# The Kaposi's Sarcoma-associated Herpes Virus G Protein-coupled Receptor Up-Regulates Vascular Endothelial Growth Factor Expression and Secretion through Mitogen-activated Protein Kinase and p38 Pathways Acting on Hypoxia-inducible Factor 1α

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# The Kaposi's Sarcoma-associated Herpes Virus G Protein-coupled Receptor Up-Regulates Vascular Endothelial Growth Factor Expression and Secretion through Mitogen-activated Protein Kinase and p38 Pathways Acting on Hypoxia-inducible Factor 1$\alpha$[1]

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## ABSTRACT

**The elucidation of the molecular mechanisms governing the transition from a nonangiogenic to an angiogenic phenotype is central for understanding and controlling malignancies. Viral oncogenes represent powerful tools for disclosing transforming mechanisms, and they may also afford the possibility of investigating the relationship between transforming pathways and angiogenesis. In this regard, we have recently observed that a constitutively active G protein-coupled receptor (GPCR) encoded by the Kaposi's sarcoma-associated herpes virus (KSHV)/human herpes virus 8 is oncogenic and stimulates angiogenesis by increasing the secretion of vascular endothelial growth factor (VEGF), which is a key angiogenic stimulator and a critical mitogen for the development of Kaposi's sarcoma. Here we show that the KSHV GPCR enhances the expression of VEGF by stimulating the activity of the transcription factor hypoxia-inducible factor (HIF)-1$\alpha$, which activates transcription from a hypoxia response element within the 5′-flanking region of the VEGF promoter. Stimulation of HIF-1$\alpha$ by the KSHV GPCR involves the phosphorylation of its regulatory/inhibitory domain by the p38 and mitogen-activated protein kinase (MAPK) signaling pathways, thereby enhancing its transcriptional activity. Moreover, specific inhibitors of the p38 (SKF86002) and MAPK (PD98059) pathways are able to inhibit the activation of the transactivating activity of HIF-1$\alpha$ induced by the KSHV GPCR, as well as the VEGF expression and secretion in cells overexpressing this receptor. These findings suggest that the KSHV GPCR oncogene subverts convergent physiological pathways leading to angiogenesis and provide the first insight into a mechanism whereby growth factors and oncogenes acting upstream from MAPK, as well as inflammatory cytokines and cellular stresses that activate p38, can interact with the hypoxia-dependent machinery of angiogenesis. These results may also help to identify novel targets for the development of antiangiogenic therapies aimed at the treatment of Kaposi's sarcoma and other neoplastic diseases.**

## INTRODUCTION

Angiogenesis is essential in many physiological processes, including embryonic development, wound healing, and tissue regeneration, and also represents a critical pathogenic mechanism in a number of human diseases, including cancer (1, 2). Indeed, tumor-associated neovascularization is now viewed as a central pathogenic step in tumor development, and angiogenesis might in fact be a prerequisite for tumor growth, invasion, and metastasis (1, 2). Emerging evidence suggests that neovascularization is a highly complex process that results from an enhanced availability of angiogenic stimulators and a concomitant decrease in the activity of angiostatic factors (1, 2). In this regard, VEGF,[3] an endothelial cell-specific mitogen, is among the most potent angiogenic stimulators, and the use of blocking monoclonal antibodies, antisense approaches, and gene knockout models has revealed that VEGF plays a central role in the regulation of neovascularization (3–5). Deregulated expression of VEGF is believed to contribute to the development of solid tumors by promoting tumor angiogenesis as well as to the etiology of a number of diseases that are characterized by abnormal growth of blood vessels (3–7).

The complexity of the mechanism controlling VEGF expression has just begun to be appreciated. For example, tissue hypoxia and exposure to compounds that mimic cellular hypoxia, such as cobalt ions, can induce a remarkable increase in VEGF expression (8) by enhancing the stability (9, 10) and transcriptional activity (11) of HIF-1$\alpha$ and by prolonging the half-life of VEGF transcripts (12). VEGF expression is also potently stimulated under normoxic conditions by growth factors and cellular stresses; by a variety of cytokines, such as IL-1, tumor necrosis factor $\alpha$, and IL-8; and by tumorigenic genes, including *ras*, *raf*, and *src* (6, 7, 13–15). Recent efforts are now helping to unveil the nature of the signaling pathways linking these oncogenes with the transcription factor HIF-1$\alpha$ (16–18).

Of interest, KS, the most common AIDS-associated neoplasm, is a neovascular tumor that has been shown to be strictly dependent on angiogenic stimulators, including VEGF (19, 20). For example, spindle cells, the dominant cell type of KS lesions, secrete a variety of proinflammatory and angiogenic factors (19, 20), among which VEGF is unique for its extensive impact on KS pathogenesis: it is expressed at elevated levels by KS spindle cells and is suggested to stimulate their growth by an autocrine mechanism (20). Compelling evidence points to KSHV or human herpes virus 8 as the infectious etiological agent of KS (21): (*a*) KSHV infection precedes KS development and overlaps with KS risks (22, 23); (*b*) KSHV infects KS spindle and endothelial cells in the KS lesion (23); and (*c*) KSHV infects and transforms cells thought to be premalignant for KS (24). Because KSHV is highly homologous to two viruses implicated in malignancies, EBV and *Herpesvirus saimiri*, it is likely that KSHV is an oncogenic virus involved in the pathogenesis of KS. Indeed, molecular characterization of the KSHV genome has shown the existence of several genes that bear potential for KS pathogenesis (22, 23). These include genes that could lead to KS angiogenesis by inducing an angiogenic phenotype in KSHV-infected cells or by

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpes virus; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; HIF, hypoxia-inducible factor; HRE, hypoxia response element; IL, interleukin; GST, glutathione *S*-transferase; GFP, green fluorescence protein; MDCK, Madin-Darby canine kidney; JNK, c-Jun-$NH_2$-terminal kinase; MEK, mitogen-activated protein/extracellular signal-regulated kinase; MKK6, MAP kinase kinase 6; MEKK1, MAP, kinase kinase kinase 1; TAD, transactivation domain; HID, HIF-1$\alpha$ regulatory/inhibitory domain; ATF2, activating transcription factor 2; HA, hemagglutinin.

promoting inflammation or the production of angiogenesis-activating inflammatory cytokines (23, 25–27).

