **, P < 0.001; ***, P < 0.0001).

Results

PGE2, mPGES, and EP1-4 receptors are detected in KS lesions. We used immunohistochemistry to first investigate the presence of mPGES, PGE2, and EP1-4 in serial sections of biopsies from three healthy subjects and three KS+ patients. The presence of KSHV in KS lesions was confirmed by the detection of characteristic nuclear staining of LANA-1 (Fig. 1A, b). Strong cytoplasmic mPGES, PGE2, and EP1-4 reactivities were detected in KS lesions (Fig. 1B–D). However, KS lesion samples exhibited distinct staining for EP2 and EP4 receptors compared with normal samples (Fig. 1C, b and D, b). Collectively, these results for the first time show the presence of inflammation-associated EP receptors in KS lesions.

KSHV infection upregulates EP1, EP3, and EP4 receptors and downregulates EP2 receptors. We next examined the role of EP receptors in maintaining latent gene expression in TIVE-LTC cells, which sustains expression of latency genes (16). In a separate study, we have observed the upregulation of COX-2 and mPGES proteins and PGE2 secretion in TIVE-LTC cells compared with control TIVE cells and downregulation of LANA-1 expression in TIVE-LTC cells treated with COX-2 inhibitor NS-398 (13). Western blot analysis shows...
that, compared with TIVE cells, EP1, EP3, and EP4 receptors were significantly upregulated in TIVE-LTC cells, whereas EP2 receptor was downregulated in TIVE-LTC cells (Fig. 2A, a–d). Confocal microscopy also confirmed the presence and cellular localization of all four EP receptors in TIVE and TIVE-LTC cells (Fig. 2B).

*KSHV uses EP receptors to maintain LANA-1 and COX-2 gene expression and PGE2 secretion.* We measured LANA-1, COX-2, and COX-1 gene expression in TIVE-LTC cells treated with noncytotoxic concentrations (Supplementary Fig. S1A–I) of well-characterized competitive EP receptor blockers SC-51322 (EP1 antagonist; 50 μmol/L), AH6809 (EP2 antagonist;
50 μmol/L, or GW 627368X (EP4 antagonist; 5 μmol/L) at 2, 8, and 24 hours posttreatment and observed no significant change in COX-1 gene expression (Fig. 2C, b). COX-1 gene expression was conducted as a control, because COX-1 promoter is constitutively active. EP1, EP2, and EP4 antagonists downregulated LANA-1 gene expression significantly by 69%, 82%, and 73% and by 55%, 64%, and 40% at 8 and 24 hours posttreatments, respectively (Fig. 2C, a). COX-2 gene expression was downregulated by EP2 and EP4 antagonists at 2 hours posttreatment and by EP4 antagonist at 8 hours posttreatment (Fig. 2C, c). At 2 hours posttreatment, EP2 and EP4 antagonists downregulated PGE2 secretion significantly with no significant changes at 8 and 24 hours posttreatments (Fig. 2D).

KSHV uses EP1 receptor–mediated cytoplasmic Ca²⁺ ([Ca²⁺]i) signaling to maintain LANA-1 gene expression. From the above results showing the downregulation of
LANA-1 gene expression by EP1 blockade (Fig. 2C, a) together with the fact that EP1 receptor is a well-characterized Ca\(^{2+}\)-inducing GPCR (10), we hypothesized that PGE2 secreted in the supernatant of TIVE-LTC cells might be inducing Ca\(^{2+}\) signaling via the EP1 receptor. Therefore, we predicted that, if we block the EP1 receptor, the potential of the PGE2 in the supernatant to induce Ca\(^{2+}\) signaling via the EP1 receptor, if any, would also be blocked. The purpose of our

