Constitutive activation of NF-κB and secretion of IL-8 induced by the G protein-coupled receptor of Kaposi’s sarcoma-associated herpesvirus involves Ga13 and RhoA*

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Running title: KSHV-GPCR couples to Ga13

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SUMMARY:

The Kaposi’s sarcoma herpesvirus (KSHV) open reading frame 74 encodes a G protein-coupled receptor (GPCR) for chemokines. Exogenous expression of this constitutively active GPCR leads to cell transformation and vascular overgrowth characteristic of Kaposi’s sarcoma. We show here that expression of KSHV-GPCR in transfected cells resulted in constitutive transactivation of nuclear factor κB (NF-κB) and secretion of IL-8, and this response involves activation of Gα13 and RhoA. The induced expression of a NF-κB luciferase reporter was partially reduced by pertussis toxin and the Gβγ scavenger transducin, and enhanced by co-expression of Gα13 and to a lesser extent, Gαq. These results indicate coupling of KSHV-GPCR to multiple G proteins for NF-κB activation. Expression of KSHV-GPCR led to stress fiber formation in NIH 3T3 cells. To examine the involvement of the Gα13 – RhoA pathway in KSHV-GPCR-mediated NF-κB activation, HeLa cells were transfected with KSHV-GPCR alone and in combination with the regulator of G protein signaling (RGS) from p115RhoGEF or a dominant negative RhoA (T19N). Both constructs, as well as the C3 exoenzyme from Clostridium botulinum, partially reduced NF-κB activation by KSHV-GPCR, and by a constitutively active Gα13 (Q226L). KSHV-GPCR-induced NF-κB activation is accompanied by increased secretion of IL-8, a function mimicked by the activated Gα13 but not by an activated Gαq(209L). These results suggest coupling of KSHV-GPCR to the Gα13 – RhoA pathway in addition to other G proteins.
Chemokine receptors belong to the superfamily of G protein-coupled receptors (GPCRs) that share a characteristic 7-transmembrane domain structure. Recent studies have demonstrated that chemokine receptors play important roles in lymphocyte homing, directed migration of phagocytes to inflammatory sites, and entry of HIV-1 into host cells (reviewed in (1,2)). These receptors bind a large number of chemokines, peptides of typically 8-10 kDa and contain cysteine residues at defined positions. Activation of chemokine receptors results in the dissociation of $G_{\alpha}$ from $G_{\beta\gamma}$ proteins, triggering a series of signaling events leading to chemotaxis and other cellular functions (1,2).

Human herpesvirus 8 is a recently identified gamma herpesvirus associated with Kaposi’s sarcoma and hence is also named Kaposi’s sarcoma-associated herpesvirus (KSHV) (3,4). Kaposi’s sarcoma is characterized by angiogenic proliferation of mesenchymal cells, resulting in abundant vascular spaces filled with red blood cells and surrounded by spindle cells. KSHV DNA is found in spindle cells as well as endothelial cells in the lesions of Kaposi’s sarcoma, suggesting that the viral DNA stimulates synthesis of proteins that contribute to overgrowth of vascular endothelial cells (4). Analysis of the KSHV sequence has led to the identification of an open reading frame (ORF-74) that encodes a putative GPCR, which bears structural similarity to CXCR1 and CXCR2, the two human receptors for IL-8 (5,6). When expressed in transfected cell lines, KSHV-GPCR binds a large number of chemokines (7). Unlike most other chemokine receptors that depend on agonist binding for activation, the KSHV-derived receptor is a constitutively active GPCR. Exogenous expression of KSHV-GPCR results in cell proliferation and foci formation (7). Furthermore, KSHV-GPCR induces oncogenic transformation of NIH 3T3 cells leading to tumor growth in nude mice (8). The transformed cells promote a switch of surrounding endothelial cells to an angiogenic phenotype due to increased secretion of vascular endothelial growth factor (VEGF) (8), which stimulates endothelial growth in Kaposi’s sarcoma lesions through a paracrine mechanism (9). Expression of KSHV-GPCR in transgenic mice produced the same pathological changes as seen in Kaposi’s sarcoma, indicating a causal relationship between KSHV-GPCR and certain lesions of Kaposi’s sarcoma (10).
There has been a great deal of interest in the signaling pathways activated by KSHV-GPCR. Whereas some chemokines bind to and further activate this receptor (7,11), others inhibit its function (12-14). As with many GPCRs whose activation is negatively regulated by G protein-coupled receptor kinases (GRKs), signaling by the KSHV-GPCR is blocked by GRK5 but not GRK2 (15). Like other chemokine receptors, KSHV-GPCR stimulates activation of several protein kinases, including related adhesion focal tyrosine kinase, extracellular signal-related protein kinases and p38 (8,16,17). However, KSHV-GPCR differs from most chemokine receptors in that it is a viral oncogene that transforms host cells and stimulates tumor growth. Therefore it is important to determine the proximal signaling events including the G proteins that couple to this constitutively active GPCR.