One of these KSHV angiogenic genes, encoded by the open reading frame 74, is a constitutively active CXC chemokine GPCR (KSHV GPCR), which is a close homologue of the one of the receptors, CXCR2, for the human angiogenic chemokine IL-8 (28, 29). KSHV GPCR contains a V138D mutation in the highly conserved DRY sequence among the GPCRs, which results in an increased agonist-independent receptor activity (29). Indeed, this KSHV GPCR harbors transforming and angiogenic potential and is sufficient to induce the secretion of VEGF from KSHV GPCR-expressing cells (25). Furthermore, expression of KSHV GPCR in transgenic mice leads to the occurrence of KS-like lesions with VEGF-driven angiogenesis and spindle cell proliferation (30, 31). These findings suggest that KSHV GPCR might participate in KS pathogenesis, driving spindle cell formation, growth, and angiogenesis in a paracrine fashion by inducing the expression and secretion of VEGF (25, 30, 31).

The complexity of the signal transduction routes initiated by GPCRs has begun to be unraveled (32–34). In this study, we have investigated the intracellular pathways controlling VEGF expression by the KSHV GPCR. These observations could provide a unique opportunity to help elucidate the still poorly understood molecular mechanism(s) whereby viral and cellular oncogenes regulate the expression and secretion of VEGF and, ultimately, govern tumor-induced angiogenesis.

## MATERIALS AND METHODS

**Expression Plasmids.** The full-length VEGF promoter and a truncated VEGF promoter lacking a HRE were excised, respectively, from the pGL2.VEGF.Kpn.Luc and pGL2.VEGF.Pst.Luc plasmids (kindly provided by G. L. Semenza; Johns Hopkins University School of Medicine, Baltimore, MD) and subcloned into the pGL3 Promoter vector (Promega) as an $Asp^{718}$-*Xho*I fragment, thus generating the pGL-VEGF/K and pGL-VEGF/P reporter plasmids, respectively. The wild-type HRE oligonucleotide and the mutated HRE oligonucleotide containing a CGT to AAA mutation in the HIF-1 binding site (35) were introduced into the pGL3 Promoter vector, generating the pGL-HRE and pGL-HREmut reporter plasmids, respectively.

pBS KS-HIF-1$\alpha$, obtained from American Type Culture Collection (EST 22495), was subcloned into the pCEFL vector as a *Bam*HI-*Xba*I fragment, thus generating the pCEFL HIF-1$\alpha$ expression plasmid. pALTER-1 HIF-1$\beta$, generously provided by E. Bradfield (Northwestern University Medical School, Chicago, IL), was subcloned into the pCEFL vector as a *Hin*dIII-*Xba*I fragment, thus generating the pCEFL HIF-1$\beta$ expression plasmid. The TADs of HIF-1$\alpha$ (amino acids 531–826; Ref. 36) and Elk-1 (amino acids 307–428; Ref. 37) transcription factors were expressed as GAL4 fusion proteins after amplification of the corresponding DNA sequences and subcloning in frame within a pcDNA III derivative expressing the DNA binding domain of the yeast transcription factor GAL4 (amino acids 1–147). The pGL3.TATA GAL-driven luciferase reporter plasmid was constructed as described previously (38). The GST-HIF-1$\alpha$ fusion protein carrying amino acids 568–702 of the HID was obtained by PCR using human HIF-1$\alpha$ cDNA as a template. The fragment was then subcloned between the *Bam*HI and *Eco*RI site of pGEX4T-3 (Pharmacia), in frame with the *GST* gene. Sequences of oligonucleotide primers will be provided on request. Other expression plasmids have already been described (38, 39).

**Cell Lines and Transfection.** Stable transfections of NIH 3T3 cells were performed using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer's protocol. NIH 3T3 fibroblasts stably expressing the KSHV GPCR or GFP, designated NIH-KS.GPCR or NIH-GFP, respectively, were maintained as described previously (25). For transient transfections for reporter gene assays, NIH 3T3 cells and MDCK cells were transfected by using the calcium-phosphate precipitation technique. COS-7 cells were transfected by the DEAE-dextran method. In each experiment, the total amount of DNA was adjusted to 3–5 $\mu$g/plate using pCEFL GFP.

**Northern Blot Analysis.** Total RNA was isolated from NIH-KS.GPCR or NIH-pCEFL using Trizol (Life Technologies, Inc.) according to the manufacturer's instructions, separated by electrophoresis on a 2% denaturing glyoxal-DMSO gel (10 mg RNA/lane), and transferred to a Nytran-N nylon membrane (Schleicher & Schuell). The cDNA probe used for analysis of the VEGF and $\beta$-actin mRNA was prepared using reverse transcription-PCR products obtained from mouse total RNA as a template. Probes were labeled with [$\alpha$-$^{32}$P]dCTP using a Random Primer DNA labeling kit (Boehringer Mannheim), and RNA hybridization was performed as described previously (39).

**Reporter Gene Assays.** NIH-KS.GPCR, NIH-GFP, and MDCK cells were transfected with the different expression plasmids together with 0.5 $\mu$g of pcDNAIII-$\beta$gal, a plasmid expressing the enzyme $\beta$-galactosidase, and 0.25 $\mu$g of either pGL-VEGF/K, pGL-VEGF/P, pGL-HRE, or pGL-HREmut, reporter plasmids expressing the luciferase gene under the control of the VEGF promoter or a HRE. After overnight incubation, cells were washed once with PBS and kept for approximately 36 h in DMEM supplemented with 10% calf serum (NIH 3T3 cells) or FCS (MDCK cells). Cells were then lysed using reporter lysis buffer (Promega). Luciferase activity and $\beta$-galactosidase activity present in cellular lysates were assayed as described previously (38). The data for luciferase activity, normalized by the $\beta$-galactosidase activity, are expressed as the fold induction with respect to control cells and are the mean $\pm$ SE of triplicate samples from a typical experiment.

COS-7 cells were transfected with different expression plasmids together with 0.5 $\mu$g of pcDNAIII-$\beta$gal, 0.5 $\mu$g of pGL3.TATA GAL, and 0.5 $\mu$g of the pcDNAIII GAL4/HIF expression plasmid. After overnight incubation, COS-7 cells were washed once with PBS and kept for approximately 36 h in DMEM supplemented with 10% FCS. Cells were then lysed using reporter lysis buffer (Promega). Additional DNAs were added to the transfection mixtures as indicated in each figure. Luciferase activity and normalization for transfection efficiency were done as described above.

