

Chemokines Activate Kaposi's Sarcoma-associated Herpesvirus G Protein-coupled Receptor in Mammalian Cells in Culture

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8, a virus that appears to be involved in the pathogenesis of Kaposi's sarcoma and primary effusion lymphomas, encodes a G protein-coupled receptor (KSHV-GPCR) that exhibits constitutive signaling. In this report, we show that two chemokines, interleukin 8 (IL-8) and growth-related protein- α , activate KSHV-GPCR over constitutive levels. Moreover, as with human receptors, the integrity of the ELR motif of these chemokines is required for activation of KSHV-GPCR. Other residues that are required for IL-8 binding to human chemokine receptors CXCR1 and CXCR2 are important for KSHV-GPCR activation also. Thus, it appears that the ELR binding site and other key domains of ELR chemokine activation have been preserved in the virus KSHV-GPCR. The results suggest that KSHV-GPCR originated from CXCR1 or CXCR2 and that activation of KSHV-GPCR by endogenous chemokines may affect the pathobiology of KSHV infection in humans. (*J. Clin. Invest.* 1998. 102:1469–1472.) **Key words:** human herpesvirus-8 • growth-related protein- α • interleukin 8 • platelet factor-4 • constitutive signaling

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV)¹/human herpesvirus 8 is a gammaherpesvirus with homology to Herpesvirus saimiri and Epstein-Barr virus, which transform lymphocytes

(1, 2). Accumulating evidence suggests that KSHV may be involved in the pathogenesis of human primary effusion lymphomas (3) and Kaposi's sarcoma (KS) (4). KSHV encodes a G protein-coupled receptor (KSHV-GPCR) (5, 6) that in the absence of agonist signals via activation of intracellular phosphoinositide-specific phospholipase C leading to formation of inositol phosphate (IP) second messengers (7). Transfer of the KSHV-GPCR gene into NIH 3T3 cells results in expression of the receptor protein and leads to a number of cellular responses. These include cell proliferation (7), transformation (8) and production of vascular endothelial growth factor (8), which is an angiogenic factor and growth factor for AIDS-KS spindle cells (9, 10). In nude mice, tumors derived from these cells exhibit a highly vascularized histopathology similar to that of human KS. Thus, KSHV-GPCR may be a mediator of human KSHV-induced tumorigenesis.

KSHV-GPCR shows similarity to human chemokine receptors including CXCR1, which binds interleukin 8 (IL-8), and CXCR2, which binds IL-8, growth-related protein- α (GRO- α), and several other CXC chemokines (11). The ELR motif (sequence Glu-Leu-Arg) preceding the first cysteine is required for binding and activation of CXCR1 or CXCR2. The chemokine interferon- γ -inducible protein-10 (IP-10) binds and activates CXCR3 via an NH₂-terminal sequence distinct from the ELR motif (12, 13). Recently, we showed that the constitutive KSHV-GPCR signaling can be inhibited by IP-10 (14). In this report, we demonstrate that ELR containing chemokines can activate KSHV-GPCR above its constitutive signaling level in rodent and monkey cells in culture.

Methods

Materials. The CXC chemokines and chemokine analogues were chemically synthesized using established procedures (15). *myo*-[³H]inositol was purchased from New England Nuclear (Boston, MA). Dulbecco's modified Eagle's medium, Nu-SerumTM, and calf serum were from Collaborative Research Inc. (Waltham, MA).

Cell culture and IP formation. Untransfected NIH 3T3 cells and NIH 3T3 cells stably expressing KSHV-GPCRs (8) were grown in Dulbecco's modified Eagle's medium containing 10% calf serum. KSHV-GPCRs were expressed transiently in COS-1 cells grown in Dulbecco's modified Eagle's medium containing 5% Nu-SerumTM by transfection with pcKSHV-GPCR as described previously (7). For experiments, NIH 3T3 and COS-1 cells were reseeded in 24- or 12-well plates and incubated in medium containing 1% calf serum and 1 μ Ci *myo*-[³H]inositol/ml for 24–48 h. IP formation was measured for 1.5 h at 37°C by methods previously described (7). IP formation was expressed as the ³H-radioactivity in IPs as a percentage of the total ³H-radioactivity in lipids plus IPs at the end of the incubation.