Recent studies conducted in this and other laboratories have demonstrated activation of nuclear factor κB (NF-κB) by GPCRs (18-23). NF-κB is a ubiquitously expressed and highly regulated dimeric transcription factor (24). Numerous environmental signals can induce NF-κB activation, which in turn regulates the expression of a large number of genes coding for cytokines, chemokines and growth factors (25). Activation of NF-κB has been suggested and recently demonstrated to play an important function in autocrine production of several CXC chemokines through the IL-8 receptors (26-28). In melanoma cells, autocrine production of growth regulated oncogene-α (GRO-α; also termed melanoma growth stimulatory activity or MGSA) is associated with cell proliferation similar to that observed in KSHV-GPCR-transfected cells (27,29). This finding prompted us to determine whether KSHV-GPCR, like its mammalian homologs, can also activate NF-κB. Here we report that KSHV-GPCR stimulates constitutive NF-κB activation and induces IL-8 secretion in transfected HeLa cells. We further demonstrate that the heterotrimeric G protein, Gα13, together with Gq, Go/i/o and Gβγ proteins, play an important role in KSHV-GPCR-mediated activation of NF-κB and secretion of IL-8. NF-κB activation by KSHV-GPCR was reported by others while this work was being completed (30) and after initial submission of the manuscript (31,32).
EXPERIMENTAL PROCEDURES:

**Reagents.** The anti-\(\text{G} \alpha q\) and anti-\(\text{G} \alpha 13\) Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-\(\text{G} \alpha i2\) antibody was prepared against a synthetic peptide with the sequence CAKNNLKDCGLF. The \(\text{G} \alpha i2\) and \(\text{G} \alpha q\) expression vectors were kindly provided by Drs. Cindy Knall and Gary Johnson (University of Colorado, Denver, CO) and were described elsewhere (33). The \(\text{G} \alpha 13\) and p115RhoGEF constructs were described in previous publications (34-36). A HA-tagged \(\text{G} \alpha 13\) construct was obtained from Dr. Silvio Gutkind (National Institutes of Health, Bethesda, MD). The IL-8 luciferase reporter was a generous gift from Dr. Naofumi Mukaida (Kanazawa University, Japan). Other reagents were described in a recent publication from our laboratory (37).

**Cell culture, transfection and luciferase reporter assay.** HeLa cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 50 \(\mu\)g/ml streptomycin. Cells (~40% confluence) in 6-well plates were transfected with plasmid expression vectors coding for a 3x \(\kappa B\)-directed luciferase reporter (37) or an IL-8 luciferase reporter (\(-272\)-Luc) (38), KSHV-GPCR and other expression constructs as indicated. The total DNA was brought to 1 \(\mu\)g/well, and pCMV\(\beta\) (\(\beta\)-galactosidase expression vector) was included for standardization of transfection and transcription efficiency. Transient transfection was performed as described (37) using the LipofectAmine Plus reagent (Life Technologies, Rockville, MD) according to manufacturer’s instructions. Twenty-eight hours after transfection, cells were serum-starved for 16-18 h, washed twice with PBS, and assayed with or without agonist stimulation. Reporter lysis buffer (Promega, Madison, WI) was added to the cells, and the expressed luciferase activity was measured in a Femtomaster FB12 luminometer (Zylux, Maryville, TN). Relative expression level of the transfected constructs was standardized against the expressed \(\beta\)-galactosidase. Luciferase assays were done with duplicate or triplicate samples, and 2-4 independent experiments were usually conducted. Normalized data were plotted using the Prism software (Version 3.0; GraphPad, San Diego, CA).
**Immunodetection.** Cell surface expression of an AU5-tagged KSHV-GPCR was measured on a Coulter Elite flow cytometer, using a monoclonal Ab against AU5 (1:500, Babco, Berkeley, CA) and a secondary, FITC-conjugated goat-anti-mouse Ab (1:200). The AU5 tag (TDFYLK) was added to the N-terminus of KSHV-GPCR by PCR (30 cycles with Pfu Turbo polymerase). The resulting PCR fragment was subcloned into the pRK5 vector (BD Pharmingen) and confirmed by DNA sequencing. The final construct contains a methonine preceding the tag, which was fused to the second residue of KSHV-GPCR (minus the initiation codon). In transfection experiments, the AU5-tagged KSHV-GPCR gave the same constitutive NF-κB activation data as with untagged KSHV-GPCR.

For Western blotting, proteins from whole cell extracts were separated on 6% to 10% acrylamide SDS-PAGE gels by electrophoresis at 30 mA. Proteins were electrotransferred to nitrocellulose membrane at 100 V for 1 h at 4°C. The membrane was pretreated with 5% non-fat milk in TTBS (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.05% Tween-20) for 1-2 hours at room temperature. Incubation with primary Ab was done at 4°C in TTBS with 5% BSA, for 16 h. The membrane was then washed 3 times with TTBS, for 10 min each, and incubated with horseradish peroxidase-conjugated secondary Ab for 1 h at room temperature (23°C). After 3 washes with TTBS, the bound Ab was detected by enhanced chemiluminescence (Pierce, Rockford, IL).

**Fluorescent microscopy.** A stable NIH 3T3 cell line was established by transfection of KSHV-GPCR cDNA in the pSFFV.neo expression vector (39). The G418-resistant clones were pooled and the expression of KSHV-GPCR was confirmed by flow cytometry using a rabbit polyclonal Ab against the amino terminal 41 residues of the receptor (Torrey Pines Biolabs, Pearland, TX). The cells were grown on gelatin-coated glass coverslips. Two days later, the cells were fixed with glutaraldehyde (4%), permeabilized with Triton X-100 (0.1%), and stained for 20 min with rhodamine-phalloidin (0.5 μM; Sigma). The stained samples were viewed using a Nikon Eclipse TE300 inverted microscope equipped with Hoffman optics and appropriate filter sets for epifluorescence microscopy. The images were
captured with a Hamamatsu CCD digital camera and SimplePCI software (C-Imaging Systems, Cranberry Township, PA).

