Kaposi’s Sarcoma-Associated Herpesvirus Induces Nrf2 Activation in Latently Infected Endothelial Cells through SQSTM1 Phosphorylation and Interaction with Polyubiquitinated Keap1

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ABSTRACT

Nuclear factor erythroid 2-related factor 2 (Nrf2), the cellular master regulator of the antioxidant response, dissociates from its inhibitor Keap1 when activated by stress signals and participates in the pathogenesis of viral infections and tumorigenesis. Early during de novo infection of endothelial cells, KSHV induces Nrf2 through an intricate mechanism involving reactive oxygen species (ROS) and prostaglandin E2 (PGE2). When we investigated the Nrf2 activity during latent KSHV infection, we observed increased nuclear serine-40-phosphorylated Nrf2 in human KS lesions compared to that in healthy tissues. Using KSHV long-term-infected endothelial cells (LTC) as a cellular model for KS, we demonstrated that KSHV infection induces Nrf2 constitutively by extending its half-life, increasing its phosphorylation by protein kinase Cζ (PKCζ) via the infection-induced cyclooxygenase-2 (COX-2)/PGE2 axis and inducing its nuclear localization. Nrf2 knockdown in LTC decreased expression of antioxidant genes and genes involved in KS pathogenesis such as the NAD(P)H quinone oxidase 1 (NQO1), gamma glutamylcysteine synthase heavy unit (γGCSH), the cysteine transporter (xCT), interleukin 6 (IL-6), and vascular endothelial growth factor A (VEGF-A) genes. Nrf2 activation was independent of oxidative stress but dependent on the autophagic protein sequestosome-1 (SQSTM1; p62). SQSTM1 levels were elevated in LTC, a consequence of protein accumulation due to decreased autophagy and Nrf2-mediated transcriptional activation. SQSTM1 was phosphorylated on serine-351 and -403, while Keap1 was polyubiquitinated with lysine-63–ubiquitin chains, modifications known to increase their mutual affinity and interaction, leading to Keap1 degradation and Nrf2 activation. The latent KSHV protein Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein (vFLIP) increased SQSTM1 expression and activated Nrf2. Collectively, these results demonstrate that KSHV induces SQSTM1 to constitutively activate Nrf2, which is involved in the regulation of genes participating in KSHV oncogenesis.

IMPORTANCE

The transcription factor Nrf2 is activated by stress signals, including viral infection, and responds by activating the transcription of cytoprotective genes. Recently, Nrf2 has been implicated in oncogenesis and was shown to be activated during de novo KSHV infection of endothelial cells through ROS-dependent pathways. The present study was undertaken to determine the mechanism of Nrf2 activation during prolonged latent infection of endothelial cells, using an endothelial cell line latently infected with KSHV. We show that Nrf2 activation was elevated in KSHV latently infected endothelial cells independently of oxidative stress but dependent on the autophagic protein sequestosome-1 (SQSTM1), which was involved in the degradation of the Nrf2 inhibitor Keap1. Furthermore, our results indicated that the KSHV latent protein vFLIP participates in Nrf2 activation. This study suggests that KSHV hijacks the host’s autophagic protein SQSTM1 to induce Nrf2 activation, thereby manipulating the infected host gene regulation to promote KS pathogenesis.

Kaposi’s sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is etiologically associated with three human malignancies: body cavity-based lymphoma (BCBL) or primary effusion lymphoma (PEL), multicentric Castleman’s disease (MCD), and Kaposi’s sarcoma (KS). PEL and MCD are lymphoproliferative disorders, whereas KS is an angioproliferative malignancy of the human skin (1–3). KS lesions are characterized by spindle-shaped endothelial cells latently infected with KSHV, inflammatory cells, and numerous secreted factors, such as inflammatory cytokines and growth and angiogenic factors (4).

Similar to other gammaherpesviruses, KSHV displays latent and lytic cycles in infected B and endothelial cells. During latency, no viral particles are produced, but the cells express KSHV-associated genes from the major latency locus, which consists of open...
reading frame 71 (ORF71) (Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein [vFLIP], also called K13), ORF72 (viral cyclin), ORF73 (latent-associated nuclear antigen-1 [LANA-1]), K12 (kaposis), ORF10.5 (LANA-2), and viral interleukin 6 (vIL-6) as well as 12 microRNAs (5). In addition to the latent and lytic cycles of KSHV, the infection-induced angiogenic and inflammatory networks are involved in KS pathogenesis. Numerous cellular pathways are regulated by viral proteins, leading to the reprogramming of the infected cells’ transcriptional machinery and consequently affecting expression of genes involved in cell proliferation, apoptosis, autophagy, immune evasion, and angiogenesis.

Nuclear factor E2-related factor (Nrf2), a member of the Cap’n’Collar family of basic-region leucine zipper (bZIP) transcription factors, plays key roles in the cellular defense against oxidative and xenobiotic stresses (6, 7). Under basal conditions, Kelch–like ECH-associated protein 1 (Keap1) negatively regulates Nrf2 by blocking Nrf2 translocation to the nucleus and by promoting its degradation. Specifically, Nrf2 interacts with Keap1 in the cytoplasm, where Keap1 acts as an adaptor for the cullin 3-based E3 ubiquitin ligase complex, leading to Nrf2 polyubiquitination and proteosomal degradation. Disruption of the Keap1–Nrf2 interaction is crucial in mediating Nrf2 stabilization and, eventually, activation. In the canonical pathway of Nrf2 activation, oxidative stress-mediated cysteine residue modifications on Keap1 induce conformational changes, thereby preventing it from binding to Nrf2. This allows for stabilization of Nrf2, which then translocates into the nucleus, binds to antioxidant response elements (ARE) in the promoter regions of more than 200 genes, and activates the transcription of a battery of antioxidant and cytoprotective genes involved in glutathione synthesis, elimination of reactive oxygen species (ROS), xenobiotic metabolism, and drug transport (8).

In a more recently discovered noncanonical pathway, Keap1-mediated Nrf2 degradation is disrupted by the autophagic protein sequestosome-1 (SQSTM1 or p62), independent of oxidative stress status (9–13). SQSTM1 is a key molecule involved in targeting specific proteins for degradation through selective autophagy (14, 15). The interaction of SQSTM1 with its targets is highly dependent on the posttranslational modifications of both proteins, notably the phosphorylation of SQSTM1 and the Lys-63 polyubiquitination of the targeted protein (9, 16–19). To activate Nrf2 signaling, phosphorylated SQSTM1 binds to the Nrf2-binding site of Lys-63–polyubiquitinated Keap1, competitively inhibiting Keap1–Nrf2 interaction and inducing Keap1 degradation (9–13, 20). Once the Keap1–Nrf2 interaction is disrupted, Nrf2 is stabilized and translocates into the nucleus to activate its target genes.

In addition to its stabilization, Nrf2 requires serine-40 (Ser-40) phosphorylation before its transcriptional properties can take effect (21, 22). Several kinases, such as extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, protein kinase RNA-like endoplasmic reticulum kinase (PERK), casein kinase II (CK2), and protein kinase C (PKC), can affect Nrf2 phosphorylation status, either directly or indirectly (22–28). In particular, the phosphorylation of Ser-40 of Nrf2 by PKC is critical for Nrf2 nuclear translocation, leading to the activation of ARE-responsive genes (21, 22).

While Nrf2 activation was originally thought to be beneficial in protecting cells from genotoxic damage caused by carcinogens, recent studies indicate that Nrf2 could be involved in carcinogenesis and chemoresistance (29, 30). Constitutive activation of Nrf2 is observed in various cancers, and the prognosis of patients carrying Nrf2-positive tumors is poorer than that of patients with Nrf2-negative tumors (31). Several cancer-linked mutations have been identified in the Keap1 and Nrf2 genes. Gain-of-function mutations in the Nrf2 gene have been found in carcinomas of the esophagus, skin, larynx, and lung (32), whereas loss-of-function mutations in the Keap1 gene have been found in breast, lung, gallbladder, ovary, liver, and stomach cancers (8). In addition to mutations in coding regions, epigenetic hypermethylation of the Keap1 and Nrf2 promoters also contributes to the Nrf2 deregulation observed in cancers (33, 34).