Figure 3. Effect of EP receptor antagonists on Ca\(^{2+}\), Src, PI3K, PKC\(\zeta/\lambda\), Akt, NF-\(\kappa\)B, and ERK 1/2 in TIVE-LTC cells. A, schematic of the experimental design for Ca\(^{2+}\) experiments. B, TIVE-LTC cells were grown on coverslips placed in six-well plates and serum starved for 48 hours. The cells were washed and treated with fresh serum-free media for 24 hours. Coverslip 1 was removed and loaded with fura-2AM (5 \(\mu\)mol/L) with or without different EP receptor antagonists. The cells were then washed and incubated with serum-free media (a) or with the supernatant produced by coverslip 2 TIVE-LTC cells with or without EP1, EP2, and EP4 antagonists or DMSO (b). Transient elevation in [Ca\(^{2+}\)]\(_i\) was measured for 35 minutes. For each graph, five representative TIVE-LTC cells were selected to portray the transient [Ca\(^{2+}\)]\(_i\) signal. d, summary results showing the peak amplitude (mean ± SEM) of transient elevation in [Ca\(^{2+}\)]\(_i\) observed in different treatments. \(n\) refers to the number of cells that showed a change in [Ca\(^{2+}\)]\(_i\) signal within the field studied. C and D, TIVE-LTC cells were serum starved for 48 hours and treated with EP1, EP2, and EP4 antagonists or DMSO. At 8 and 24 hours posttreatment, total cell lysates were prepared, immunoblotted for p-Src (C, a), p-PI3K (C, b), p-PKC\(\zeta/\lambda\) (C, c), p-Akt (D, a), p-NF-\(\kappa\)B (D, b), and p-ERK 1/2 (D, c), and normalized with respect to the levels of total protein levels. The fold change was calculated with respect to the signal intensity for untreated (UN) cells for each time point.
The experiment was not to test the effect of downregulating PGE2 secretion in the supernatant by EP receptor antagonists and consequently the calcium signal because, even at 2 hours posttreatment with EP2 and EP4 antagonists, PGE2 is present within the range of 80 to 100 pg/mL (Fig. 2D). However, our goal was to investigate the effect of blocking EP receptors on the supernatant-induced calcium signal, if any. By doing so, we are answering the role...
of PGE2 in the supernatant in inducing calcium signal through EP receptors.

To test our hypothesis, TIVE-LTC cells were grown on coverslips and [Ca\textsuperscript{2+}]i was measured as outlined in Fig. 3A. To measure [Ca\textsuperscript{2+}]i, coverslip 1 was removed and loaded with the Ca\textsuperscript{2+} indicator fura-2AM for 30 minutes with and without EP receptor antagonists in Hanks solution. However, whereas being loaded with fura-2AM, the cells in coverslip 1 are not exposed to the physiologic supernatant of TIVE-LTC cells. Therefore, to test whether the supernatant of TIVE-LTC can induce Ca\textsuperscript{2+} signaling in coverslip 1 cells, we used the supernatant produced by cells grown in parallel on coverslip

![Image]

**Figure 5.** Effect of Ca\textsuperscript{2+}, p-Src, p-Pi3K, p-PKC\(\zeta/\lambda\), p-Akt 1/2, p-NF-\(\kappa\)B, and p-ERK 1/2 inhibition on PGE2-mediated LANA-1 promoter activity. A and B, 293 cells transfected with p-LANA-1-Luc was pretreated with indicated pharmacologic signal inhibitors for 2 hours and supplemented with 10 \(\mu\)mol/L PGE2 for 4 hours or left untreated and assayed for RLU. The fold change was calculated with respect to untreated cells. C, 293 cells serum starved for 24 hours were loaded with fura-2AM, washed by perfusion, and treated with 10 \(\mu\)mol/L PGE2 or HBSS (pH 7.4), and the [Ca\textsuperscript{2+}] levels were measured for 25 minutes. Representative cells from a field of \(\sim\)50 to 60 cells. D, 293 cells serum starved for 24 hours were treated with 100 ng of LPA and 10 \(\mu\)mol/L PGE2, infected with 20 DNA copies per cell of KSHV, or left untreated (UN) for 4 hours. Total cell lysate was prepared, immunoblotted for p-Src, p-Pi3K, p-PKC\(\zeta/\lambda\), Akt 1/2, NF-\(\kappa\)B, and ERK 1/2, and normalized with respect to the levels of total protein levels, and the fold change was calculated with respect to (UN) cells for each time point.
2. The supernatant of coverslip 2 TIVE-LTC cells incubated in the presence of DMSO (Fig. 3B, b) induced a significant transient elevation in \([\text{Ca}^{2+}]_i\). We did not observe any significant change between the observed peak amplitude of transient elevation in \([\text{Ca}^{2+}]_i\), induced by the supernatant alone or with solvent control (DMSO; Fig. 3B, d). In contrast, serum-free medium used as a negative control had no significant effect on \([\text{Ca}^{2+}]_i\) (Fig. 3B, a), indicating that factors