**Detection of IL-8 secretion.** IL-8 secreted from cultured HeLa cells was detected using an ELISA kit and following the protocol supplied by the manufacturer (Biosource, Camarello, CA). For each experiment, duplicate samples were taken and standard curves were generated using the manufacturer-supplied reagents.

**Data analysis.** Luciferase reporter activities were normalized against β-galactosidase activities from the coexpressed pCMVβ (luc / β-gal), and expressed as relative luciferase activities (RLA) over basal (set as 1). Data were plotted using the Prism software (ver. 3.0) from GraphPad (San Diego, CA). Where indicated, statistical analysis was conducted with one-way ANOVA using the same software.

**RESULTS:**

**Expression of KSHV-GPCR induces constitutive activation of NF-κB.** Recent studies have shown that certain GPCRs can activate NF-κB, leading to transcription of genes coding for cytokines and growth factors. To investigate whether the constitutively active KSHV-GPCR plays a role in NF-κB activation and cytokine secretion, HeLa cells were transiently transfected with expression vectors coding for an N-terminal tagged KSHV-GPCR and a κB-driven luciferase reporter (37). HeLa was chosen because the cell line has been extensively characterized for NF-κB activation by cytokine receptors, and also because the cell does not contain SV40 large T antigen that can cause overexpression of transfected genes when certain vectors are used. To correct variations in transfection efficiency, an expression vector coding for β-galactosidase under a CMV promoter was co-transfected with the above constructs, and the expressed β-galactosidase activity was used for normalization of κB luciferase data. Forty-eight hours after transfection, cells were collected and the induced κB luciferase reporter activities were measured.

In a typical experiment (Figure 1), expression of KSHV-GPCR in HeLa cells induced an increase in the κB luciferase activity indicating transactivation of NF-κB. KSHV-GPCR-induced κB luciferase

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activities range from 2.5- to 27-fold over baseline, and correlate with the amount of input receptor DNA (from 20 to 800 ng, Fig. 1A). KSHV-GPCR had no effect on the expression of a luciferase reporter lacking functional NF-κB binding sites (κB-). To determine whether the induced NF-κB luciferase activity is a function of cell surface receptor expression, the transfected HeLa cells were subject to flow cytometry analysis using an anti-AU5 monoclonal Ab, that recognizes the N-terminal AU5-tagged-KSHV-GPCR (see Experimental Procedures). As shown in Figure 1B, the AU5 tag could be detected when the DNA construct was used at ≥ 400 ng. Tranfection of the cells with 800 ng of DNA resulted in a cell surface expression that is nearly twice as much as transfecting with 400 ng of DNA (mean fluorescent channel number of 0.21 vs. 0.11). Thus, expression of KSHV-GPCR leads to dose-dependent activation of NF-κB in the transfected cells.

The ability of TNFα to stimulate NF-κB activation in HeLa cells has been well recognized. We therefore compared KSHV-GPCR-induced NF-κB luciferase activity with that of TNFα. Transfection of the cells with 200 ng of the KSHV-GPCR expression vector resulted in slightly higher κB luciferase activity than the activity induced by 10 ng/ml of TNFα (Fig. 1C). Furthermore, the NF-κB activity induced by KSHV-GPCR was additive to the TNFα-induced NF-κB activation (Fig. 1C), suggesting that potentially different signaling pathways are utilized by TNFα and KSHV-GPCR.

KSHV-GPCR couples to multiple G proteins for NF-κB activation. KSHV-GPCR is a structural homolog of the human chemokine receptors CXCR1 and CXCR2. These receptors have been shown to mediate chemotaxis and other leukocyte functions through functional coupling to pertussis toxin-sensitive G proteins (40). Although nearly all chemokine receptors are known for coupling to Gαi proteins, the identity of the G proteins that mediate the cellular functions of KSHV-GPCR remained unclear when this study was initiated. To determine whether KSHV-GPCR also couples to Gi/o, we treated the transfected cells with pertussis toxin (PTX, 500 ng/ml, 4 h). This treatment resulted in a 20-25% reduction in the ability of KSHV-GPCR to induce the κB luciferase reporter activity (Fig. 2A), suggesting that Gi/o is one of the G proteins that are activated by KSHV-GPCR. To identify other G
proteins that contribute to the remainder of KSHV-GPCR-induced NF-κB activation, we used a gain-of-function approach that has been proven effective by recent studies (37,41). HeLa cells were transfected to express selected wild type Gα proteins including Gαi2, Gαq and Gα13, in addition to the κB reporter and the KSHV-GPCR expression construct. These G proteins are endogenously expressed in HeLa cells (Fig. 2B), but it was predicted that coexpression of a given Gα protein that couples to the receptor would further enhance the induced κB reporter activity. Our results demonstrate that coexpression of Gαi2 did not further increase the κB luciferase activity, but coexpression of Gαq and Gα13 potentiated the KSHV-GPCR-induced response by 25% (P ≤ 0.05) and 60% (P ≤ 0.01), respectively (Fig. 2C). These effects were not due to variation of transfection efficiency since such differences were overcome by normalization of data with the co-transfected β-galactosidase construct. In comparison, expression of these Gα proteins without KSHV-GPCR did not significantly alter the κB luciferase activity. As an additional control, experiments were conducted under the same conditions to examine the effect of these G proteins on B2 bradykinin receptor (B2BKR)-mediated NF-κB activation. Our results indicate that Gαq produced a more potent enhancement that Gα13 of the BK-induced response (Fig. 2D). These results combined suggest that KSHV-GPCR preferentially couples to Gα13, in addition to Gαi/o and Gαq, for NF-κB activation.