Nrf2 target genes encoding antioxidant enzymes may confer a great advantage to cancer cell survival by maintaining the redox balance; by protecting against high levels of oxidative stress, Nrf2 can increase resistance to chemotherapy (35). Nrf2 may also directly regulate cell proliferation. The first evidence indicating that Nrf2 is involved in cell proliferation and differentiation was observed in Keap1 knockout mice. These mice, in which Nrf2 is constitutively activated, die due to hyperplastic keratinization of the esophagus and forestomach (36). In addition, Nrf2 was shown to regulate murine hematopoietic and Drosophila intestinal stem cell survival (37, 38). A high-throughput sequencing approach identified numerous new Nrf2 target genes involved in cell proliferation, cell cycle regulation, and survival (39). Nrf2 regulates cellular differentiation by modulating Notch1 expression through ARE binding sites located in its promoter (40, 41). Furthermore, a recent study demonstrated that Nrf2 activates genes involved in the pentose phosphate pathway, which is necessary for DNA and RNA synthesis during cell proliferation (42). Finally, Nrf2 has been shown to aid in cell proliferation by augmenting PI3K/Akt signaling, inhibiting apoptosis through Bcl-2 and Bcl-x-L induction, creating an angiogenic environment through HIF-1 and vascular endothelial growth factor (VEGF) expression, and participating in cell migration or invasion by altering matrix metallopeptidase 9 (MMP9) expression (42–47).

We have previously shown that de novo KSHV infection of endothelial cells (human dermal microvascular endothelial cells [HMVEC-d]) induced the formation of ROS that are critical for virus entry (48). In a subsequent study, we demonstrated that this KSHV-mediated ROS induction, along with the activation of Src, PI3K, and PKCζ, resulted in enhanced Nrf2 activity during the early stages of de novo infection (49). In addition, in the postentry stages of early de novo infection, we observed that the paracrine and autocrine signaling activated by KSHV infection-induced cyclooxygenase-2 (COX-2) product prostaglandin E2 (PGE2) participated in Nrf2 activation and that KSHV latent protein vFLIP may have a role in such an induction (50–53).

In the present study, we investigated the activation of Nrf2 during prolonged latency using a cellular model for KS and determined the mechanisms involved in this activation. Endothelial cell lines in which KSHV episomes are maintained indefinitely in the absence of selection were obtained after de novo infection of telomerase-immortalized human umbilical vein endothelial (TIVE) cells (54). These long-term-infected TIVE cells (LTC) express latent viral genes and can generate tumors in mice with characteristics similar to those of KS (54). Here we demonstrate that Nrf2 phosphorylation levels are elevated in human KS lesions
and in KSHV \(^{+}\) LTC. Compared to control cells, Nrf2 was constitutively activated at higher levels in the LTC, which participated in the regulation of host genes involved in KSHV pathogenesis. This Nrf2 activation was independent of oxidative stress but dependent on the noncanonical pathway involving the host autophagic protein SQSTM1, which degraded the Nrf2 inhibitor Keap1, all of which was facilitated by the KSHV latent protein vFLIP.

**MATERIALS AND METHODS**

**Cells.** TIVE cells and LTC, gifts from Rolf Renne (University of Florida), were cultured in endothelial basal medium 2 (EBM-2) with growth factors (Lonza, Walkersville, MD). Human dermal microvascular endothelial cells (HMVEC-d) (CC-2543; Lonza) were cultured in EBM-2 with growth factors. Human embryonic kidney HEK293T cells were grown in DMEM containing 10% fetal serum (Lonza). Human dermal microvascular endothelial cells (HMVEC-d) (CC-2543; Lonza) were cultured in EBM-2 with growth factors (Lonza). Human embryonic kidney HEK293T cells were maintained in Dulbecco’s modified eagle medium (DMEM) containing 1 mM pyruvate, 2 mM Glutamax, 50 U/ml of penicillin, 50 mg/ml of streptomycin (Lonza, Walkersville, MD). Human dermal microvascular endothelial cells (HMVEC-d) (CC-2543; Lonza) were cultured in EBM-2 with growth factors (Lonza). Human embryonic kidney HEK293T cells were maintained in Dulbecco’s modified eagle medium (DMEM) containing 1 mM pyruvate, 2 mM Glutamax, 50 U/ml of penicillin, 50 mg/ml of streptomycin (Lonza, Walkersville, MD). Human embryonic kidney HEK293T cells were maintained in Dulbecco’s modified eagle medium (DMEM) containing 1 mM pyruvate, 2 mM Glutamax, 50 U/ml of penicillin, 50 mg/ml of streptomycin (Lonza, Walkersville, MD).

**Reagents.** Phospho-Nrf2 (pNrf2) (Western blotting [WB], 1:2,000; immunofluorescence assay [IFA], 1:250), SQSTM1 (WB, 1:2,000), lamin B1 (WB, 1:2,000) and TATA-binding protein 1 (TBP-1) (WB, 1:2,000) antibodies were from Abcam, Cambridge, MA. Total Nrf2 (nNrf2) (WB, 1:1,000), Keap1 (WB, 1:1,000), PKCx (WB, 1:2,000), and PKCh (WB, 1:1,000) antibodies were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. SQSTM1 (IFA, 1:100) antibody was from GeneTex, Irvine, CA. LC3 antibody (WB, 1:200; IFA, 1:100) was from AbD Serotec, Raleigh, NC. The LC3 antibody recognizes an epitope within the N-terminal regions of both LC3A and LC3B, which are widely used for the monitoring of autophagy. pSer-403 SQSTM1 (WB, 1:1,000), total ubiquitin FK1 (IFA, 1:100) and ubiquitin Lys-63 (WB, 1:500) antibodies were from Millipore, Billerica, MA. pSer-351 SQSTM1 (WB, 1:1,000) was from MBL International, Woburn, MA. α-Actin (WB, 1:1,000) and tubulin (WB, 1:1,000) antibodies were from Sigma-Aldrich, St. Louis, MO. 4',6-Diamidino-2-phenylindole (DAPI), Alexa Fluor 488 (1:500), and Alexa Fluor 594 (1:1,000) antibodies were from Life Technologies. Anti-mouse and anti-rabbit secondary antibodies linked to horseradish peroxidase (HRP) were from KPL, Inc., Gaithersburg, MD. Myc-PKCh and PGE2 were from Cayman Chemicals, Ann Arbor, MI. Celcexib was purchased from Tocris Biosciences (Ellisville, MO).

**Production of lentiviral gene transfer vectors.** Plasmids expressing a short hairpin RNA (shRNA) against Nrf2 (shNrf2) (TRCN0000007558) and shSQSTM1 (TRCN0000007236 and TRCN0000007237) were purchased from Thermo Fisher Scientific, Waltham, MA. Lentiviral vectors were produced using a four-plasmid transfection system, as previously described (55). Briefly, HEK293T cells were transfected with vector and packaging plasmids and the medium was changed 16 h after transfection. The supernatants containing the lentiviral vectors were collected 24 h later, passed through a 45-µm filter, and used to transduce TIVE cells and LTC in the presence of Polybren (5 µg/ml; Pierce).

**Luciferase assay.** HEK293T cells were transiently transfected by the calcium phosphate method with the plasmid expressing an individual KSHV latent gene, an ARE-luciferase reporter plasmid, and a vector expressing a β-galactosidase (β-Gal) reporter gene controlled by a Rous sarcoma virus promoter (used to normalize for transfection efficiency) (55). Thirty-six hours posttransfection, the cells were harvested and lysed in lysis reporter solution (Promega, Madison, WI). Soluble extracts were assayed for luciferase and β-Gal activities using Steady-Glo and Beta-Glo (Promega, Madison, WI), by following the manufacturer’s instructions.

**WB analysis.** Cells were harvested in RIPA lysis buffer (125 mM NaCl, 0.01 M sodium phosphate [pH 7.2], 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, and 50 mM sodium fluoride) with protease and phosphatase inhibitor cocktails (Sigma). Cellular debris was removed by centrifugation at 13,000 \(\times g\) for 10 min at 4°C, and equal amounts of protein samples were resolved by SDS-10% PAGE and subjected to WB with the antibodies indicated in the figures. To confirm equal protein loading, blots were also probed with antibodies against human β-actin or tubulin. Secondary antibodies conjugated to horseradish peroxidase were used for detection, and immunoreactive bands were developed by enhanced chemiluminescence (Lumi-light PLUS Western blotting substrate; Roche).