**Kinase Assays.** The phosphorylating activity of epitope-tagged MAPK, JNK p38$\alpha$, p38$\gamma$, and p38$\delta$ was assayed as described previously (38). *In vitro* kinase assays were performed using either 5 $\mu$g of myelin basic protein (Sigma; for MAPK, p38$\alpha$, and p38$\gamma$), 5 $\mu$g of purified bacterially expressed GST-ATF2 (for JNK and p38$\delta$), or 5 $\mu$g of purified, bacterially expressed GST-HID as substrates. Samples were analyzed by SDS-gel electrophoresis on 12% acrylamide gels, and autoradiography was performed with the aid of an intensifying screen. GST-fusion proteins were expressed in bacteria and purified by affinity chromatography using standard procedures.

**Western Blot.** HA immunoprecipitates from transiently transfected COS-7 cells carrying HA-MAPK, JNK, extracellular signal-regulated kinase 5, p38$\alpha$, p38$\gamma$, and p38$\delta$ DNAs were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis using an anti-HA monoclonal antibody as described previously (38).

**Kinase Inhibitors.** The p38 inhibitor (SKF86002) and the MEK inhibitor (PD98059; Calbiochem, Inc.) were dissolved in DMSO as 1000-fold concentrated stock solutions and used at the indicated concentrations. Cells were then treated with either four doses of SKF86002 (one every 3 h for 12 h) or a single dose of PD98059 (for 8 h) before lysis. In each case, the final concentration of DMSO was $<$0.1%.

**ELISA.** Conditioned media from either NIH-KS.GPCR cells or NIH-GFP cells were collected after 24 h. VEGF secretion was detected in the media using a VEGF immunoassay kit (R&D Laboratories) as indicated in the standard protocol provided by the manufacturer.

## RESULTS

**The KSHV GPCR Induces VEGF Expression by Activating Transcription from the VEGF Promoter through the HIF-1 Response Element.** We have previously shown that NIH 3T3 cells stably expressing the KSHV GPCR (NIH-KS.GPCR) secrete high levels of VEGF (25). As shown in Fig. 1*A*, these cells exhibit remarkably increased levels of VEGF mRNA when compared with control cells expressing the empty pCEFL vector (NIH-pCEFL). Similar high levels of VEGF mRNA could be observed in cells transformed by the KSHV GPCR on prolonged culture, as well as in

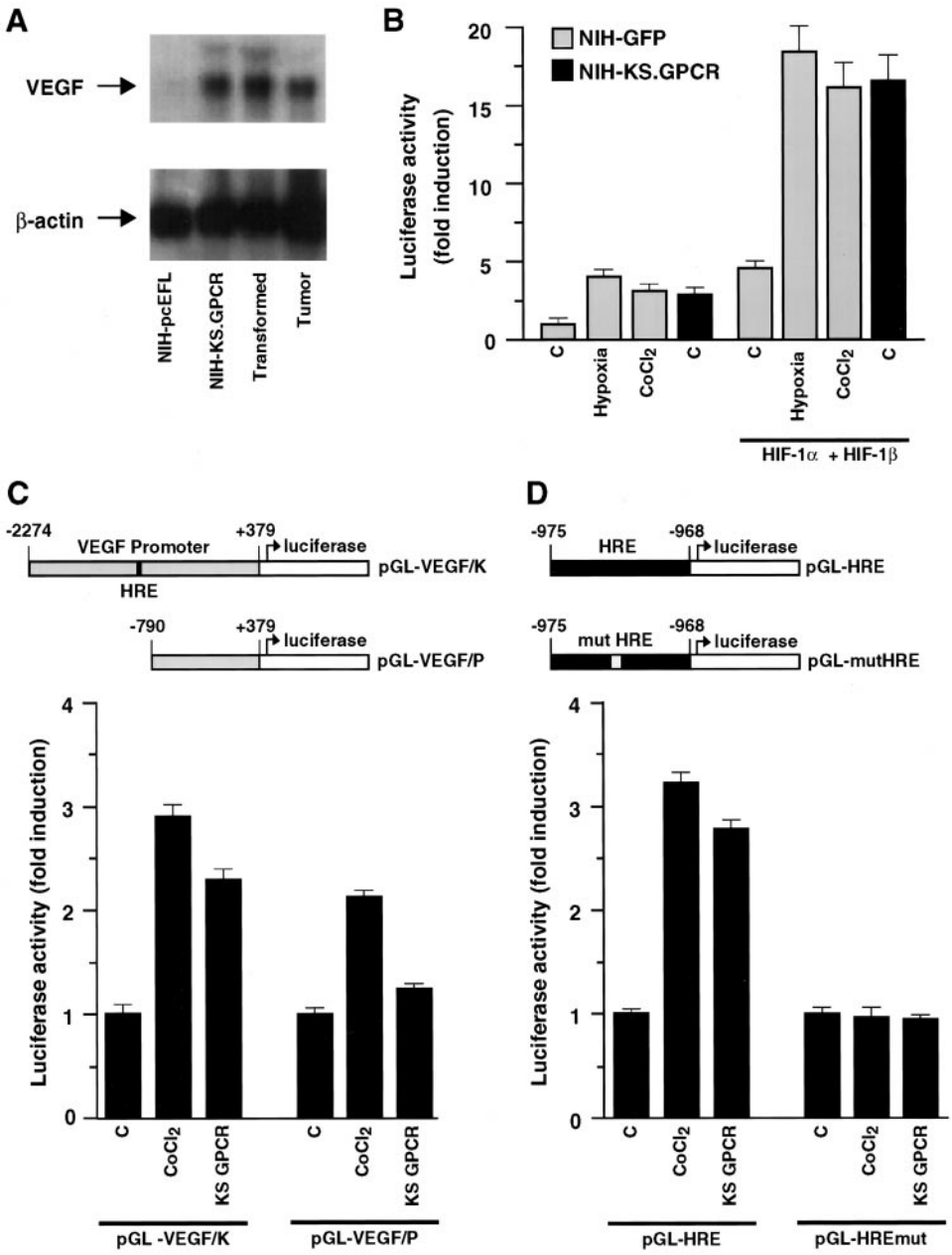


Fig. 1. KSHV GPCR induces expression of VEGF by increasing VEGF transcription through HIF-1. *A*, Northern blot of VEGF mRNA from NIH-pCEFL control cells, a NIH-KS.GPCR stable cell line, and NIH-KS.GPCR cells transformed upon prolonged culturing (*Transformed*) or grown as a tumor in nude mice (*Tumor*). A probe for $\beta$-actin mRNA was hybridized with the same membrane as a control. *B*, induction of transcription from the VEGF promoter in NIH-GFP control cells or NIH-KS.GPCR cells. A histogram compares the relative induction of transcription from the pGL-VEGF/K reporter plasmid in the absence or presence of cotransfected HIF-1$\alpha$ and HIF-1$\beta$. Induction with hypoxia or $CoCl_2$ (36 h) was used as a control. *C* and *D*, KSHV GPCR induction of transcription from the VEGF promoter and from an isolated HRE, respectively, in MDCK cells. The four reporter constructs [the full-length VEGF promoter (pGL-VEGF/K), a truncated VEGF promoter lacking the HRE (pGL-VEGF/P), the HRE (pGL-HRE), and a mutated HRE lacking the HIF-1 binding site (pGL-HREmut)] are depicted. *Bottom*, a histogram compares the relative induction of transcription from these reporter constructs in response to the KSHV GPCR, using induction with $CoCl_2$ as a control.