Figure 6. PGE2-responsive regions of LANA-1 promoter. A, PGE2-responsive TF binding sites on p-LANA-1-Luc. The LANA-1 promoter sequence (14) was analyzed by Alibaba 2.1 software. The schematic shows the different TFs predicted by Alibaba 2.1 software, the TF binding sites predicted by ref. 14, and by both. The numbers for each TF refer to the beginning and end sequences of the binding site within the 774-bp LANA-1 promoter sequence studied, with 128,674 bp and 127,900 bp as the start and end sites of the LANA-1 promoter, respectively, in the KSHV genome. The site of origin of each LANA-1 promoter deletion construct is marked. B, identification of PGE2-responsive regions of LANA-1 promoter. Sequential series of LANA-1 promoter deletion constructs with luciferase reporter or empty vector (pGL3.0) were transfected into 293 cells and treated with 10 \(\mu\)mol/L PGE2 for 4 hours or left untreated. The cells were harvested, lysed, and assayed for RLU. The fold change and corresponding statistics (t test) were calculated with respect to untreated cells for each time point. C, schematic model depicting the potential mechanism by which PGE2 and EP receptor play roles in KSHV pathogenesis. During latency, KSHV induces proinflammatory COX-2 gene expression and PGE2 secretion (5), resulting in the activation of EP receptors in an autocrine and paracrine fashion. Activated EP receptors influence specific signal cascades that in turn influence specific TFs converging on the LANA-1 promoter. EP2-mediated and EP4-mediated signaling might also be involved in the regulation of this process by initiating a positive feedback loop to maintain COX-2 gene expression and therefore PGE2 synthesis/secretion resulting in the sustenance of the characteristic chronic proinflammatory environment created by KSHV infection.
present in the physiologic supernatant of TIVE-LTC cells can induce Ca\(^{2+}\) signaling.

Next, we examined whether the transient [Ca\(^{2+}\)], induced by TIVE-LTC cell supernatant is due to activation of EP1 receptor by the supernatant PGE2. Therefore, we treated the cells on coverslip 1 with EP1, EP2, or EP4 antagonist while being treated with fura-2-AM and the supernatant from coverslip 2 cells, as outlined in Fig. 3A. Treatments with EP1 but not EP2 or EP4 antagonists significantly abolished the supernatant-mediated Ca\(^{2+}\) signal in TIVE-LTC cells (Fig. 3B, c and d). These observations clearly showed that PGE2 present in the supernatant of TIVE-LTC cells could induce a Ca\(^{2+}\) signal through the EP1 receptor.

**Inhibition of EP receptors downregulates p-Src, p-PI3K, p-PKC\(\zeta/\lambda\), p-Akt, and p-NF-\(\kappa\)B and upregulates p-ERK in KSHV-infected TIVE-LTC cells.** We next examined the signal cascades regulated by EP receptors. TIVE-LTC cells serum starved for 48 hours were treated with EP1, EP2, and EP4 antagonists or DMSO. EP2 and EP4 antagonists downregulated p-Src by 17% and 47%, and 29% and 75% at 8 and 24 hours posttreatments, respectively (Fig. 3C, a). At 24 hours posttreatment, EP1, EP2, and EP4 antagonists downregulated PI3K phosphorylation by 23%, 33%, and 34%, respectively, with no effect at 8 hours posttreatment (Fig. 3C, b). EP2 and EP4 antagonists downregulated p-PKC\(\zeta/\lambda\) by 12% and 20%, and 23% and 69% at 8 and 24 hours posttreatments, respectively (Fig. 3C, c). Compared with DMSO treatment, we did not observe any changes on p-Akt by EP receptor antagonists at 8 and 24 hours posttreatment (Fig. 3D, a). At 8 hours posttreatment, p-NF-\(\kappa\)B was downregulated by 39%, 46%, and 66% with EP1, EP2, and EP4 antagonists, respectively, and by 41% with EP2 antagonist 24 hours posttreatment (Fig. 3D, b). With EP4 antagonist, p-ERK 1 and p-ERK 2 were upregulated by 1.3-fold and 2.0-fold, and 1.2-fold and 2.0-fold at 8 and 24 hours posttreatment, respectively (Fig. 3D, e).