**RNA extraction, reverse transcription, and real-time PCR.** Total RNA was extracted using Trizol reagent (Life Technologies) and quantified by absorbance analysis at 260 nm. One to two micrograms of total RNA was treated with DNase for 1 h (DNA-free kit; Life Technologies) and then reverse transcribed into cDNA using a high-capacity cDNA reverse transcription (RT) kit (Life Technologies). cDNA was used as a template with primer sets as indicated in Table 1. PCR was performed using an ABI Prism 7500 real-time PCR system utilizing SYBR green PCR master mix (Life Technologies).

**Nuclear extraction.** Nuclear extracts were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA) as per the manufacturer’s instructions. After protein concentrations were measured with Bradford reagent (Bio-Rad), proteins were stored at −70°C. Tubulin and lamin B1 or TBP-1 were used to demonstrate the purity and equal loading of cytoplasmic and nuclear fractions, respectively.

**ROS measurement.** TIVE cells and LTC were cultured until confluent. Cells were loaded with dye by replacing medium with fresh EGM-2 containing 10 µM CM-H2DCFDA (Life Technologies) or 10 mM dihydroethidium (DHE; Life Technologies) for 1 h at 37°C in a 5% CO\(_2\) environment. The cells were recovered by trypsinization and resuspended in phosphate-buffered saline (PBS) before analysis by flow cytometry (LSRII; BD Biosciences) at the Flow Cytometry Facility at Rosalind Franklin University of Medicine and Science.

**Immunohistochemistry.** Sections from skin biopsy samples of healthy and Kaposis’s sarcoma patients were obtained from the AIDS and Cancer Specimen Resource (ACSR), National Cancer Institute (NCI), and staining was performed by the Pathology Core Facility, Northwestern University, Chicago, IL. Sections were deparaffinized with HistoChoice clearing reagent, hydrated with water after microwave treatment in 1
mM EDTA (pH 8.0) for 15 min for antigen retrieval, and then blocked with blocking solution (2% donkey serum and 0.3% Triton X-100 in PBS). Sections were incubated with the primary antibody against pNrf2 overnight at 4°C. These sections were incubated with rabbit polymer-HRP (Biocare Medical, Concord, CA) for 15 min, washed, and developed using 3,3’-diaminobenzidine reagent (Dako). Counterstaining was done by hematoxylin.

Immunofluorescence staining of tissue sections. Formalin-fixed, paraffin-embedded tissue samples from healthy subjects and patients with KS were obtained from the ACSR. Sections were deparaffinized with HistoChoice clearing reagent and rehydrated through washes in decreasing grades of ethanol to water. For antigen retrieval, the sections were microwaved in 1 mM EDTA (pH 8.0) for 15 min, permeabilized with 0.5% Triton X-100 for 5 min, and then blocked with blocking solution (Image-iT FX signal enhancer; Life Technologies) for 20 min at room temperature. Immunostaining was performed using anti-rabbit pNrf2 and anti-mouse LANA-1 antibodies, followed by Alexa Fluor 488- and Alexa Fluor 594-conjugated secondary antibodies. Nuclei were stained with DAPI (Molecular Probes, Life Technologies), and stained cells were viewed under a Nikon 80i fluorescence microscope with a 40x objective and Nikon NIS-Elements imaging software.

IFA. TIVE cells and LTC were seeded onto 8-well chamber slides (Nalge Nunc International, Naperville, IL), fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked with Image-iT FX signal enhancer (Life Technologies) for 20 min at room temperature. The cells were incubated with primary antibodies against the specific proteins, followed by Alexa Fluor dye-conjugated secondary antibodies. Nuclei were visualized using DAPI (Life Technologies). These cells were viewed with the appropriate filters under a Nikon 80i fluorescence microscope, and data were analyzed using Nikon MetaMorph digital imaging software. All experiments were performed at least three times.

Cyto-ID autophagy detection. The Cyto-ID autophagy detection kit (Enzo Life Sciences, Farmingdale, NY) uses a cationic amphiphilic tracer (CAT) dye that selectively labels autophagic vacuoles. TIVE cells and LTC were seeded onto 8-well chamber slides (Nalge Nunc International) and grown with growth media or starved overnight in Hanks’ balanced salt solution (HBSS; Life Technologies) to induce autophagy. IFA cells and LTC were stained by following the manufacturer’s instructions. Briefly, cells were washed twice with PBS and incubated 30 min at 37°C in the dark with microscopy dual-detection reagent containing the autophagic dye as well as Hoechst 33342 nuclear stain. After three PBS washes, the cells were fixed with 4% paraformaldehyde for 20 min. Stained cells were viewed with the appropriate filters under a Nikon 80i fluorescence microscope with a 40x objective, and data were analyzed using Nikon NIS-Elements imaging software.

RESULTS
Human KS lesions have high levels of phosphorylated and nuclear Nrf2. Nrf2 phosphorylation on Ser-40 and nuclear localization in uninfected bystander cells (Fig. 1B, white arrows). The specificity of staining was demonstrated by the absence of reactivity by secondary antibodies alone in the healthy and KS tissue sections (Fig. 1C). These results strongly suggested that KSHV infection is essential for Nrf2 activation and that Nrf2 is activated in LANA-1-positive cells within KS lesions.

KSHV+ LTC have enhanced Nrf2 stabilization and phosphorylation. Because KS lesions cannot be experimentally manipulated, we chose to examine Nrf2 activation in TIVE cells latently infected with KSHV (KSHV+ LTC) (54). These cells are considered the closest in vitro model mimicking a KS lesion (54). We determined the levels of Nrf2 activation in control KSHV-negative TIVE and KSHV+ LTC by WB analysis. Compared to TIVE cells, LTC exhibited a 12-fold-higher level of total Nrf2 expression (tNrf2) and an 8-fold-higher level of Nrf2 phosphorylation (pNrf2) (Fig. 2A, lanes 1 and 2). Analysis of Nrf2 mRNA expression revealed no difference between the two cell lines (Fig. 2B), suggesting protein stability as a likely reason for the observed difference. To determine if the increase in Nrf2 protein levels was due to increased protein stability, we halted protein synthesis by using cycloheximide (CHX) at 100 ng/ml for the times indicated in the figure and determined the half-life of Nrf2 in TIVE cells and LTC through WB (Fig. 2C). While Nrf2 protein levels quickly decreased in TIVE cells (Fig. 2C, lanes 1 to 6), they were significantly more stable in LTC (Fig. 2C, lanes 7 to 12). Specifically, we observed 11, 27, 61, 92, and 86% reductions in tNrf2 protein levels in TIVE cells at 15 min, 30 min, 1 h, 2 h, and 4 h post-CHX treatment, respectively. In contrast, tNrf2 was more stable in LTC, with 16, 24, 34, 28, and 38% decreases observed at the same time points (Fig. 2C). We estimated the half-life of Nrf2 to be ~45 min in TIVE cells and greater than 4 h in LTC. Taken together, these results showed that LTC exhibited increased protein stability and phosphorylation compared to those of TIVE cells.

PKCζ is important for Nrf2 phosphorylation in LTC. Several kinases have been implicated, directly or indirectly, in the phosphorylation of Nrf2 (22–28). In particular, atypical PKCs directly phosphorylate Nrf2 on Ser-40 and promote Nrf2 nuclear translocation and transcriptional activity (57). Among the atypical PKCs, PKCζ is activated at 30 min following de novo KSHV infection of endothelial cells, which contributes to Nrf2 phosphorylation (49, 58). Furthermore, PKCζ is also induced beyond 24 h postinfection (p.i.), when latency is established (59). To determine whether PKCζ is activated in LTC, we analyzed the level of PKCζ activation in TIVE cells and LTC by measuring the phosphorylation of its activation loop site (Ser-410). We observed a 5.5-fold-higher level of pPKCζ and a 2-fold-higher level of total PKCζ in LTC than in TIVE cells (Fig. 2D, lanes 1 and 2). To determine if PKCζ is involved in Nrf2 phosphorylation in LTC, we treated the cells overnight with a myristoylated PKCζ pseudosubstrate inhibitor (Myc-PKCζ; 10 μM) or with a solvent control (dimethyl sulfoxide [DMSO]). As shown in Fig. 2E, Myr-PKCζ treatment significantly reduced Nrf2 phosphorylation, by 50% (Fig. 2E, top blot, lanes 1 and 2). Interestingly, the inhibitor had no effect on protein stability, as tNrf2 levels were not reduced (Fig. 2E, middle blot, lanes 1 and 2). Collectively, these results indicated that PKCζ was important for Ser-40 Nrf2 phosphorylation in KSHV+ LTC.