tumors arising from NIH-KS.GPCR cells injected into nude mice (25). To explore whether this enhanced steady-state level of VEGF mRNA results from an increase in VEGF transcription, we transfected NIH-KS.GPCR cells with pGL-VEGF/K, a reporter plasmid expressing luciferase under the control of the full-length VEGF promoter (35). As shown in Fig. 1*B*, the VEGF promoter was highly active in NIH-KS.GPCR cells, similar to that of control cells exposed to hypoxia or $CoCl_2$, which mimics hypoxia (40). Furthermore, we observed a synergistic increase in expression from the VEGF promoter when plasmids for the transcriptional enhancers HIF-1$\alpha$ and HIF-1$\beta$ were cotransfected into these cells (Fig. 1*B*), thus indicating that the KSHV GPCR, as well as hypoxia (35), can activate HIF-1 to stimulate the VEGF promoter.

We next set out to determine the mechanism by which the KSHV GPCR was inducing VEGF transcription. For these experiments, we used transiently transfected MDCK cells, which were found to respond in a manner similar to that of NIH 3T3 cells but displayed much greater transfection efficiency, thus enabling us to cotransfect multiple DNAs without observing changes in the expression of control plasmids driven by constitutive promoters. As shown in Fig. 1*C*, in these cells, both $CoCl_2$ and the KSHV GPCR induced transcription from the VEGF promoter effectively. However, the stimulation by the KSHV GPCR and $CoCl_2$ was markedly reduced when we used a truncated VEGF reporter construct lacking the HRE, pGL-VEGF/P, suggesting that the ability of the KSHV GPCR to stimulate expression from the VEGF promoter requires an intact HRE. We next asked whether the KSHV GPCR could activate transcription from an isolated HRE. For these experiments, we engineered a reporter plasmid expressing the luciferase gene under the control of a single VEGF HRE consensus sequence, which was designated pGL-HRE. As shown in Fig. 1*D*, the viral-encoded GPCR strongly induced the pGL-HRE to a level similar to that achieved by $CoCl_2$ treatment. However, both KSHV GPCR and $CoCl_2$ failed to stimulate a control reporter plasmid, pGL-

HREmut, which is identical to pGL-HRE but contains a mutation in the HIF-1 binding site (35), supporting the specificity of the transcriptional response. Taken together, these results indicate that the KSHV GPCR can stimulate expression from the VEGF promoter by activating transcription from a HRE.

**The KSHV GPCR Induces the MEK/MAPK and MKK6/p38 Signaling Pathways: A Potential Role in the Activation of the VEGF Promoter.** Because the KSHV GPCR was able to stimulate the activity of the VEGF promoter through its HRE, we next sought to investigate the nature of the biochemical pathways linking this viral GPCR to HIF-1. We focused our attention on the MAPK superfamily of proline-directed serine-threonine kinases because they are central components of many signaling routes communicating cell surface receptors to the nucleus (32–34). To determine which such kinase pathways might be stimulated by the KSHV GPCR in our cellular system, we screened representative members of each MAPK family for enhanced activity in the presence of cotransfected KSHV GPCR. The expression of this receptor was able to enhance the activity of MAPK, p38$\alpha$, p38$\gamma$, and p38$\delta$ but had a more limited effect on JNK (Fig. 2*A*). We then explored whether any of these MAPK pathways could stimulate the activity of the VEGF promoter, taking advantage of the availability of activated molecules acting upstream from each of the MAPK cascades, including MEK, which activates MAPK (41), MKK6, which activates p38$\alpha$, p38$\gamma$, and p38$\delta$ (37), and MEKK1, which activates JNK (42). As shown in Fig. 2*B*, expression of activated mutants of both MEK and MKK6, but not of MEKK1, provoked a potent induction of the pGL-VEGF/K reporter plasmid. Interestingly, induction of the truncated pGL-VEGF/P, which lacks the HRE, by these kinases was greatly diminished, thus suggesting a role for HIF-1 in both MEK and MKK6 stimulation of transcription from the VEGF promoter. Furthermore, the activated forms of both MEK and MKK6 also strongly induced the HRE reporter plasmid, pGL-HRE, but showed no induction of the pGL-HREmut (Fig. 2*C*). These data indicated that both MEK and MKK6 and their downstream MAPKs can induce HIF-1-dependent transcription from the VEGF promoter.

**MAPK and p38 Kinases Phosphorylate HIF-1$\alpha$ in Its Regulatory/Inhibitory Domain and Enhance Its Transcriptional Activity.** Kinase activation of transcriptional enhancers can occur through direct phosphorylation of transcription factors (43, 44). For the HIF-1$\alpha$/HIF-1$\beta$ heterodimer, the HIF-1$\alpha$ subunit has been shown to be the essential element for the hypoxic regulation of the HRE (11), whereas HIF-1$\beta$, although unaffected by hypoxia, appears to be required for DNA binding. HIF-1$\alpha$ contains a DNA binding domain (amino acids 1–390) that includes the heterodimerization domain (amino acids 1–166) and a TAD (amino acids 531–826). This last domain includes a negative regulatory region (amino acids 575–786) that inhibits the transcriptional activity of the adjacent $NH_2$-terminal and COOH-terminal transactivating regions under normoxic conditions (see Fig. 3*A*, *N-TAD* and *C-TAD*; Ref. 36). Within this inhibitory domain, there are eight serine residues that may be putative targets for members of the MAPK superfamily. To explore whether kinase-induced HIF-1-dependent transcription was mediated by activation of HIF-1$\alpha$ through phosphorylation, we constructed a fusion protein containing the $NH_2$-terminal domain of GST and the HID. As shown in Fig. 3*B*, activated MAPK, p38$\alpha$, and p38$\gamma$ were able to phosphorylate the GST-HID fusion protein but not the GST protein alone. Activated p38$\delta$ and JNK, however, were unable to use the GST-HID as a substrate, although both were able to phosphorylate GST-ATF2 when used as a control. These data suggest that HIF-1$\alpha$ can be a direct target for phosphorylation by MAPK, p38$\alpha$, and p38$\gamma$, thus providing a possible mechanism for the induction of HIF-1-dependent transcription by MEK and MKK6.