**KSHV infection and exogenous PGE2 activates the LANA-1 promoter.** Based on our data from TIVE-LTC cells, we hypothesized that PGE2-mediated signaling can upregulate LANA-1 promoter activity. To test this, 293 cells were transfected with a luciferase reporter gene under the control of 774-bp LANA-1 promoter (p-LANA-1-Luc; Fig. 4A, a; ref. 14). The efficacy of our system was first shown by the induction of p-LANA-1-Luc activity by primary KSHV infection, whereas less induction of p-LANA-1-Luc activity was observed with entry of incompetent heparin-treated and UV-inactivated virus (Fig. 4A, b).

To determine the effect of exogenous PGE2 on p-LANA-1-Luc, we first confirmed the presence of EP1-4 receptors by FACS (Fig. 4A, c). Exogenous PGE2 (10 \(\mu\)mol/L) induced p-LANA-1-Luc activity by 8.7-fold at 4 hours posttreatment with no significant effect on the empty vector (Fig. 4B, a). Treatments with varying concentrations of PGE2 show that 10 \(\mu\)mol/L of PGE2 or more were necessary to activate p-LANA-1-Luc significantly with no significant difference between the effects of 10 and 100 \(\mu\)mol/L of PGE2 (Fig. 4B, b and c). Well-characterized agonists for EP1-4 receptors were also able to induce p-LANA-1-Luc activity significantly at 4 hours but not at 24 hours posttreatment (Fig. 4B, c).

**De novo KSHV infection of 293 cells induces the COX-2/PGE2 pathway.** To validate whether the 293 cells used above were ideal to study the paradigm, we showed that de novo KSHV infection of 293 cells induces COX-2 and mPGES proteins (Fig. 4C, a), PGE2 secretion (Fig. 4C, b), and LANA-1 expression (Fig. 4D, a). To determine whether the COX-2/PGE2 pathway is important for maintaining LANA-1 gene expression in 293 cells, we showed that 10 \(\mu\)mol/L PGE2 can restore the reduction in LANA-1 expression caused by COX-2–specific inhibitor NS-398 (100 \(\mu\)mol/L; Fig. 4D, b).

**Inhibition of Ca\(^{2+}\), p-Src, p-PI3K, p-PKC\(\zeta/\lambda\), p-Akt, and p-NF-\(\kappa\)B, and p-ERK 1/2 blocks PGE2-mediated LANA-1 promoter.** We next examined the role of Ca\(^{2+}\), Src, PI3K, PKC\(\zeta/\lambda\), Akt 1/2, NF-\(\kappa\)B, and ERK 1/2 in PGE2-mediated LANA-1 transcriptional regulation by measuring the LANA-1 promoter activity in 293 cells pretreated with specific inhibitors for 2 hours followed by PGE2 (10 \(\mu\)mol/L) treatment for 4 hours and then incubated with PGE2. We used pharmacologic inhibitors of Ca\(^{2+}\) (BAPTA-AM and TMB-8), PI3K (wortmannin and Ly290042), Src kinase (PP2), PKC (GF109203X and GO69877), Akt 1/2 (Akt 1/2 inhibitor), NF-\(\kappa\)B (Bay 11-7085), and ERK 1/2 (PD98059 and U0126) at the indicated nontoxic concentrations.