Previous studies have demonstrated the ability of Gα13 to activate serum responsive factor (42), but its function in NF-κB activation was not known at the time these experiments were conducted. We therefore examined the ability of Gα13 to mediated NF-κB activation by co-transfection of a construct encoding a GTPase-deficient (constitutively active) form of Gα13 (Q226L) together with the κB luciferase reporter. As shown in Fig. 3A, the constitutively active Gα13 induced a potent and dose-dependent increase of the κB luciferase activity, in the absence of KSHV-GPCR. Like KSHV-GPCR, the activated Gα13 (Q226L) also enhanced TNFα-induced NF-κB transactivation (Fig. 3B).
The above findings suggest that G\(\alpha\)13 is involved in KSHV-GPCR-induced NF-\(\kappa\)B activation. To test this possibility further, a number of loss-of-function experiments were conducted. We compared the abilities of relevant inhibitors to block NF-\(\kappa\)B activation by KSHV-GPCR and by G\(\alpha\)13(Q226L). Regulators of G protein signaling (RGS) are a group of recently identified proteins that contain GTPase activating protein (GAP) domains. RGS proteins are effective inhibitors for G protein activation (43). The p115RhoGEF has been identified as a guanine nucleotide exchange factor that mediates activation of RhoA by G\(\alpha\)13 and G\(\alpha\)12 (35,44). p115RhoGEF contains an RGS domain that exhibits specificity for G\(\alpha\)13 and G\(\alpha\)12, and has been used as an inhibitor of these G\(\alpha\) proteins (45). We therefore speculated that the RGS domain of p115RhoGEF (p115RGS) would inhibit KSHV-GPCR-induced NF-\(\kappa\)B activation if G\(\alpha\)13 were involved in coupling this receptor. Our experimental results demonstrated that p115RGS indeed partially (~50%) blocked the induced NF-\(\kappa\)B activation (Fig. 4A). The inhibition was targeted primarily to G\(\alpha\)13 as p115RGS also inhibited NF-\(\kappa\)B activation by G\(\alpha\)13(Q226L).

We have shown above that KSHV-GPCR couples to more than one G\(\alpha\) protein. Activation of G\(\alpha\) results in the dissociation of G\(\beta\)\(\gamma\), which can directly stimulate several downstream effectors and result in NF-\(\kappa\)B activation (37). To determine the involvement of G\(\beta\)\(\gamma\), we co-transfected HeLa cells with a G\(\beta\)\(\gamma\) scavenger, bovine transducin. Expression of transducin led to a partial inhibition of KSHV-GPCR-mediated NF-\(\kappa\)B activation, while having no effect on NF-\(\kappa\)B activation by the constitutively active G\(\alpha\)13(Q226L) (Fig. 4B). These results combined indicate that KSHV-GPCR-induced NF-\(\kappa\)B activation involves multiple G proteins including G\(\alpha\)i/o, G\(\alpha\)q, G\(\alpha\)13 as well as G\(\beta\)\(\gamma\) proteins that are released when G\(\alpha\) is activated by the receptor.

**RhoA is a downstream effector of KSHV-GPCR that contributes to the induced NF-\(\kappa\)B activation.** G\(\alpha\)13 activates the small GTPase RhoA through p115RhoGEF, a guanine nucleotide exchange factor (35,44). To determine whether RhoA is downstream of KSHV-GPCR-induced signaling pathway, we took advantage of the ability of RhoA to stimulate formation of stress fibers in serum-starved fibroblast (46). A stable transfectant of NIH 3T3 cells was generated that expressed moderate
levels of KSHV-GPCR (Fig. 5A). This cell line exhibited NF-κB-activating property similar to the transiently transfected HeLa cells (Fig. 5A, inset). The cells were serum-starved, fixed and stained with Rhodamine-phalloidin for the detection of actin cytoskeleton. Most of the receptor-transfected cells displayed actin stress fibers indicative of RhoA activation (Fig. 5B, left panels), as compared to the control NIH 3T3 cells which lacked stress fiber formation (Fig. 5B, right panels). This result suggests RhoA activation by KSHV-GPCR.

Given that RhoA is a downstream effector of Gα13, we speculated that RhoA might function downstream of KSHV-GPCR to activate NF-κB. To test this hypothesis, HeLa cells were cotransfected with KSHV-GPCR and a dominant negative form of RhoA, RhoA(T19N). Expression of RhoA(T19N) partially inhibited KSHV-GPCR-induced NF-κB activation (Fig. 6A). This mutant RhoA also inhibited NF-κB activation by Gα13(Q226L) to a greater extent. Moreover, the C3 exoenzyme from Clostridium botulinum, a Rho-specific inhibitor (47), inhibited NF-κB activation induced by both KSHV-GPCR and Gα13(Q226L) (Fig. 6B). In contrast, the C3 exoenzyme had no effect on TNFα-induced NF-κB activation (not shown). Taken together, these data suggest that KSHV-GPCR activate the Gα13 – RhoA pathway.