KSHV infection-induced COX-2/PGE2 is important for PKCζ-mediated Nrf2 phosphorylation. Latent and de novo KSHV infection of several cell types induces the proinflammatory
molecule COX-2, which leads to elevated production of PGE2 and its powerful paracrine signaling (50–52). In a recent study, we demonstrated that COX-2/PGE2 participated in Nrf2 phosphorylation through the activation of PKCζ during the early stages of de novo infection of HMVEC-d (49,59). Studies by Sharma-Walia et al. (52) have also shown that KSHV induces sustained COX-2 gene and protein expression early during de novo infection as well as during latency in LTC. It has also been demonstrated that KSHV latency-associated vFLIP induces the activation of COX-2 (53), leading to the secretion of its principal metabolite PGE2, which activates several signaling pathways, including PKCζ, in KSHV-infected and uninfected cells.

We hypothesized that PKCζ is activated in latently infected LTC through the vFLIP-mediated activation of COX-2 and the subsequent secretion of PGE2. To determine whether the COX-2/PGE2 axis was involved in PKCζ/pNrf2 activation during prolonged KSHV latency, we first treated the uninfected control TIVE cells with increasing doses of PGE2 for 4 h. We observed that PKCζ phosphorylation increased in a dose-dependent manner, 4.0-, 10.2-, and 15.2-fold with 1, 10, and 100 μM concentrations of PGE2 treatment, respectively (Fig. 2F, blot 3). In contrast, the total PKCζ levels remained unchanged (Fig. 2F, blot 4). Furthermore, Nrf2 phosphorylation was increased 4.7-, 4.8-, and 8.0-fold while total Nrf2 was increased 2.0-, 8.0-, and 14.7-fold with 1, 10, and 100 μM PGE2 treatment, respectively (Fig. 2F, blots 1 and 2).

To confirm the presence of a COX-2/PGE2/PKCζ axis mediating Nrf2 phosphorylation in LTC, we treated LTC with the COX-2 inhibitor celecoxib (10 μM) for 8 and 24 h (Fig. 2G). We observed ~50% and 70% decreases in PKCζ phosphorylation and ~70% and 80% decreases in Nrf2 phosphorylation after 8 h and 24 h of celecoxib treatment, respectively (Fig. 2G, blots 1 and 3). The level of total PKCζ was not significantly changed (Fig. 2G, blot 4).
addition, we observed ~70% and 90% decreases in tNrf2 levels after 8 h and 24 h of celecoxib treatment, respectively (Fig. 2G, blot 2), which probably reflects the reduction in Nrf2 gene expression induced by the decrease in transcriptionally active pNrf2 on its own promoter in the presence of celecoxib (as discussed in references 49 and 60) as well as a probable increase in Keap1 binding to nonphosphorylated Nrf2 resulting in proteosomal degradation (22).

All together, these results suggested that KSHV infection-induced COX-2-mediated secretion of PGE2 participates in PKCζ...
activation, which, in turn, is involved in the constitutive phosphorylation of Nrf2 in KSHV latently infected LTC.

**tNrf2 and pNrf2 are elevated and predominantly nuclear in LTC compared to TIVE cells.** Because it is a transcription factor, Nrf2 nuclear localization is crucial for its activity. We therefore examined the subcellular localization of tNrf2 and pNrf2 by WB analysis of nuclear and cytoplasmic fractions (Fig. 3A). We observed higher levels of tNrf2 and pNrf2 in both cytoplasmic and nuclear fractions of LTC than in their respective fractions in TIVE cells. Indeed, in the cytoplasm, pNrf2 and tNrf2 levels were 12- and 8-fold higher, respectively, whereas in the nucleus, pNrf2 and tNrf2 levels were 6-fold higher (Fig. 3A). Tubulin and lamin B1 were used to demonstrate the purity and equal loading of cytoplasmic and nuclear fractions, respectively. (B) TIVE cells and LTC were subjected to immunofluorescence analysis for pNrf2 (red). Cell nuclei were visualized by DAPI (blue). Boxed areas are enlarged. White arrowheads indicate pNrf2 located in the cytoplasm, whereas yellow arrowheads indicate pNrf2 located in the nuclei. Magnification, ×40.

**FIG 3 Analysis of Nrf2 subcellular localization in TIVE cells and LTC.** (A) Nuclear and cytoplasmic fractions were isolated and subjected to analysis by WB using anti-pNrf2 and Nrf2 antibodies. Tubulin and lamin B1 were used to demonstrate the purity and equal loading of cytoplasmic and nuclear fractions, respectively. (B) TIVE cells and LTC were subjected to immunofluorescence analysis for pNrf2 (red). Cell nuclei were visualized by DAPI (blue). Boxed areas are enlarged. White arrowheads indicate pNrf2 located in the cytoplasm, whereas yellow arrowheads indicate pNrf2 located in the nuclei. Magnification, ×40.

**FIG 4 Analysis of Nrf2 influence on host gene expression.** (A) Analysis of Nrf2 target gene expression in TIVE cells and LTC. Real-time PCR was performed on cDNA from TIVE cells and LTC using the indicated primers and normalized to β-actin. Each bar represents the mean increase in gene expression ± SEM relative to TIVE cells (set to 100%) from 6 independent experiments. Statistical analysis was conducted using a two-tailed Student t test. *, P < 0.05; **, P < 0.01. (B and C) Efficiency of Nrf2 knockdown. LTC were transduced with a lentiviral vector expressing an shRNA control (shC) or an shRNA against Nrf2 (shNrf2). mRNA levels were quantified by real-time PCR (B), and protein levels were observed by WB analysis (C). (D) Role of Nrf2 in LTC gene expression. LTC were transduced with a lentiviral vector expressing shC or shNrf2. mRNA levels were quantified by real-time PCR using the primers indicated and normalized to tubulin. Each bar represents the mean gene expression ± SEM compared to the value for shC-transduced cells (set to 100%) from 3 independent experiments. Statistical analysis was conducted using a two-tailed Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.005.

**Nrf2 target genes are upregulated in LTC.** Once phosphorylated, Nrf2 translocates into the nucleus, where it binds to promoters containing ARE (61). We next determined whether the elevated nuclear pNrf2 levels observed in LTC correlated with an increase in target gene mRNA levels. The genes for NAD(P)H quinone oxidase 1 (NQO1), gamma glutamylcysteine synthase heavy unit (γGCSH), and the cysteine transporter (xCT) are three characteristic Nrf2 antioxidant target genes (8). In LTC, we observed 2-, 3-, and 3-fold-higher levels of γGCSH, NQO1, and xCT expression, respectively, than in TIVE cells (Fig. 4A).

Recently, in addition to its antioxidative functions, Nrf2 has been involved in several other cellular pathways, such as angiogenesis and inflammation. For example, a role for Nrf2 in cancer angiogenesis has recently been documented; knockdown of Nrf2 by small interfering RNA (siRNA) in human colon cancer cells
reduced blood vessel formation and VEGF-A expression (44). A role for Nrf2 in angiogenesis has been confirmed in endothelial cells in aging and hypoxic models (62, 63). To determine whether Nrf2 participated in KSHV-associated angiogenesis, we examined VEGF-A mRNA levels in TIVE and LTC. We observed an 18-fold-higher level of VEGF-A mRNA levels in LTC than in TIVE cells (Fig. 4A).

Nrf2 also participates in the inflammatory response. DNA array analysis suggested that Nrf2 regulates proinflammatory and anti-inflammatory factors, including IL-6 activation (39, 64, 65). KSHV infection of endothelial cells induces secretion of proinflammatory and anti-inflammatory factors (52). In alignment with these studies, we observed a 9-fold-higher level of IL-6 mRNA in LTC than in TIVE cells (Fig. 4A). Collectively, these experiments established an increased expression of Nrf2 target genes in LTC compared to that in TIVE cells.