Ca\(^{2+}\) chelation (Fig. 5A, a), Src inhibition (Fig. 5A, b), and PKC inhibition (Fig. 5A, c) decreased PGE2-mediated p-LANA-1-Luc activity significantly (Fig. 5A, a–c). Similarly, PI3K inhibitors wortmannin (1.0 \(\mu\)mol/L) and Ly290042 (25 and 50 \(\mu\)mol/L) reduced PGE2-induced p-LANA-1-Luc activity significantly (Fig. 5A, b). Akt 1/2 and NF-\(\kappa\)B inhibition downregulated PGE2-mediated p-LANA-1-Luc activity significantly (Fig. 5A, d). Although, PGE2-mediated LANA-1 promoter activity was downregulated by Akt 1/2 inhibitor, we did not observe any effect on Akt phosphorylation by EP receptor antagonists (Fig. 3D, a). Furthermore, exogenous PGE2 was able to induce Akt 1/2 in 293 cells. The dichotomy between TIVE-LTC and 293 cells indicates that, in KSHV latent TIVE-LTC cells, Akt phosphorylation might also be under the control of EP2/EP4 receptor-independent mechanisms. However, in serum starved 293 cells, PGE2 might be acting as a powerful signal inducer through EP receptors to induce Akt that may subsequently activate the LANA-1 promoter. ERK inhibition by PD98059 (10 and 20 \(\mu\)mol/L) and U0126 (5 and 10 \(\mu\)mol/L) reduced PGE2-induced p-LANA-1-Luc activity significantly by 50% to 55% with no significant inhibition on the basal activity (Fig. 5A, e). Our promoter studies using ERK 1/2 inhibitors are further validated by the observation that de novo KSHV infection and exogenous PGE2 activate ERK 1/2 in 293 cells (Fig. 5D, f). This is in contrast to the EP4 antagonist–induced upregulation of ERK 1/2 phosphorylation in TIVE-LTC cells. This could be due to the differences in the cell systems used, as the determining factors regulating ERK phosphorylation in TIVE-LTC could be different from that of 293 cells. Under serum-starved conditions, PGE2 might be acting as a power signal inducer in 293 cells through EP receptors (Fig. 5D, f). Therefore, PGE2-induced LANA-1 promote activity is inhibited by ERK inhibitors. Therefore, in TIVE-LTC cells,
the presence of viral proteins and a cytokine/chemokine-rich supernatant might be altering the signal transduction profile of the cell to such an extent that EP2 and EP4 receptors might be responsible for inhibiting ERK phosphorylation.

To explore further the signal molecules studied here, next we used different combinations of signal inhibitors (Fig. 5B) at noncytotoxic concentrations (Supplementary Fig. S1J). Blocking of PI3K and Ca2+ simultaneously showed a significant additive effect of 80% (Fig. 5B) on the decrease in LANA-1 promoter activity compared with 5 μmol/L of BAPTA-AM (Fig. 5A, a) and 12.5 μmol/L of Ly290042 (Fig. 5A, b), when used alone.

**Induction of Ca2+, p-Src, p-PI3K, p-PKCζ/λ, p-Akt 1/2, p-NF-κB, and p-ERK 1/2 by PGE2 in 293 cells.** To validate the capacity of PGE2 to induce LANA-1 promoter activity through the signal molecules that were blocked in Fig. 5A, we examined whether exogenous PGE2 (10 μmol/L) and KSHV infection can induce them. To test whether PGE2 can induce Ca2+, we treated serum-starved 293 cells loaded with fura-2AM with PGE2 (10 μmol/L) that evoked an oscillatory Ca2+ signal for 25 minutes (Fig. 5C, a). As a negative control, we also measured the basal levels of Ca2+ in untreated cells, LPA treatment (positive control), KSHV infection, and PGE2 (10 μmol/L) that evoked no intracellular Ca2+ signals (Fig. 5C, b). Compared with untreated cells, LPA treatment (positive control), KSHV infection, and PGE2 (10 μmol/L) increased the phosphorylation of p-Src, p-PI3K, p-PKCζ/λ, Akt 1/2, NF-κB, and ERK 1/2 (Fig. 5D).

**Identification of candidate PGE2-responsive elements on LANA-1 promoter.** To determine the minimal LANA-1 promoter region responsive to exogenous PGE2, a sequential series of LANA-1 promoter deletion constructs was assayed in a luciferase reporter experiment in 293 cells (Fig. 6A and B). We then examined the p-LANA-1-Luc sequence using Alibaba 2.1 TF software to characterize the transcription factor (TF) binding site profile (Fig. 6A). Exogenous PGE2 (10 μmol/L) activated pGL3.6, pGL3.4, and pGL3.3 promoter constructs at a similar level, whereas the pGL3.2 and pGL3.1 promoter constructs had significantly lower activities (Fig. 6B). Taken together, these results suggested that the promoter region located between −262 and −159 bp with candidate TFs, such as YY1, Sp1, Oct-1, Oct-6, C/EBP, and c-Jun, is required for PGE2-mediated LANA-1 promoter activity (Fig. 6A and B).