The Gα13 – RhoA pathway is involved in KSHV-GPCR-induced IL-8 secretion. The expression of a number of chemokines, including IL-8 and GRO-α/MGSA, are controlled in part by NF-κB (25,48-50). IL-8 and GRO-α/MGSA also bind and stimulate KSHV-GPCR (11). To determine whether KSHV-GPCR-induced NF-κB activation affects IL-8 expression, HeLa cells were cotransfected with a luciferase reporter driven by the human IL-8 promoter (-272 base pairs upstream of transcription initiation site) that contains functional sites for NF-κB, NF-IL6 and AP-1 (38). KSHV-GPCR induced potent expression of the IL-8 luciferase reporter, which was dependent on the input DNA concentration (Fig. 7A). The KSHV-GPCR-induced IL-8 gene expression was accompanied by increased production of IL-8 as detected in the culture medium by ELISA (Fig. 7B).
In Fig. 2 above we reported a ~20% reduction of the κB luciferase activity when the KSHV-GPCR-transfected cells were treated with PTX. Similar reductions (~16%) were observed in IL-8 luciferase reporter assay (Fig. 8A) and in IL-8 secretion as determined by ELISA (Fig. 8B), indicating that a PTX-sensitive G protein is partially responsible for KSHV-GPCR-induced IL-8 gene expression and protein synthesis. A longer treatment with PTX (100 ng, 16 h) reduced the IL-8 luciferase activity by 24% (data not shown).

KSHV-GPCR has been reported to activate a PKC-responsive promoter that contains an AP-1 binding motif (7). In addition, the receptor induces inositol phosphate accumulation in transfected COS-1 cells (7). These findings suggest that KSHV-GPCR couple to Goq. Since the IL-8 promoter also contains an AP-1 binding motif (49), and Goq is known to activate NF-κB (37), we sought to determine the relative contribution of Goq and Gq13 to KSHV-GPCR-induced IL-8 gene expression and protein secretion. In the transfected cells, a constitutively active Goq(Q209L) induced nearly twice as much expression of the IL-8 luciferase reporter as did either KSHV-GPCR or Gq13(Q226L) (Fig. 8A). This activity, however, did not translate into a potent IL-8 production as cells transfected with Goq(Q209L) secreted little IL-8 (Fig. 8B). In comparison, IL-8 secretion was detected from cells transfected to express KSHV-GPCR and the activated Gq13 (Fig. 8B). There is a good correlation between the abilities of these two constructs to induce the IL-8 luciferase reporter and their abilities to stimulate IL-8 secretion.

The above observation suggests the involvement of Gq13 in KSHV-GPCR-induced IL-8 secretion. To test whether RhoA is part of the Gq13 signaling pathway for this function and whether NF-κB is required for KSHV-GPCR-induced IL-8 expression, HeLa cells were cotransfected with the dominant negative RhoA (T19N) or an IκBα “super repressor” which is devoid of inducible serine phosphorylation (51). As shown in Figure 8C, expression of RhoA (T19N) partially inhibited IL-8 secretion induced by KSHV-GPCR and by the activated Gq13, whereas expression of IκBαM nearly completely blocked the induced IL-8 secretion. This result is consistent with the notion that NF-κB is
essential for IL-8 expression (49). It also suggests the presence of a Gα13 activated pathway that may not involve RhoA.

KSHV-GPCR binds a large number of chemokines (7), including both agonists (11) and antagonists for this receptor (12,13). We investigated whether these chemokines affect KSHV-GPCR-induced secretion of IL-8 by treatment of the transfected HeLa cells with GRO-α/MGSA, an activator of KSHV-GPCR, or with interferon-γ-inducible protein 10 (IP-10), a negative antagonist for the receptor. As shown in Figure 9, KSHV-GPCR-induced IL-8 secretion was further enhanced by stimulating the cells with GRO-α/MGSA, but diminished by treating the cells with IP-10. The two chemokines did not affect Gα13(Q226L)-induced IL-8 secretion, indicating that these chemokines regulate IL-8 secretion at the receptor level. Because expression of GRO-α/MGSA, like IL-8, is also controlled by NF-κB, these findings suggest a possible regulatory mechanism for KSHV-GPCR-induced NF-κB activation and chemokine production.

DISCUSSION:

In this study we have demonstrated that expression of KSHV-GPCR results in constitutive NF-κB transactivation. This function is mediated by several G proteins including the PTX-sensitive Gαi/o, the PTX-insensitive Gαq and Gα13, and Gβγ proteins. Because the roles of Gαi/o, Gαq and Gβγ in NF-κB activation have been characterized previously with another GPCR (37) and recently with KSHV-GPCR (30,32), our study is focused on the activation of Gα13 and RhoA by KSHV-GPCR. The involvement of Gα13 and RhoA in KSHV-GPCR signaling is evidenced by the following observations: (1) Expression of wild type Gα13 enhances KSHV-GPCR-induced NF-κB activation. (2) Inhibition of Gα13 by p115RGS, a RGS specific for Gα13 and Gα12, reduces NF-κB activation by KSHV-GPCR. (3) Stress fiber formation has been observed in stably transfected NIH 3T3 cells that express KSHV-GPCR, indicating RhoA activation. (4) A dominant negative mutant of RhoA partially inhibits KSHV-GPCR-induced NF-κB activation. (5) The C3 exoenzyme, a specific inhibitor of RhoA, reduces NF-κB
activation by KSHV-GPCR. (6) The above inhibitors and dominant negative constructs also effectively inhibit NF-κB activation stimulated by a constitutively active Gα13 mutant. We do not observe complete inhibition by any of the above agents except IκBαM, indicating the involvement of multiple G proteins in KSHV-GPCR-induced NF-κB activation.