To investigate whether the ability to phosphorylate the HID could affect the activity of the HIF1-$\alpha$ TAD, we constructed a chimeric GAL4-core HIF-1$\alpha$ fusion molecule (Gal4/HIF) containing both the $NH_2$-terminal and COOH-terminal TADs and the regulatory/inhibitory domain (Fig. 3*C*; Ref. 36). This GAL4/HIF chimera was found to be stable and localized to the nucleus under normoxic conditions (data not shown), thus permitting the investigation of HIF-1$\alpha$ transactivation activity independently of other mechanisms affecting protein

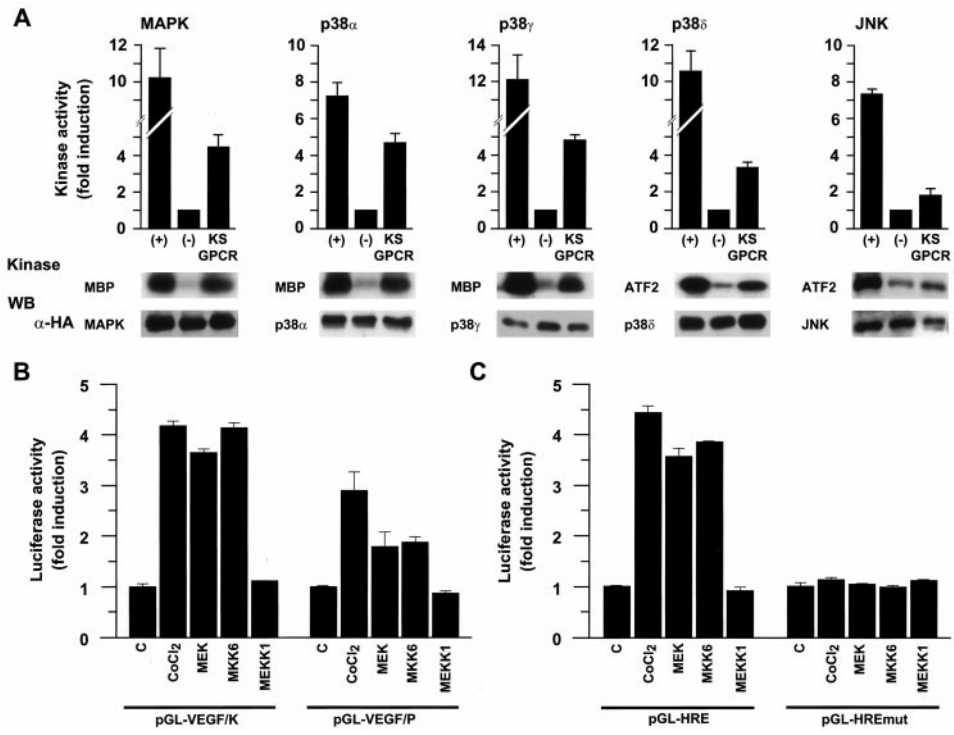


Fig. 2. KSHV GPCR induces the MEK/MAPK and MKK6/p38 signaling pathways, both of which can induce transcription from the VEGF promoter. *A*, KSHV GPCR induction of various signaling pathways. COS-7 cells were transfected with HA-tagged kinases for MAPK assays, together with pCEFL GFP vector (−) or pCEFL KSHV GPCR. Cotransfection with the mutationally activated Ras (RasV12; 1 $\mu$g/plate; for MAPK) or the mutationally activated cdc42 (cdc42QL; 1 $\mu$g/plate; for JNK) or treatment of cells with 10 $\mu$g/ml anisomycin for 20 min (for p38$\alpha$) or 300 mM NaCl for 15 min (for p38$\gamma$ or p38$\delta$) was used as a positive control (+). Kinase reactions were performed in anti-HA immunoprecipitates from the corresponding cellular lysates. Data represent the mean ± SE of three independent experiments, expressed as the fold increase with respect to pCEFL GFP-transfected cells. Autoradiograms correspond to representative experiments. Western blot analysis (*WB*) was performed in the corresponding cellular lysates and immunodetected with the antibody to HA. *B* and *C*, constitutively active MAPK kinases stimulate transcription from the VEGF promoter and from a HRE, respectively, in MDCK cells. A histogram compares relative induction of transcription from the reporter plasmids in response to constitutively active forms of the MAPK kinases, MEK, MKK6, or MEKK1.