**Discussion**

The novelty of our comprehensive study is the demonstration for the first time that a proinflammatory lipid metabolite, such as PGE2 and its receptors, plays a crucial role in herpes virus latency. Previous reports have indicated the role of Ca2+ in KSHV lytic cycle (17–20). In contrast, our studies showing the downregulation of LANA-1 expression by EP1 receptor antagonist, the blockage of supernatant-induced [Ca2+]i signal by EP1 antagonist, and the downregulation of PGE2-induced LANA-1 promoter activity by calcium chelators are the first demonstration of a role for [Ca2+]i in KSHV latency program. Unlike calcium, previous reports have shown the role of Src, PI3K, PKCζ/λ, and NF-κB in KSHV latency program (21–23). However, the novelty of our study is that the data linking PGE2/EP receptors with KSHV LANA-1 expression and LANA-1 promoter through PGE2 via Src, PI3K, PKCζ/λ, and NF-κB signal induction provides a new framework to understand the host mechanisms used by KSHV to induce these signal cascades. Furthermore, the promoter region we studied accounts for the transcripts of LANA-1, vFLIP, vCyclin, and some of the viral microRNAs (24, 25) and together with its induction by PGE2 suggests that KSHV might be using PGE2 via EP receptors for regulating the expression of other latency genes.

The downregulation of COX-2 gene expression by EP2 and EP4 antagonists could either be due to the direct effect of signal cascades on the COX-2 gene, which has an inducible promoter (26), or due to the downregulation of COX-2 mRNA transcript half-life that has been shown to be mediated by p38/MK2–dependent signaling acting on the ARE sequences in the 3′ untranslated region of the COX-2 mRNA (27). The absence of any effect on COX-2 gene expression by EP receptor antagonism 24 hours posttreatment could be due to COX-2 promoter induction by other factors and suggests that KSHV uses multiple pathways with specific levels of temporal hierarchy to ensure the sustained activity of COX-2/PGE2/EP axis of inflammation, including a positive feedback loop mediated through EP2 and EP4 receptor signaling.

The effect of exogenous PGE2 on LANA-1 promoter is the eventuality of numerous distinct yet related signal cascades converging on specific TFs (Fig. 6C), which are also probably used for the maintenance of host gene expression, such as COX-2, which is crucial for KSHV survival. Besides COX-2/PGE2, KSHV must be also using several signature proinflammatory and angiogenic molecules that are induced during infection for the sustained induction of these signal networks (2, 28–32). Nevertheless, the present study showing the PGE2/EP receptor utilization for latent gene expression is unique due to the fact that PGE2 is a lipid signal inducer that functions by autocrine and paracrine fashion at the site of synthesis with a circulating half-life of ~30 seconds and normal plasma levels varying from 3 to 15 pg/mL (33). Despite the short half-life, signaling events initiated by PGE2 through EP receptors are proposed to initiate an avalanche of temporal effects on cellular signaling such as Src, PI3K, PKCζ/λ, NF-κB, and Ca2+ with immense oncogenic potential (6, 34, 35), which are the same signal pathways that are identified here to be regulated by PGE2/EP receptors in KSHV latency program.

The hallmark of KSHV interaction in human host, like in other herpes viruses, is the establishment of lifelong latency with periodic reactivation and reinfection. Successful reactivation and reinfection, however, is the consequence of the continuous tug of war between KSHV and the host immune system. Regardless of the outcome of this battle, maintenance and establishment of latency are crucial for herpes virus survival. Therefore, in the course of evolution, KSHV might have recalibrated the very purpose of inflammation from being a host response to eliminate the virus to the host mechanism that enables viral survival through the continuous production of inflammatory cytokines and...
growth factors. Pirating the proinflammatory lipid metabolite PGE2 and EP receptors for maintaining latency gene expression is a hallmark of such a phenomenon and thus gives a glimpse of the plasticity of the KSHV genome and also a novel paradigm shift in comprehending host mechanisms underlying KSHV latency. More excitingly, it adds a new paradigm in the understanding of the pathogenic mechanisms underlying chronic inflammation of KSHV-associated KS lesions. Furthermore, the study also exposes a potential “Achilles heel” of KSHV pathogenesis, wherein the use of anti-COX-2 and anti-EP receptor therapy could ameliorate KS by simultaneously controlling latency gene expression and chronic inflammation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


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Piracy of Prostaglandin E2/EP Receptor–Mediated Signaling by Kaposi’s Sarcoma-Associated Herpes Virus (HHV-8) for Latency Gene Expression: Strategy of a Successful Pathogen

Arun George Paul, Neelam Sharma-Walia, Nagaraj Kerur, et al.

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