The observation that activated Gα13, but not activated Gαq, stimulates IL-8 secretion provides additional evidence for a role of Gα13 in functional coupling with KSHV-GPCR. The underlying mechanism is not clear at this time, but may include the possibilities of translational inhibition of the IL-8 message by Gαq and lack of a necessary component for efficient translation. This finding is similar to an earlier report indicating that C5a induces transcription of the IL-1 message but does not provide a translational signal (52). The C5a receptor is known for coupling to Gαi and Gα16, a member of the Gαq family, but not to Gα13. Further experiments will be necessary to thoroughly investigate the differences between Gαq and Gα13 in stimulating IL-8 biosynthesis. Gαq has been shown to mediate KSHV-GPCR-induced accumulation of inositol phosphate as this function is resistant to PTX treatment (7). Using the –272 IL-8 luciferase reporter, we demonstrated that Gαq is a potent inducer of IL-8 transcription. Therefore, Gαq may indirectly participate in IL-8 biosynthesis by upregulating the IL-8 message.

Results obtained from this study suggest activation of RhoA by KSHV-GPCR. RhoA is a member of the Rho small GTPases that regulate cytoskeleton rearrangement and other important cellular functions such as cell cycle progression through G1 (53). Thus, the stimulated cell proliferation by KSHV-GPCR may be attributed in part to Rho-mediated alteration of cell cycle. Consistent with a previous observation that the Rho GTPases activate c-Jun N-terminal kinase (JNK) and p38 MAP kinases but not extracellular signal-regulated kinase (ERK) (53), KSHV-GPCR has been shown to activate JNK and p38 but not ERK (8). These findings combined suggest that KSHV-GPCR stimulates cell proliferation through activation of Rho GTPases, but probably not ras, which has been known to stimulate ERK activation through Raf and ERK kinase. We are currently investigating the role of other Rho GTPases, Cdc42 and Rac1, in KSHV-GPCR-mediated functions.
RhoA is an effector of G\(\alpha\)13, and GPCRs that couple to G\(\alpha\)13 activate cellular functions in part through this small GTPase. A recent study demonstrates that G2A, a stress-inducible GPCR expressed in immature T and B lymphocyte progenitors (54), couples to G\(\alpha\)13 and activates RhoA (55). Expression of G2A in NIH 3T3 cells results in oncogenic transformation characterized by loss of contact inhibition, anchorage-independent growth, and tumorigenicity in mice (45). These studies indicate that G2A is a GPCR that utilizes G\(\alpha\)13 and RhoA for oncogenic transformation. Like G2A, KSHV-GPCR also induces cellular proliferation, cell transformation and tumorigenicity in nude mice (7-9). In addition, both receptors are absent from cells under resting condition, but are induced to express by either environmental stress or viral infection. These similarities suggest that the two receptors share certain components of their signaling pathways.

NF-\(\kappa\)B is a ubiquitous transcription factor that regulates the expression of a large number of cytokine and growth factor genes (24). NF-\(\kappa\)B also regulates the expression of several genes coding for anti-apoptotic factors such as Bcl-2 (24). Thus, activation of NF-\(\kappa\)B may be partially responsible for the stimulated proliferation of cells that express KSHV-GPCR (7). Of potential interest is that NF-\(\kappa\)B activation by KSHV-GPCR leads to expression of IL-8, a CXC chemokine that binds KSHV-GPCR as well as the chemokine receptors CXCR1 and CXCR2 (11). Expression of another CXC chemokine, GRO-\(\alpha\)/MGSA, is also induced by NF-\(\kappa\)B (50,56), although the cells used in the current study did not secrete GRO-\(\alpha\)/MGSA when stimulated by a variety of NF-\(\kappa\)B activators including TNF\(\alpha\) (data not shown). In several cell models, binding of the above two CXC chemokines to CXCR2 induces activation of the receptor and is responsible for the proliferation of these cells through an autocrine mechanism (29,57-59). Since both IL-8 and GRO-\(\alpha\)/MGSA have been shown to bind and activate KSHV-GPCR, a potential function of the secreted IL-8 could be autocrine stimulation through KSHV-GPCR similar to that seen with CXCR2 (59). Thus, it is likely that KSHV-GPCR-induced activation of NF-\(\kappa\)B and secretion of IL-8 contribute to the proliferation of cells that express this constitutively active receptor.
A characteristic feature of Kaposi’s sarcoma is overgrowth of vascular endothelial cells. It has been shown in transgenic mice that KSHV-GPCR is primarily responsible for the vascular overgrowth found in Kaposi’s sarcoma (10). KSHV-GPCR activates hypoxia-inducing factor 1α, resulting in increased production of VEGF (17). VEGF has been shown to play an important role in KSHV-GPCR-stimulated angiogenesis (8,10). In this regard, it is notable that IL-8 and a number of CXC chemokines bearing the N-terminal Glu-Leu-Arg (ELR) sequence can stimulate proliferation of vascular endothelial cells and therefore are angiogenic factors (60,61). These CXC chemokines bind and activate CXCR2, expressed in vascular endothelial cells, and stimulate the growth of these cells (62). Therefore, KSHV-GPCR-stimulated activation of NF-κB and the resultant secretion of IL-8 may contribute to angiogenesis and vascular overgrowth as seen in Kaposi’s sarcoma. Interestingly, the ELR+ GRO-α/MGSA could further stimulate IL-8 secretion, whereas the angiostatic and ELR CXC chemokine IP-10 (61) inhibited the induced IL-8 secretion. These results suggest that chemokines can positively and negatively regulate angiogenic factor production in cells that express KSHV-GPCR.