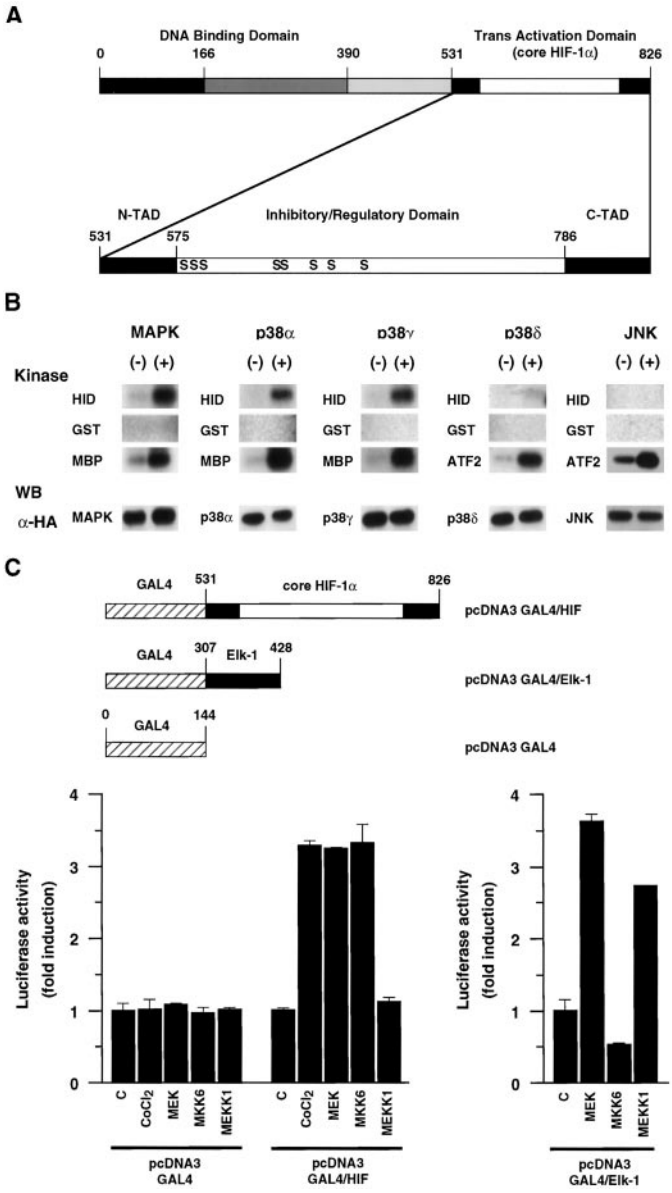


Fig. 3. MAPK, p38$\alpha$, and p38$\gamma$ can phosphorylate HIF-1$\alpha$ and activate the transactivating activity of HIF-1$\alpha$. *A*, the structure of full-length HIF-1$\alpha$ depicting the DNA binding domain and the TAD, the latter contains eight putative proline-directed serine phosphorylation sites in the inhibitory/regulatory domain within the TAD. *B*, phosphorylation of HID by MAPKs. COS-7 cells were transfected with HA-tagged kinases as described above. Kinase reactions were performed using either the GST-HID fusion protein or GST alone as a substrate. Phosphorylation of either myelin basic protein (MAPK, p38$\alpha$, and p38$\gamma$) or ATF2 (p38$\delta$ and JNK) was used as a control. Autoradiograms correspond to representative experiments. Western blot analysis (*WB*) was performed in the corresponding cellular lysates and immunodetected with the antibody to HA. *C*, the structure of the proteins encoded by pcDNA3. GAL4/HIF, pcDNA3.GAL4/Elk-1, and pcDNA3.GAL4 plasmid constructs are depicted. *Bottom*, a histogram compares the relative induction of transcription from the pGL3.TATA GAL reporter plasmid by GAL4, GAL4/HIF, or GAL4/Elk-1 in response to constitutively active forms of the MAPK kinases, MEK, MKK6, or MEKK1.

stability and nuclear localization. We then expressed activated mutants of MEK, MKK6, and MEKK1 together with GAL4/HIF and the pGL3-TATA GAL reporter plasmid. As shown in Fig. 3*C*, both the activated mutants of MEK and MKK6 potently induced the transcriptional activity of GAL4/HIF. Additional molecules acting upstream of MAPK, such as Raf and Ras, were also able to potently stimulate the activity of the GAL4/HIF chimera (data not shown). In contrast, the activated mutant of MEKK1 had little effect on GAL4/HIF, although it effectively activated a GAL4/Elk-1 fusion construct when used as control (Fig. 3*C*). Thus, these data indicate that signaling routes activating MAPK, p38$\alpha$, and p38$\gamma$ can stimulate the transcriptional activity of HIF1-$\alpha$ by direct phosphorylation of its regulatory domain.

**p38 and MAPK Signaling Pathways Mediate the Stimulating Effects of the KSHV GPCR on the Transactivating Activity of HIF-1$\alpha$.** To examine whether the KSHV GPCR could also act through the HIF-1$\alpha$ transactivating domain, we investigated the effect of transiently expressing increasing amounts of KSHV GPCR cDNA on the activity of the GAL4/HIF chimera, cotransfecting the corresponding DNAs along with the pGL3.TATA GAL reporter plasmid. As shown in Fig. 4*A*, the KSHV GPCR was able to induce the transcriptional activity of GAL/HIF in a dose-dependent manner but had little effect on the GAL4 protein alone. To further elucidate the biochemical route used by this viral receptor to activate HIF-1$\alpha$, we took advantage of the availability of pharmacological agents that specifically block either the p38 (SKF86002) or MAPK (PD98059) pathways (45, 46). Thus, we studied the KSHV GPCR-mediated induction of GAL4/HIF transcriptional activity in the presence of increasing concentrations of either compound. Fig. 4, *B* and *C*, shows a clear dose-dependent inhibition of KSHV GPCR activation of GAL4/HIF when cells were treated with either SKF86002 or PD98059, respectively. However, these compounds did not affect GAL4/Elk-1 activation by MEKK1 when used as a control. These data suggest that the KSHV GPCR enhances the transcriptional activity of HIF-1$\alpha$ through the p38 and MAPK signal transduction pathways.

**Inhibitors of the p38 and MAPK Pathways Diminish the Expression and Secretion of VEGF Induced by the KSHV GPCR.** Finally, we decided to look further into the physiological relevance of the activation of these MAPK pathways by the KSHV GPCR. For that purpose, we investigated the effect of the SKF86002 or PD98059 inhibitors on the transcription and secretion of VEGF in cells overexpressing the receptor. Fig. 5, *A* and *B*, shows the percentage of inhibition of transcription from the VEGF promoter (pGL-VEGF/K) in response to increasing concentrations of SKF86002 or PD98059, with respect to control-treated cells. Both kinase inhibitors reduced transcription from the full-length VEGF promoter in NIH-KS.GPCR cells in a dose-dependent manner. Moreover, the inhibition of the VEGF promoter activity affected the KSHV GPCR-induced VEGF secretion, as the treatment with SKF86002 or PD98059 also decreased the levels of VEGF secreted from NIH-KS.GPCR cells. As shown in Fig. 5, *A* and *B*, a clear dose-dependent inhibition of VEGF secretion by SKF86002 or PD98059 was observed in NIH-KS.GPCR cells, paralleling the effects of these kinase inhibitors on the VEGF promoter activity. To further control for the specificity of the pharmacological inhibitors, we ran a parallel control experiment showing that VEGF transcription and secretion by NIH 3T3 cells transformed by RasV12, an oncogene that activates MAPK and fails to activate p38, were inhibited by PD98059 but not by SKF86002 (data not shown). Taken together, these data strongly suggest that the KSHV GPCR induces the expression and secretion of VEGF by stimulating the activity of the VEGF promoter through the p38 and MAPK signaling pathways, acting on the transactivating domain of HIF-1$\alpha$.