To confirm that the induction of NQO1, γGCSH, xCT, VEGF-A, and IL-6 in LTC is dependent on Nrf2, we knocked down Nrf2 expression using shRNA. LTC were transduced with lentiviral vectors expressing an shRNA control (shC) or an shRNA against Nrf2 (shNrf2). We observed ~75% reduction in Nrf2 mRNA expression by real-time PCR and ~40% reduction in tNrf2 protein levels in LTC with shNrf2 (Fig. 4B and C, respectively). We observed no change in the expression levels of the transcription factor NF-κB/p65, arguing against the possibility of nonspecific targeting by the shNrf2 vector (Fig. 4C). Interestingly, the levels of expression of NQO1, γGCSH, xCT, VEGF-A, and IL-6 were reduced by 75%, 50%, 77%, 60%, and 25%, respectively, in shNrf2 LTC (Fig. 4D). Taken together, these results demonstrated that activated Nrf2 in KSHV + LTC participates in antioxidant gene expression as well as in the expression of genes involved in KSHV-associated angiogenesis and inflammation.

**Nrf2 activation in LTC is independent of ROS levels.** The canonical model inducer of Nrf2 activation is oxidative stress, which mediates cysteine residue modifications on Keap1, inhibiting its direct binding to newly synthesized Nrf2 and abrogating its inhibitory capacity (66). We have recently shown that during the binding and entry stages of de novo infection of HMVEC-d, KSHV induces ROS which not only facilitated entry and productive infection but also were important for the early stages of Nrf2 induction (48, 49). To determine whether ROS are involved in Nrf2 activation in LTC, we first evaluated the levels of ROS in TIVE cells and LTC by flow cytometry using two widely used dyes. The CM-H$_2$DCFDA dye is oxidized by a wide range of ROS, and the resulting 2′,7′-dichlorofluorescein (DCF) product fluoresces green, whereas dihydroethidium (DHE) is oxidized specifically by superoxide anions and consequentially fluoresces red (Fig. 5A and B). To our surprise, but in concordance with the antioxidant gene expression profile observed in Fig. 4A, TIVE cells presented a high level of DCF green fluorescence, indicating the presence of high oxidative stress, whereas LTC exhibited lower ROS levels (Fig. 5A). These results were confirmed with the DHE dye: the level of red fluorescence produced by DHE was higher in TIVE cells than in LTC (Fig. 5B). These results showed decreased ROS levels in LTC compared to those in TIVE cells and suggested that Nrf2 activation in LTC was not due to increased ROS levels.

To further exclude the role of oxidative stress in Nrf2 activation in LTC, we treated the cells with the powerful antioxidants N-acetylcysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) and analyzed the levels of tNrf2 and pNrf2 by WB (Fig. 5C). We observed no significant changes in tNrf2 and pNrf2 levels in cells treated with NAC or PDTC compared to those in the untreated cells (Fig. 5C). Taken together, these results demonstrated that Nrf2 activation in LTC was independent of oxidative stress.

**Autophagic sequestosome-1 (SQSTM1, p62) protein is increased in LTC.** Since the canonical pathway is not involved in Nrf2 activation in LTC (Fig. 5), we focused on the recently described noncanonical pathway, in which Nrf2 activation is mediated via the autophagic protein SQSTM1, which directly binds Keap1 and prevents its inhibitory activity on Nrf2 (9–13). To investigate the possible role of SQSTM1 in Nrf2 stability in LTC, we assessed the level of SQSTM1 protein in TIVE cells and LTC by WB analysis. We observed that SQSTM1 is highly expressed (9-fold higher) in LTC compared to TIVE cells (Fig. 6A). To determine whether increased SQSTM1 was due to an increase in gene expression, we measured SQSTM1 mRNA levels in TIVE cells and LTC by real-time reverse transcription-PCR (RT-PCR), and we observed significantly higher levels of SQSTM1 mRNA in LTC than in TIVE cells (Fig. 6B). Interestingly, it has been proposed that Nrf2 positively regulates SQSTM1 gene expression, creating a positive-feedback loop between these two agents (67–69). To determine whether increased SQSTM1 expression was dependent on Nrf2 in LTC, we measured SQSTM1 gene expression in cells transduced with shC or shNrf2 by real-time RT-PCR (Fig. 6C). Knockdown of Nrf2 significantly reduced SQSTM1 gene expression, indicating that SQSTM1 expression in LTC was at least partially dependent on Nrf2 (Fig. 6C).

SQSTM1 is punctate and colocalizes with ubiquitinated proteins in LTC. IFA was done to confirm SQSTM1 protein accumulation. We observed a weak and diffuse SQSTM1 staining in TIVE cells and, in contrast, strongly elevated and punctate SQSTM1...
staining in LTC (Fig. 6D, arrowheads). We quantified the percentage of cells with SQSTM1 speckles and observed 3 times as many positive cells in LTC as in TIVE cells (Fig. 6E). Because the formation of cytoplasmic inclusions containing both SQSTM1 and ubiquitinated proteins has previously been observed (70), we examined the TIVE cells and LTC by IFA using anti-SQSTM1 and anti-ubiquitin antibodies (Fig. 6F). The higher level of SQSTM1 punctate staining in LTC colocalized strongly with ubiquitin puncta, with little interaction in TIVE cells (Fig. 6F). Taken together, these results demonstrated that SQSTM1 accumulated in ubiquitin-positive inclusion bodies in LTC.

**Autophagy is inhibited in LTC.** Accumulation of SQSTM1 protein in ubiquitin-positive inclusion bodies is associated with decreased autophagy (13). Because KSHV latently infected cells have been shown to be protected from autophagy and the KSHV latent protein vFLIP negatively regulates autophagy during latency, we next determined whether the increased presence of SQSTM1 inclusion bodies in LTC was due to autophagy inhibition (71, 72).

To monitor the levels of autophagy, we used microtubule-associated protein 1A/1B-light chain 3 (LC3), a marker of autophagy (73). During autophagy induction, the cytosolic LC3 (LC3-I) is converted into phosphatidylethanolamine-bound LC3 (LC3-II), which migrates faster than LC3-I due to its hydropho-
We measured the basal level of autophagy of TIVE cells and LTC by WB analysis with LC3 antibody, and we observed 80% lower levels of both LC3-I and LC3-II bands in LTC than in TIVE cells, suggesting an inhibition of autophagy in these cells (Fig. 7A). As LC3-II is recruited to autophagosomal membranes, LC3 punctate staining is representative of the autophagosome formation in IFA. We observed abundant formation of LC3 puncta in TIVE cells, and these puncta were strikingly reduced in LTC (Fig. 7B). We found that the LC3 puncta were present in 48% of TIVE cells but in only 10% of LTC (Fig. 7C), which further suggested that autophagy is decreased in LTC.

To confirm these results, TIVE cells and LTC were stained with Cyto-ID autophagy reagent, which specifically detects autophagosomes and autolysosomes. As shown in Fig. 7D, the autophagic staining was high in TIVE cells and reduced in LTC. To confirm the specificity of the dye, TIVE cells and LTC were starved overnight to induce autophagy, and as expected, Cyto-ID staining was increased in both TIVE cells and LTC after induction of autophagy compared to uninduced cells (compare Fig. 7E to D). Collectively, these results showed that basal autophagy is reduced in KSHV/H11001 LTC compared to control TIVE cells, explaining the increased number of inclusion body-associated SQSTM1 and its ubiquitinated cargo protein in LTC observed in Fig. 6.

**Keap1 is degraded in LTC.** An important difference between the canonical (ROS-dependent) and noncanonical (SQSTM1-dependent) pathways of Nrf2 activation is the fate of Keap1. In the canonical pathway, ROS structurally modifies Keap1, preventing it from interacting with Nrf2 without affecting overall Keap1 pro-

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**FIG 7** Autophagy levels in TIVE cells and LTC. (A) Reduced autophagy in LTC. TIVE cell and LTC extracts were analyzed by WB with anti-LC3 and β-actin antibodies. (B) LC3 puncta in TIVE and LTC. TIVE cells and LTC fixed with 4% paraformaldehyde were subjected to IFA for LC3 (red). Arrowheads indicate the presence of LC3 puncta. Boxed areas are enlarged. Magnification, ×40. (C) LC3 puncta were quantified in 12 fields containing 10 to 40 cells, and the percentage of cells with LC3 puncta was calculated. The data represent means ± SEM. Statistical analysis was conducted using a two-tailed Student t test. ***, P < 0.005.** (D and E) Autophagy was assessed in both TIVE cells and LTC via Cyto-ID green fluorescence staining. The cells were grown in media containing all supplemental growth factors (full media) (D) or starved overnight in HBSS (E). Cell nuclei were visualized by DAPI (blue). Boxed areas are enlarged. Magnification, ×40.
Keap1 protein level in TIVE cells and LTC. TIVE cell and LTC extracts were analyzed by WB using a Keap1 antibody. β-Actin was used as a loading control. (B) Keap1 mRNA expression in TIVE cells and LTC. Real-time PCR was performed on cDNA from TIVE cells and LTC using Keap1 primers and normalized to β-actin. Each bar represents the mean fold increase in Keap1 gene expression ± SEM compared to expression in TIVE cells (set to 100%) from 6 independent experiments. (C) Effect of the proteosome inhibitor MG132 on Nrf2 and Keap1 expression. LTC were treated overnight with a 10 μM concentration of the proteosome inhibitor MG132. As expected, Nrf2 protein levels increased after MG132 treatment (Fig. 8C, top blot). In contrast, we observed a decrease in Keap1 expression in MG132-treated cells (Fig. 8C, second blot). This decrease in Keap1 correlated with a substantial increase in SQSTM1 expression (Fig. 8C, third blot). Taken together, these results showed that Keap1 is degraded by a proteosome-independent mechanism in KSHV+ LTC and that Keap1 and SQSTM1 expression are inversely correlated.