While this paper was in review, Schwarz and Murphy reported secretion of several proinflammatory cytokines, chemokines and growth factors in cells transfected to express KSHV-GPCR (31). Although the study does not lead to identification of the G proteins that couples KSHV-GPCR, the findings suggest that more than one G protein is involved in KSHV-GPCR signaling. This work also demonstrates that the ability of KSHV-GPCR to activate NF-κB and AP-1 is preserved in several cell lines. In another recent publication, Couty et al demonstrated with strong evidence that KSHV-GPCR can couple to Gi/o and Gq. They also displayed constitutive NF-κB activation in the transfected mouse lung endothelial cells (MLEC), that could be further induced by the KSHV-GPCR agonist GRO-α (32). Since Gαq and the PLCβ pathway is not constitutively activated by this receptor in MLEC, the inability of PTX and transducin to completely block constitutive activation of NF-κB in MLEC suggests the presence of another signaling molecule, possibly a G protein, in coupling the receptor for this function. Furthermore, Couty et al demonstrated that KSHV-GPCR stimulates phosphatidylinositol-3 kinase via a PTX-
insensitive mechanism. Thus, our finding that Gα13 functionally couples KSHV-GPCR for NF-κB activation and IL-8 secretion complements these recent reports and demonstrates for the first time that a chemokine receptor can utilize Gα13 for transcriptional regulation. With the recent identification of US28 as another constitutively active chemokine receptor of viral origin (41), it will be interesting to determine whether expression of a constitutively active GPCR is a general mechanism by which viruses and environmental stress regulate homeostasis of host cells.

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The abbreviations used are: GPCR, G protein-coupled receptor; KSHV, Kaposi’s sarcoma herpesvirus; VEGF, vascular endothelial growth factor; GRK, G protein-coupled receptor kinase; NF-κB, nuclear factor kappa B; GRO-α, growth regulated oncogene-α; MGSA, melanoma growth stimulating activity; PTX, pertussis toxin; RGS, regulator of G protein signaling; IP-10, interferon-γ-inducible protein 10; RLA, relative luciferase activity; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.
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LEGENDS FOR FIGURES:

Figure 1. Constitutive activation of NF-κB in cells expressing KSHV-GPCR. A, HeLa cells transfected with variable amounts of the KSHV-GPCR expression plasmid display increased κB luciferase activity in a gene dose-dependent manner. The AU5-tagged vector (shown) and the untagged vector (not shown) gave similar results. Ctrl, control without KSHV-GPCR. KB-, a mutant κB luciferase reporter that does not contain the functional κB binding sequence 5’-GGGACTTTCC-3’. All values were normalized against the coexpressed β-galactosidase, and expressed as relative luciferase activity (RLA, fold induction over baseline). B, Cell surface expression of KSHV-GPCR. Cells transfected with 400 ng and 800 ng of the AU5-tagged receptor construct were stained with an anti-AU5 mAb followed by a FITC-conjugated secondary Ab. Histograms of flow cytometry analysis were compared to cells transfected with the untagged KSHV-GPCR (control). The differences between mean fluorescent channel numbers are 0.11 (400 ng DNA vs. control) and 0.21 (800 ng DNA vs. control), indicating dose-dependent expression of the receptor. C, Cells expressing KSHV-GPCR responded to TNFα with an enhanced κB luciferase activity compared to cells with TNFα or KSHV-GPCR alone. TNFα was used at 10 ng/ml and the cells were stimulated for 4 h. Duplicate samples were included in each experiment. Data shown in A and C are mean ± S.D. from one representative experiment of a total of three.

Figure 2. KSHV-GPCR couples to multiple G proteins for NF-κB activation. A, Effect of pertussis toxin (PTX) on KSHV-GPCR-induced NF-κB transactivation. PTX was added to 500 ng/ml for 4 h. B, Western blotting showing the expression of Gα proteins in HeLa cells without (−) and with (+) transfection of the individual wild type G proteins. Antibodies against Gα13, Gαq and Gαi2 were used for immunoblotting (I.B.). Arrowhead marks Gα13. Because only about 30% of the cells were transfected and all the cells were collected for Western blotting, actually G protein expression level in the transfected cells could be higher. C and D, Expression of Gα proteins differentially alters the induced κB
luciferase activity in cells transfected with the KSHV-GPCR expression plasmid (C) or a B2BKR expression plasmid (D). The Gα proteins were used at 200 ng per sample. The B2BKR-transfected cells were either left unstimulated (−), or stimulated (+) with bradykinin (BK) at 10 nM for 4 h. All experiments were conducted 3 times, and a representative set of data was shown. Data were collected in triplicate, normalized against the coexpressed β-galactosidase to overcome variations in transfection efficiency, and expressed as mean ± S.E.M.

**Figure 3. A constitutively active Gα13 induces NF-κB activation.** A, Expression of Gα13 (Q226L) induces dose-dependent increases of the κB luciferase activity. B, Gα13 (Q226L) potentiated the induction of NF-κB activation by TNFα. The expression plasmid of Gα13 (Q226) was used at 200 ng / sample (B), or at the indicated amount (A). TNFα was added to 10 ng/ml for 4 h. Data were collected in duplicate, normalized against the coexpressed β-galactosidase activity, and expressed as mean ± S.D. A total of three experiments were conducted, and a representative set of results is shown.