## DISCUSSION

The expression of dominant acting viral and cellular oncogenes contributes to tumor development by subverting basic regulatory mechanisms controlling normal cell proliferation, differentiation, and survival. However, recently available evidence indicates that the formation of new blood vessels constitutes a prerequisite for the growth of solid tumors (1). Indeed, expression of many oncogenes promotes tumor neovascularization by inducing the release of angiogenic fac-

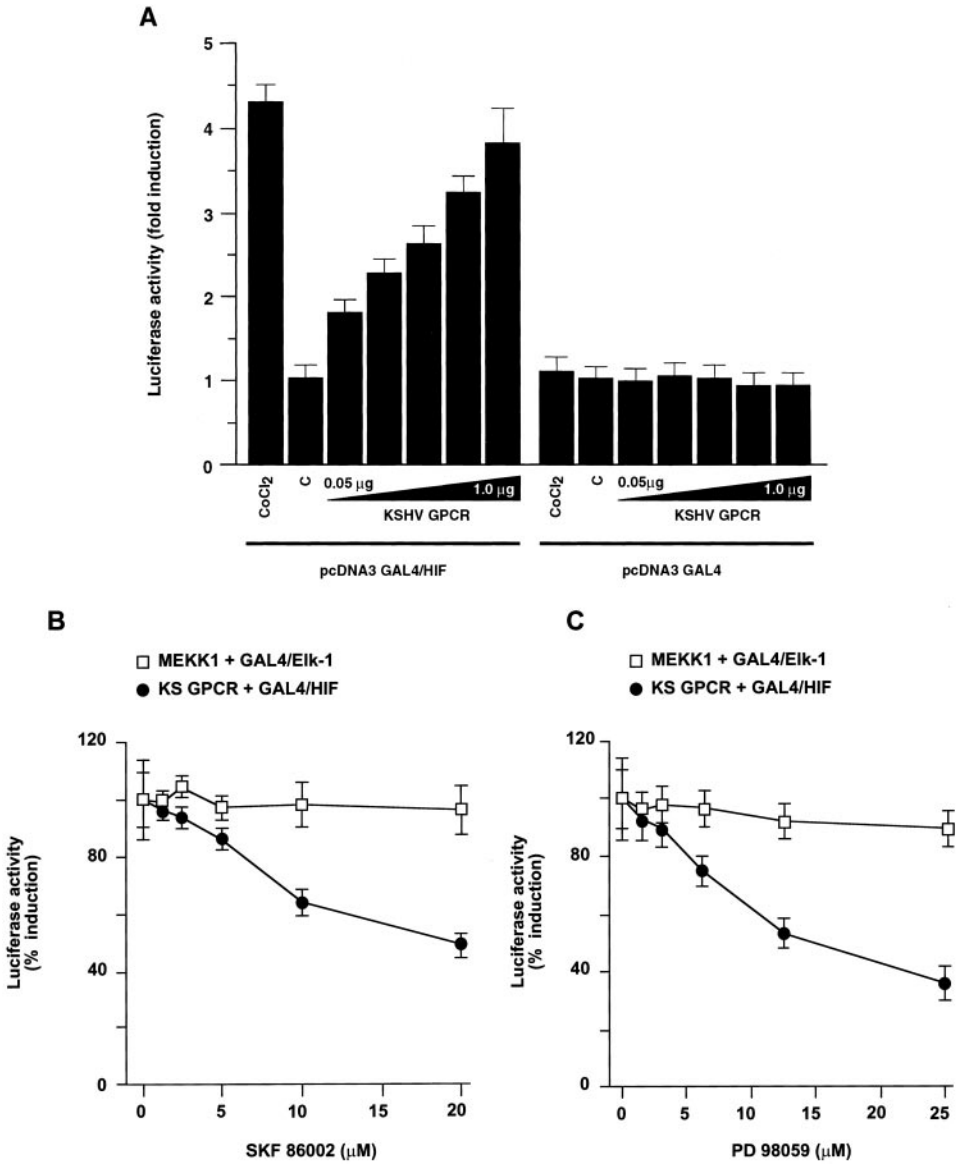


Fig. 4. Pharmacological agents reveal that the MEK/MAPK and MKK6/p38 signaling pathways mediate the stimulating effects of the KSHV GPCR on the transactivating activity of HIF-1$\alpha$. *A*, KSHV GPCR activation of the TAD of HIF-1$\alpha$ in COS-7 cells. A histogram compares the relative induction of transcription from the pGL3.TATA GAL reporter plasmid by GAL4/HIF or GAL4 in response to increasing concentrations of KSHV GPCR (0.05, 0.1, 0.25, 0.5, or 1.0 $\mu$g). *B* and *C*, inhibition of KSHV GPCR activation of the TAD of HIF-1$\alpha$ in COS-7 cells by blocking p38 and MEK, respectively. Data represent the fold induction of the GAL4/HIF transcriptional activity in the presence of increasing concentrations of p38 inhibitor SKF86002 and MEK inhibitor PD98059, expressed as a percentage of the KSHV GPCR-induced response. Lack of inhibition of MEKK1-induced GAL4/Elk-1 transcriptional activity by either kinase inhibitor served as a control for specificity.

tors, among which VEGF has been shown to play a central role (7). Viral oncogenes are powerful tools for disclosing cellular mechanisms of oncogenesis. In that regard, KSHV GPCR, an angiogenic oncogene encoded by a virus implicated in the pathogenesis of the angioproliferative disorder KS, appears as a unique model to link oncogenic signaling cascades and the expression of angiogenic factors.

Here we show that the KSHV GPCR induces expression of VEGF by stimulating transcription from a HRE, which binds the HIF-1$\alpha$/HIF-1$\beta$ heterodimer, within the 5′-flanking region of the VEGF promoter (11). We found that the KSHV GPCR could directly stimulate the transcriptional activity of HIF-1$\alpha$ using a GAL4-core HIF-1$\alpha$ fusion protein that contains both $NH_2$- and COOH-terminal TADs of HIF-1$\alpha$ and a HID domain that inhibits the activity of these transactivating regions. Furthermore, we provide evidence that the activation of HIF-1$\alpha$ may involve the direct phosphorylation of this transcription factor by the p38 and MAPK signaling pathways within the HID domain (amino acids 575–786). Finally, we also demonstrate that specific inhibitors of the p38 and MAPK pathways (SKF86002 and PD98059) are able to diminish the expression and secretion of VEGF induced by the KSHV GPCR, indicating that these pathways are necessary and sufficient for the viral receptor to stimulate transcription from the VEGF promoter. These results indicate that this viral oncogene is able, under normoxic conditions, to subvert the hypoxia response pathway, which is the strongest physiological regulator of VEGF expression.