Keap1 is ubiquitinated in LTC and interacts with SQSTM1. Quinone treatment causes Keap1 polyubiquitination, leading to its proteosome-independent degradation (74). Cells transfected with wild-type (WT) ubiquitin or a construct containing only Lys-63–ubiquitin, and not Lys-48–ubiquitin, led to Keap1 polyubiquitination, indicating that the type of ubiquitination was through Lys-63–ubiquitin chains (74). In addition, the SQSTM1 ubiquitin-associated (UBA) domain mediated the interaction of SQSTM1 with ubiquitinated proteins, preferentially binding to Lys-63–polyubiquitinated substrates (16) As a result, deletion of SQSTM1 leads to the accumulation of Lys-63-polyubiquitinated proteins, indicating that SQSTM1 targets Lys-63-polyubiquitinated proteins for degradation (19). To determine if the reduction of Keap1 in LTC was mediated through SQSTM1, we first examined whether Keap1 was Lys-63 polyubiquitinated in LTC. Keap1 protein was immunoprecipitated from TIVE cell and LTC lysates and WB was performed with Lys-63–polyubiquitin-specific antibodies, and we observed higher levels of Lys-63-polyubiquitinated Keap1 in LTC than in TIVE cells (Fig. 8D, second blot, arrows). To control for the efficiency of immunoprecipitation, we performed Keap1 WB (Fig. 8D, top blot), and we observed the presence of Keap1 in both TIVE cell and LTC immunoprecipitates (arrowhead). Interestingly, we observed a second Keap1 band at a higher molecular weight in LTC (arrow). Taken together, these results demonstrated that LTC exhibit higher levels of Lys-63 polyubiquitination than do TIVE cells.

SQSTM1 recognizes ubiquitinated proteins through its UBA domain (18). In addition, it has been shown in vivo that SQSTM1 binds preferentially to Lys-63-polyubiquitinated substrates and sequesters them to inclusion bodies for autophagosome-mediated degradation (16). To determine whether SQSTM1 interacts with Keap1 in LTC, WB was performed from the immunoprecipitated proteins with anti-SQSTM1 antibody, and as shown in Fig. 8D (bottom blot), SQSTM1 communoprecipitated with Keap1 at a higher degree in LTC than in TIVE cells (arrowhead). Collectively, these results demonstrated that Keap1 is Lys-63 polyubiquitinated in LTC and interacts with SQSTM1.

SQSTM1 phosphorylation at Ser-351 and -403 is increased in LTC. Phosphorylation of SQSTM1 regulates its interaction with other proteins. Particularly, phosphorylation of Ser–403, located in its UBA domain, mediates its interaction with Lys-63-polyubiquitinated proteins, inducing their degradation (19, 75, 76). Furthermore, SQSTM1 phosphorylation on Ser-351 has a crucial role in SQSTM1 interaction with Keap1 (9, 17). We determined the level of phosphorylation of both serines in TIVE cells and LTC (Fig. 8E). We observed 16- and 2-fold–higher levels of phosphorylation of SQSTM1 at Ser-351 and Ser-403, respectively, in LTC cells. This decrease in protein level was not due to a decrease in mRNA levels, as we did not observe a significant difference in the quantity of Keap1 mRNA between the two cell lines (Fig. 8B). To determine if the proteasome was involved in Keap1 degradation, LTC were treated overnight with a 10 μM concentration of the proteosome inhibitor MG132. As expected, Nrf2 protein levels increased after MG132 treatment (Fig. 8C, top blot). In contrast, we observed a decrease in Keap1 expression in MG132-treated cells (Fig. 8C, second blot). This decrease in Keap1 correlated with a substantial increase in SQSTM1 expression (Fig. 8C, third blot). Taken together, these results showed that Keap1 is degraded by a proteosome-independent mechanism in KSHV+ LTC and that Keap1 and SQSTM1 expression are inversely correlated.

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cells than in TIVE cells (Fig. 8E, first and second blots). Taken together, these results showed that SQSTM1 phosphorylation and Keap1 polyubiquitination, which increase their mutual affinity, are enhanced in LTC compared to TIVE cells.

**SQSTM1 participates in Nrf2 activation in LTC.** To confirm the role of SQSTM1 in Nrf2 activation in LTC, we reduced SQSTM1 expression using shRNA. LTC were transduced with lentiviral vectors expressing an shRNA control (shC) or shRNA against SQSTM1 (shSQSTM1). To minimize potential off-target effects, two independent shSQSTM1 vectors were used (shSQSTM1 no. 1 and no. 2). The knockdown efficiency was confirmed by WB, and we observed 40% and 55% decreases in SQSTM1 protein with shSQSTM1 no. 1 and no. 2, respectively (Fig. 9A, first blot). Consistent with a role for SQSTM1 in Nrf2 activation, SQSTM1 knockdown also reduced tNrf2 and pNrf2 levels (Fig. 9A, second and third blots, lanes 1 to 3). Specifically, we observed ~30% and 50% decreases in pNrf2 and ~20% and 30% decreases in tNrf2 with shSQSTM1 no. 1 and no. 2, respectively. To determine whether SQSTM1 participated in Nrf2 activation through the degradation of Keap1, we determined the level of Keap1 in shC and shSQSTM1 LTC. The decrease of SQSTM1 expression was concomitant with an increase in Keap1 levels: we observed 1.4- and 2.8-fold increases in Keap1 in shSQSTM1 no. 1 and no. 2, respectively. To confirm the specificity of shSQSTM1, we monitored the expression levels of the transcription factor NF-κB/p65 and observed no change (Fig. 9A, fifth blot). These data strongly suggested that SQSTM1 participates in Keap1 degradation in a dose-dependent manner and leads to Nrf2 activation in LTC.

**Autophagy participates in SQSTM1 regulation and Nrf2 activation in LTC.** To clarify the role of autophagy in SQSTM1 and Nrf2 activation, we chemically modulated the level of autophagy in LTC. The most typical trigger of autophagy is nutrient starvation. As shown in Fig. 7E, overnight treatment of LTC with HBSS efficiently induced autophagy as measured by Cyto-ID staining. The induction of autophagy by HBSS correlated with an ~50% decrease in SQSTM1 levels (Fig. 9B, upper left side, lanes 1 and 2). Interestingly, the decrease in SQSTM1 correlated with an ~1.1-fold increase in Keap1 expression (Fig. 9B, fourth left blot, lanes 1 and 2) and concomitant 60% and 40% decreases in pNrf2 and tNrf2 levels, respectively (Fig. 9B, second and third left blots, lanes 1 and 2).

Wortmannin is an inhibitor of phosphatidylinositol 3-kinase (PI3K), whose activity is required for autophagy (77). Overnight treatment of LTC with wortmannin induced a 2-fold increase in SQSTM1 expression (Fig. 9C, first right blot, lanes 1 and 2). As a consequence of the stabilization of SQSTM1, we observed an ~40% decrease in Keap1 levels (Fig. 9C, fourth right blot, lanes 1 and 2), while pNrf2 and tNrf2 levels were induced 1.6- and 1.4-fold, respectively (Fig. 9C, second and third right blots, lanes 1 and 2). All together, these results demonstrated that autophagy inhibition in LTC leads to SQSTM1 accumulation and, consequently, Nrf2 activation, whereas autophagy induction has the opposite effect.