**Figure 4. Negative regulation of KSHV-GPCR-induced NF-κB activation by signaling inhibitors of G protein pathways.** A, HeLa cells were transfected with KSHV-GPCR or Gα13(Q226L), with and without p115RGS. For each sample, 200 ng of DNA was used. A schematic drawing of p115RhoGEF depicts the relative locations of the RGS domain (filled bar), the Dbl-homology domain (DH) and the pleckstrin-homology domain (PH). An anti-myc mAb was used for detection of the myc-tagged p115RGS by Western blotting. B, The cells were transfected similarly as above, except that a bovine transducin expression vector was used in place of p115RGS. The expression of transducin was detected by Western blotting. For both A and B, inhibition of the κB luciferase activity is expressed as % of the maximal response induced by KSHV-GPCR (9.5-fold increase over baseline). Triplicate data were collected from one of the three similar experiments, normalized against the coexpressed β-galactosidase activity, and shown as mean ± S.E.M.
Figure 5. Stable expression of KSHV-GPCR results in NF-κB transactivation and actin stress fiber formation. A, Histogram showing the expression of KSHV-GPCR in stably transfected NIH 3T3 cells (KSHV-GPCR), as compared to untransfected cells (3T3). A polyclonal Ab against the N-terminal 41 amino acids was for flow cytometry analysis. Inset, expression of the κB luciferase reporter in the stably transfected NIH 3T3 cells (KS) but not in untransfected cells (3T3). B, Representative multi-cell images (top panels) and single-cell images (bottom panels) showing formation of actin stress fibers in the stably transfected NIH 3T3 cells (left panels) but not in untransfected NIH 3T3 cells (right panels). The cells were serum-starved and stained with Rhodamine-phalloidin. Images were taken by fluorescence microscopy. Since the transfected cells consist of a collection rather than clonal cells, approximately 2/3 (but not all) cells displayed actin stress fibers similar to the ones shown in the left panels.

Figure 6. Inhibition of RhoA reduces NF-κB activation by KSHV-GPCR and Gα13(Q226L). A, Effects of coexpression of a dominant negative RhoA (T19N) on κB luciferase activities induced by KSHV-GPCR and Gα13(Q226L). HeLa cells were transfected with KSHV-GPCR or Gα13(Q226L), with and without RhoA(T19N). B, Similar to A except that an expression vector encoding the C3 exoenzyme (C3 toxin) was used in place of the dominant negative RhoA. Inhibition of the κB luciferase activity is expressed as % of the maximal response induced by KSHV-GPCR (9.5-fold increase over baseline). Triplicate data were collected from one of the three similar experiments, normalized against the coexpressed β-galactosidase activity, and shown as mean ± S.E.M.

Figure 7. KSHV-GPCR induces IL-8 gene expression. A, Dose-dependent activation of an IL-8 luciferase reporter in transfected HeLa cells. The –272 Luc reporter containing cis-acting sites for NF-κB, NF-IL6 and AP-1 was used at 200 ng/sample. B, Production of IL-8 in KSHV-GPCR-transfected cells, as measured from culture medium with an IL-8 ELISA. For both A and B, data were collected in
duplicate and normalized against the coexpressed β-galactosidase activity. Shown in the figure were values of mean ± S.D. from one of the three experiments, each with similar results.

Figure 8. Involvement of Gα13 in KSHV-GPCR-induced IL-8 secretion. HeLa cells were transfected with the –272 IL-8 luciferase reporter, together with an expression vector for either KSHV-GPCR, Gα13(Q226L), or Gαq(Q209L). Some samples were treated with PTX (500 µM, 4 h) prior to assays. After 48 h, the induced luciferase activities were measured, normalized against the coexpressed β-galactosidase activities, and expressed as fold induction over basal (A). In (B), IL-8 secreted into the culture media was determined by ELISA. The data were normalized against the coexpressed β-galactosidase activities and are shown as means ± S.D. All expression constructs were used at 200 ng per sample. C, Inhibition of IL-8 secretion by a dominant negative RhoA and by a IκBα “super repressor”. The cells were transfected similarly as above, with 200 ng each of the RhoA(T19N) and IκBαM. Expression of these molecules were determined by Western blotting. Expression of the Gα13(Q226L) protein was determined using a HA-tagged construct. Duplicate data were collected from the above experiments and are shown as values of mean ± S.D. from one of the three experiments, all giving similar results.

Figure 9. Regulation of IL-8 secretion by ELR+ and ELR− chemokines in transfected HeLa cells. Each DNA construct was used at 200 ng/sample. Forty-eight hours after transfection, the cells were treated with or without GRO-α/MGSA (A), or IP-10 (B) for an additional 4 h. The chemokines were used at 100 nM each. The secreted IL-8 was detected by ELISA. Results were expressed as mean ± S.D. Data shown are derived from one representative experiment of a total of three.
Shepard et al., Fig. 4
A) KSHV-GPCR

RLA (% of basal)

Rhoa(T19N) − +

B) C3 toxin

RLA (% of basal)

C3 toxin − +
A

IL-8 Luciferase reporter

RLA (fold induction)

KSHV-GPCR (ng)

Ctrl 25 50 100 200

B

IL-8 ELISA

Secreted IL-8 (ng/ml)

KSHV-GPCR (ng)

Ctrl 20 50 100 200
Shepard et al., Fig. 9

**A**

| Secreted IL-8 (ng/ml) |
|----------------------|
| KSHV-GPCR | − | − | + | + | − | − |
| Gα13QL | − | − | − | + | + | + |
| GRO-α | − | + | − | + | − | + |

**B**

| Secreted IL-8 (ng/ml) |
|----------------------|
| KSHV-GPCR | − | − | + | + | − | − |
| Gα13QL | − | − | − | + | + | + |
| IP-10 | − | + | − | + | − | + |
Constitutive activation of NF-κB and secretion of IL-8 induced by the G protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus involves G α(13) and RhoA

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