These findings may have a broad impact on our understanding of the pathophysiological mechanisms involved in the acquisition of the angiogenic phenotype. Our results showing that KSHV GPCR activates HIF-1-mediated transcription of the VEGF gene via MAPK and p38 under normoxic conditions point to a general mechanism explaining how oncogenes and inflammation up-regulate VEGF and could synergize with hypoxia in tumors. Indeed, expression and secretion of VEGF are also induced by activated oncogenes such as *ras*, *raf*, and *src* (14, 15), which activate MAPK, and by cytokines and chemokines such as IL-1, tumor necrosis factor $\alpha$, and IL-8 (7, 13), which stimulate p38 (47, 48). Furthermore, these observations are also in line with recently published reports implicating the MAPK signaling pathway in increasing the transcriptional activity of HIF-1$\alpha$ independent of its protein stability (16). Of interest, the KSHV-GPCR may also regulate the stability of HIF-1$\alpha$, for example, through the Akt pathway as reported for other oncogenes (17, 18), a possibility that is currently under investigation.

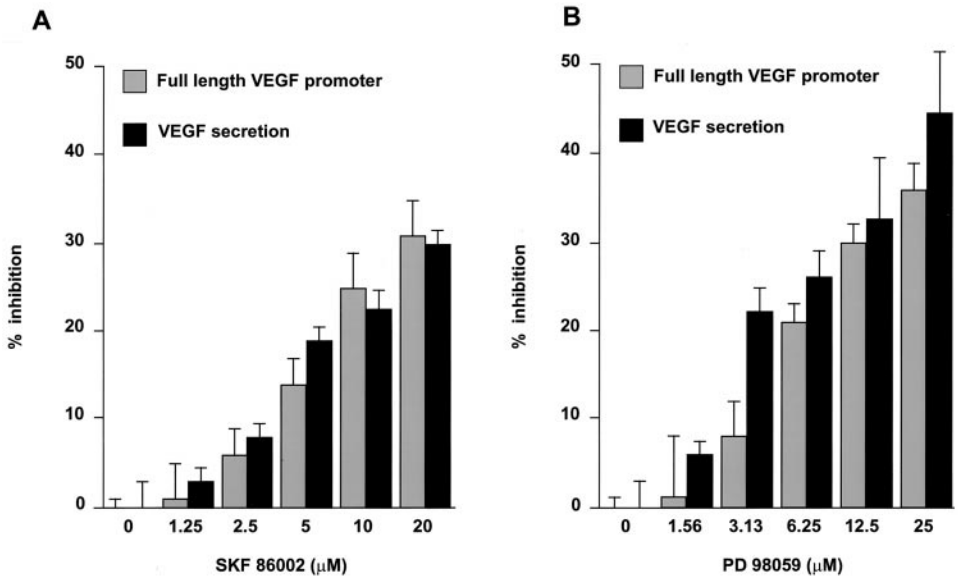


Fig. 5. Pharmacological agents reveal that the MEK/MAPK and MKK6/p38 signaling pathways mediate the stimulating effects of the KSHV GPCR on the enhanced transcription and secretion of VEGF. *A* and *B*, inhibition of KSHV GPCR-induced transcription from the VEGF promoter and secretion of VEGF protein in NIH-KS.GPCR cells by blocking p38 and MEK, respectively. A histogram compares the percentage of inhibition of transcription from the VEGF promoter (pGL-VEGF/K) or secretion of VEGF in response to increasing concentrations of p38 inhibitor SKF86002 and MEK inhibitor PD98059, with respect to cells treated with vehicle alone.

Using the KSHV GPCR as a model system for oncogenic and cytokine-induced VEGF secretion, we are now able to state that at least two distinct signaling pathways regulate the transactivating activity of the transcriptional enhancer HIF-1$\alpha$. As depicted in Fig. 6, this novel mechanism, together with those described previously such as increased half-life of HIF-1$\alpha$ (9) and stabilization of VEGF mRNA (12), may ultimately control VEGF secretion and thus play a critical role in angiogenesis, in normal physiological situations as well as in neoplastic diseases. Interestingly, the inhibition of angiogenesis is considered one of the most promising strategies for the development of novel antineoplastic therapies because targeting of the tumor vasculature might have minimal side effects even after prolonged treatment and should not lead to the development of resistance (49). Because systemic chemotherapy treatment for advanced disseminated forms of KS has been shown to be extremely difficult to tolerate, not only in AIDS patients but also in patients with other clinical varieties of this neoplasm (50), the development of rational or pathogenesis-based therapies might provide useful alternatives or additions to the use of cytotoxic drugs (50). In this regard, antiangiogenic therapies have shown promising results in the treatment of KS (51–53). Thus, our findings establishing a molecular mechanism whereby the KSHV GPCR stimulates the expression and secretion of VEGF might help to identify novel therapeutic targets for KS and other neoplastic and angioproliferative diseases.

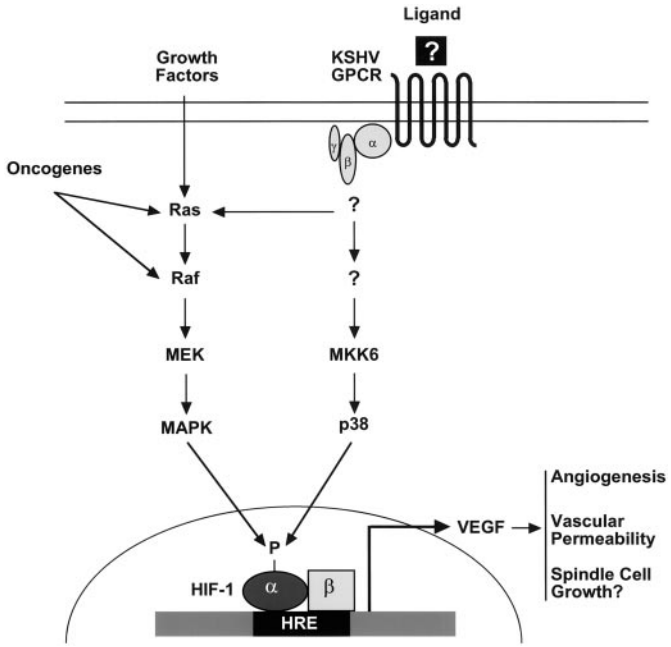


Fig. 6. Proposed mechanism whereby the GPCR of KSHV stimulates VEGF transcription through HIF-1$\alpha$. The KSHV GPCR activates an as yet unidentified G protein, which, in turn, activates downstream kinases, thereby causing the phosphorylation and activation of both p38 and MAPK. Subsequently, p38 and/or MAPK phosphorylate and activate HIF-1$\alpha$, allowing HIF-1-dependent transcription of VEGF. Additional possibilities, including the potential role of these MAPK pathways in mediating the enhanced expression of VEGF in response to growth factor, inflammatory cytokines, or the expression of oncogenic molecules, are discussed in the text.

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