**KSHV vFLIP activates Nrf2 and increases SQSTM1 expression.** KSHV latent infection of endothelial cells results in reexpression of lytic gene expression and constitutive expression of its latency locus, which includes LANA-1, viral cyclin, vFLIP, and kaposin. To determine if one or more latent KSHV proteins participate in Nrf2 activation during latency, we performed a luciferase reporter experiment with HEK293T cells transfected with a reporter plasmid in which the luciferase gene is under ARE control (HBSS). WB analysis was performed with antibodies against SQSTM1, pNrf2, tNrf2, and Keap1. p65 was used as a control for shSQSTM1 off-target effects. β-Actin was used as a control loading. The fold change of each protein is relative to the levels in shC LTC (set to 1). (B) Effect of autophagy induction on SQSTM1 levels and Nrf2 activation. TIVE cells or LTC were cultured overnight in growth medium (EGM-2) or starved in Hanks' balanced salt solution (HBSS). WB analysis was performed with antibodies against pNrf2, tNrf2, SQSTM1 and Keap1. β-Actin was used as a loading control. The fold change of each protein is relative to the levels in the EGM condition (set to 1). (C) Effect of autophagy inhibition on SQSTM1 levels and Nrf2 activation. TIVE cells or LTC were mock treated or treated overnight with 0.5 μM wortmannin (Wort). WB analysis was performed with antibodies against pNrf2, tNrf2, SQSTM1, and Keap1. β-Actin was used as a loading control. The fold change of each protein is relative to the levels without drug treatment (set to 1).
FIG 10. Role of vFLIP in Nrf2 activation and SQSTM1 expression. (A) Effect of KSHV latency proteins on an ARE-luciferase reporter. HEK293T cells were transfected with 1 μg of the ARE-luciferase plasmid and 0.5 μg of β-galactosidase reporter in the presence of 1 μg of an empty vector (pSIN) or vectors expressing vFLIP, viral cyclin, LANa-1, or kaposin. Thirty-six hours after transfection, luciferase and β-galactosidase activities were measured in triplicate; the data represent the mean fold induction ± SD. Statistical analysis was conducted using a two-tailed Student t test. ***, P < 0.005. (B) Effect of vFLIP on Nrf2 activation. HMVEC-d were transfected with a control lentivector (pSIN) or a lentivector expressing vFLIP. Three days posttransduction, nuclear and cytoplasmic fractions were isolated and subjected to WB analysis using pNrf2 and tNrf2 antibodies. Tubulin and TATA-binding protein 1 (TBP-1) were used to demonstrate the purity and equal loading of cytoplasmic and nuclear fractions, respectively. (C) Effect of vFLIP on SQSTM1 expression. HMVEC-d were transfected with a control lentivector (pSIN) or a lentivector expressing vFLIP. Three days later, cell extracts were analyzed by WB with an anti-SQSTM1 antibody. Tubulin was used as a loading control.

cyclin, LANa-1, and kaposin did not induce Nrf2-dependent luciferase activity, indicating that vFLIP activates the Nrf2 pathway. This is in concordance with a recent study by Gjyshi et al. (49), which found that vFLIP overexpression in HEK293T cells induced Nrf2 protein expression.

To confirm this result, we analyzed the subcellular localization of tNrf2 and pNrf2 in HMVEC-d transfected with an empty lentivector or a lentivector expressing vFLIP by WB (Fig. 10B). Compared to the empty vector, vFLIP expression induced ~2.8- and 10-fold increases in pNrf2 in the cytoplasmic and nuclear fractions, respectively (Fig. 10B, first blot, compare lane 2 to 1 and lane 4 to 3). In addition, vFLIP increased the quantity of tNrf2 in the cytoplasm 1.8-fold and in the nuclear fraction 6.5-fold (Fig. 10B, second panel, compare lane 2 to 1 and lane 4 to 3). Tubulin and TATA-binding protein 1 (TBP-1) were used as purity and loading controls for each fraction (Fig. 10B, third and fourth blots). Collectively, these results demonstrated that vFLIP expression induced the phosphorylation, nuclear translocation, and transcriptional activity of Nrf2.

Our results showing that autophagy is inhibited and SQSTM1 protein accumulates in LTC (Fig. 6 and 7), together with the ability of vFLIP to negatively regulate autophagy during KSHV latency (71, 72), prompted us to investigate the role of vFLIP in SQSTM1 accumulation in HMVEC-d (Fig. 10C). Compared to empty-vector-transduced cells, HMVEC-d transduced with vFLIP showed a significant (~4.5-fold) increase in SQSTM1 protein levels (Fig. 10C, lanes 1 and 2). Taken together, these results demonstrated that KSHV latent protein vFLIP activated Nrf2 in endothelial cells, which is in part probably mediated by its ability to induce SQSTM1 expression.

DISCUSSION

Our comprehensive studies presented here demonstrate for the first time that the transcription factor Nrf2 is constitutively activated in KSHV latently infected LTC (Fig. 2 to 4). From our studies, together with recent literature, we derive a model of complex interrelated pathways for Nrf2 activation in KSHV latently infected LTC (Fig. 11), which is summarized below.

Our results show that the KSHV latent protein vFLIP plays crucial roles in the observed constitutive Nrf2 activation in LTC by its ability to inhibit autophagy and activate COX-2 and PGE2 secretion, as well as by inducing the autophagic SQSTM1 protein. Our studies demonstrate that Nrf2 is activated through the non-canonical pathway, independent of ROS induction but dependent on the protein SQSTM1 (Fig. 5 and 9), and vFLIP induces SQSTM1 accumulation (Fig. 10). In LTC, SQSTM1 is phosphorylated on Ser-305 and -403 by a mechanism that remains to be determined, and Keap1 is Lys-63 polyubiquitinated. These modifications enhance the interaction between Keap1 and SQSTM1, leading to Keap1 degradation (Fig. 8) and Nrf2 activation. This activation via the Ser-40 phosphorylation of Nrf2 is mediated by PKCζ, which is, in turn, activated by the KSHV-induced COX-2/ PGE2 signal pathway (Fig. 2). Our previous study has demonstrated that vFLIP induces COX-2 gene and protein expression and, consequently, PGE2 secretion in LTC (53). Nrf2 is then stabilized and translocates into the nucleus, where it activates ARE-dependent promoters of antioxidant genes (NQO1, γGCSH, and xCT genes), as well as genes involved in KSHV pathogenesis (VEGF-A and IL-6 genes) (Fig. 4). Nrf2 also induces SQSTM1 gene expression, resulting in a feed-forward loop of constitutive Nrf2 activation, which plays roles in KSHV pathogenesis.

In the following sections, we summarize the potential implication of Nrf2 in latently infected LTC.

Nrf2 activation during viral infection and the oxidative stress pathway. We have very recently demonstrated that Nrf2 is activated during early de novo infection of HMVEC-d through the canonical, oxidative stress-dependent pathway (49). In the present study, we focused on a prolonged KSHV latency model and found that Nrf2 activation in LTC is mediated through the non-canonical, oxidative stress-independent pathway. The activation of Nrf2 independently of oxidative stress during viral infection has previously been reported. HBV activation of Nrf2 was triggered by the viral regulatory proteins HBx and LHBs (78). The activation of
Nrf2 by HBV was not affected by the radical scavenger NAC, suggesting that a virus-induced increase in ROS levels was not involved in Nrf2 activation. Rather, HBV-induced Nrf2 activation was triggered by c-Raf and MEK (78). HCV activation of Nrf2 was dependent on both oxidative stress and calcium responses (79); 5 distinct HCV proteins (core protein, E1, E2, NS4B, and NS5A) activated Nrf2, and this activation was only partially dependent on ROS production (80). In addition, infection of human foreskin fibroblasts with human cytomegalovirus (HCMV/HHV-5) led to the activation of Nrf2 (81). While a previous study reported that HCMV infection led to an increased level of ROS, Nrf2 activation was not mediated by oxidative stress but was rather due to HCMV gene expression (81,82). Finally, Marburg virus was shown to hijack the Nrf2 pathway through its protein VP-24 (83, 84). Two independent studies demonstrated that VP-24 directly interacted with Keap1, releasing Nrf2 from Keap1 inhibition. It is interesting to note the vastly diverse mechanisms of virus-mediated Nrf2 activation, through either ROS-dependent or independent mechanisms, arguing for an important role for Nrf2 in the viral life cycle.

**Role of phosphorylation in Nrf2 activation.** Different kinases have been shown to be involved in Nrf2 activation. In a previous study, we demonstrated that Src and the atypical PKCζ were involved in Nrf2 Ser-40 phosphorylation during de novo KSHV infection of endothelial cells (49). In the present study, we established that atypical PKCζ was also involved in Nrf2 phosphorylation in LTC cells during prolonged latency. While some kinases directly regulate Nrf2 phosphorylation, other kinases regulate its transcriptional activity and could act indirectly to activate Nrf2. For example, Nrf2 phosphorylation induced by HCV is mediated through p38 and JNK MAPKs (79). PKC was involved in...
Nrf2 activation induced by the HCV core, E1, E2, NS4B, and NS5A proteins, whereas casein kinase 2 (CK2) and phosphoinositide-3 kinase activation of Nrf2 was specific to the induction by core protein and NS5A (80). In contrast, HCMV-induced Nrf2 activity was reduced in the presence of a CK2 inhibitor (81).

In addition to Nrf2 phosphorylation, we observed an increase in SQSTM1 phosphorylation on Ser-351 and -401. Mass spectrometry analysis has shown that SQSTM1 is phosphorylated on several serine residues (75). Phosphorylation of Ser-403, located in the UBA domain, enhanced SQSTM1 affinity for polyubiquitinated chains and promoted the formation of SQSTM1 bodies (75). CK2 and TANK-binding kinase-1 (TBK-1) have been proposed to directly phosphorylate SQSTM1 on Ser-403 (75, 76). SQSTM1 can also be phosphorylated on Ser-351, located in its KIR domain (9). While the mammalian target of the rapamycin complex 1 (mTORC1) has been shown to directly phosphorylate Ser-351, inhibitors of mTORC1 only partially decrease SQSTM1 phosphorylation in human hepatocellular carcinoma cells, suggesting that other kinases are also involved in Ser-351 phosphorylation (9). Interestingly, several KSHV proteins have been shown to activate the mTOR pathway, and mTOR inhibitors were proposed to have antitumor activity against Kaposi’s sarcoma (85, 86). Future studies are required to determine the role of mTOR in Nrf2 activation and to examine the mechanism of SQSTM1 accumulation and phosphorylation in KSHV-infected cells.

Viral infection and antioxidant gene expression. We observed that Nrf2 activation in LTC leads to the induction of the NQO1, γGCSH, and xCT antioxidant genes. KSHV has evolved different mechanisms to facilitate its persistence in an environment of oxidative stress. xCT exchanges intracellular glutamate for extracellular cystine, which is reduced into cysteine and incorporated into glutathione, protecting the cells from oxidative stress and death (87). xCT expression is regulated by Nrf2 through an ARE in its promoter, and its expression is increased in more advanced KS lesions (88, 89). A KSHV microRNA (miR12-11) up-regulates xCT expression in macrophages and endothelial cells through suppression of Bach-1, a negative regulator of Nrf2 (89). KSHV-infected macrophages are protected from death triggered by reactive nitrogen species (RNS). Future studies are necessary to determine the synergistic role of miR12-11 with the activation of Nrf2 in regulating ARE-containing promoters.

vFLIP potently prevents the accumulation of superoxide in endothelial cells and consequently prevents apoptosis induced by oxidative stress (90). vFLIP upregulates the expression of manganese superoxide dismutase (MnSOD) through activation of the transcription factor NF-κB (90). Consequently, MnSOD reduces cellular oxidative stress by eliminating superoxide molecules. In addition to this mechanism, in this study, we showed that vFLIP could repress oxidative stress through the activation of Nrf2. Our results suggest that the reduced level of ROS in LTC is likely the result of strong Nrf2 activation and increased expression of numerous antioxidant genes, including, but not limited to, the NQO1, γGCSH, and xCT genes.

Level of oxidative stress and viral gene regulation. Modulation of oxidative stress directly regulates the life cycles of viruses. The oxidative stress triggered by herpes simplex virus 1 (HSV-1) and Epstein-Barr virus (EBV) has opposite outcomes on their replication: while HSV-1 replication was inhibited by the antioxidant compound ebselen, H2O2 participated in the maintenance of EBV latency by reducing the expression of EBV immediate early lytic genes (91, 92). In a previous study, we demonstrated that KSHV binding to endothelial cells induced a very early increase in ROS production necessary for viral entry by macrophagocytosis (48). Oxidative stress has also been shown to regulate KSHV reactivation from latency in PEL and endothelial cells (93–95). An increase in intracellular H2O2 production and the activation of ERK1/2, Jun N-terminal protein kinase (JNK), and p38 MAPK pathways participated in KSHV lytic replication following exposure to tetradecanoyl phorbol acetate (TPA), cytokines, and hypoxia induction (93–95).

KSHV has evolved mechanisms to modulate many cellular pathways to favor the maintenance of its latency. In a previous study, we observed that Nrf2 colocalizes with the KSHV genome as well as with the latency protein LANA-1 after de novo infection (49). We observed that Nrf2 knockdown decreased the early KSHV lytic gene expression burst, and we suggested that Nrf2 could play roles in the establishment of latency. In LTC, we observed that Nrf2 activation leads to the expression of antioxidant genes that could be among the mechanisms used by KSHV to prevent its reactivation. Further detailed studies are essential to decipher the role of Nrf2 activation in the maintenance of KSHV latency.

Nrf2 and KSHV pathogenesis. In addition to its antioxidant functions, Nrf2 was more recently shown to be involved in the regulation of genes participating in oncogenesis. Numerous cytokines and angiogenic factors are highly expressed in KS lesions and participate in oncogenesis (96, 97). In a similar fashion, these secreted factors are highly expressed in LTC (54). In the present study, we focused on the expression of IL-6 and VEGF-A and demonstrated that their expression is partially dependent on Nrf2. IL-6, a cytokine involved in B cell growth and differentiation, is implicated in PEL pathogenesis and in the development of KS lesions (98). We confirmed that IL-6 expression is increased in LTC, and we demonstrated that this expression was partially dependent on Nrf2. Interestingly, vFLIP has been shown to increase IL-6 expression (99, 100). While a previous study showed that IL-6 expression induced by vFLIP is dependent on NF-κB and AP1 (99), our results indicate that vFLIP also activates the transcription factor Nrf2, potentially contributing to the upregulation of IL-6. Furthermore, KS lesions are histologically characterized by neovascularization with a high level of VEGF-A (101). We confirmed an increased level of VEGF-A expression in LTC and demonstrated that this expression was dependent on Nrf2 activation. In addition, Nrf2 participates in endothelial and cancer-associated angiogenesis (43, 102, 103). We therefore propose that inhibition of Nrf2 could inhibit KS-associated angiogenesis.

We observed that Nrf2 upregulates SQSTM1, which, in turn, participates in Nrf2 activation, creating a positive-feedback loop (Fig. 11). Because of this, we propose a role for SQSTM1 in LTC oncogenesis. SQSTM1 accumulation in aggregates is commonly observed in human tumors such as hepatocellular carcinoma and malignant glioma (104–107). Furthermore, the Ser-351 phosphorylation of SQSTM1 was found to have a crucial role in this Nrf2 activation and the growth of tumor cells (9). Collectively, our results suggest that KSHV activation of Nrf2 participates in the tumorigenesis of KS lesions.

In conclusion, the work presented here describes, for the first time, the constitutive activation of Nrf2 in KSHV long-term-infected endothelial cells and demonstrates that this activation is dependent on the interaction of SQSTM1 with Keap1, followed by...
Keap1 degradation (Fig. 11). As induction of ROS has been shown to activate the KSHV lytic cycle (93–95), results of our studies suggest that KSHV has evolved to use the noncanonical pathway of SQSTM1 to inactivate Keap1 and activate Nrf2 during latency in the LTC rather than the canonical ROS pathway. Our studies provide the initial framework of the different pathways of Nrf2 activation in latently infected endothelial cells. Further detailed analyses of these pathways in endothelial as well as PEL cells are essential to fully comprehend the role of Nrf2 in KSHV latency and pathogenesis, which is beyond the scope of this work. Nevertheless, our studies give an initial indication that inhibiting Nrf2 signaling may serve as a potential therapeutic approach to ameliorate KS pathology.

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