Statistical analysis. Statistical analyses were performed by *t* test. *P* < 0.05 was considered significant.

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1. **Abbreviations used in this paper:** ELR, sequence Glu-Leu-Arg; GRO- α , growth-related protein- α ; IP, inositol phosphate; IP-10, interferon- γ -inducible protein-10; KS, Kaposi's sarcoma; KSHV, KS-associated herpesvirus; KSHV-GPCR, KSHV G protein-coupled receptor; PF-4, platelet factor-4.

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Results and Discussion

Previous studies in monkey kidney COS-1 cells transiently expressing KSHV-GPCRs failed to identify a chemokine that would activate KSHV-GPCR even though several CXC and CC chemokines bound to the receptor (7). However, we found that the level of IP formation generated constitutively by KSHV-GPCRs in COS-1 cells was similar to the level stimulated by IL-8 in COS-1 cells expressing CXCR1 or CXCR2 under the conditions originally used. Therefore, it was possible that COS-1 cells expressing KSHV-GPCRs were already stimulating very high levels of IP formation and, therefore, putative agonists were not readily detected. More recently, we have used stably transfected NIH 3T3 cells incubated under different conditions to test the biological effects of expression of the receptor (8, 14). In these cells, two human chemokines, IL-8 and GRO- α , were found to stimulate KSHV-GPCR signaling. Fig. 1 illustrates the concentration-dependent stimulation of IP formation by IL-8 and GRO- α ; the half-maximally effective concentrations were 39 and 15 nM, respectively. The amino acid sequences of these chemokines are shown in Fig. 2A. The potency of GRO- α in stimulating this receptor was similar to its potency in neutrophil activation. IL-8 had lower potency than GRO- α as an activator of KSHV-GPCR but exhibits higher potency in neutrophil activation. Both IL-8 and GRO- α are ELR-containing CXC chemokines. In contrast, CXC chemokines that do not contain the ELR motif, for example platelet factor-4 (PF-4) (Fig. 2), Mig (monokine induced by interferon- γ), and IP-10 (14), as well as CC chemokines, RANTES (regulated on activation, normal T expressed and secreted), and I-309, did not activate KSHV-GPCR (data not shown).

To show that the ELR motif is an important determinant for agonist activation of KSHV-GPCR signaling more directly, we tested several CXC chemokine analogues (Fig. 2A). IL-8AAR is a CXCR1 and CXCR2 antagonist (16) and GRO- α (8-73) is a CXCR2 antagonist (17). Fig. 2B illustrates that

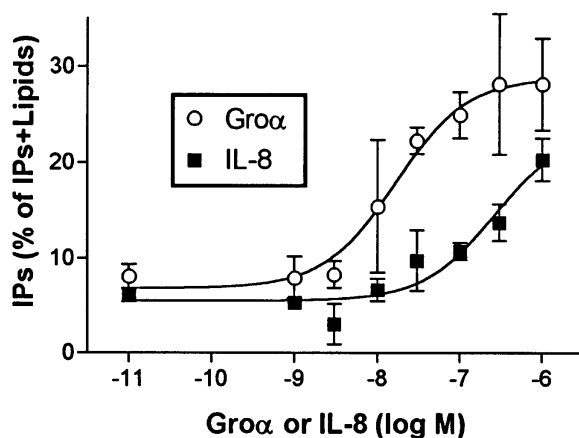


Figure 1. Concentration-dependent effects of IL-8 and GRO- α on KSHV-GPCR signaling. IP accumulation in NIH 3T3 cells stably transfected with plasmid encoding KSHV-GPCR was measured as described in Methods. Chemokine analogues were added at the concentrations indicated along with LiCl (10 mM). NIH 3T3 cells that do not express KSHV-GPCR do not signal in response to IL-8 or GRO- α (data not shown). The data represent the mean \pm SD of triplicate determinations in a representative of two experiments.

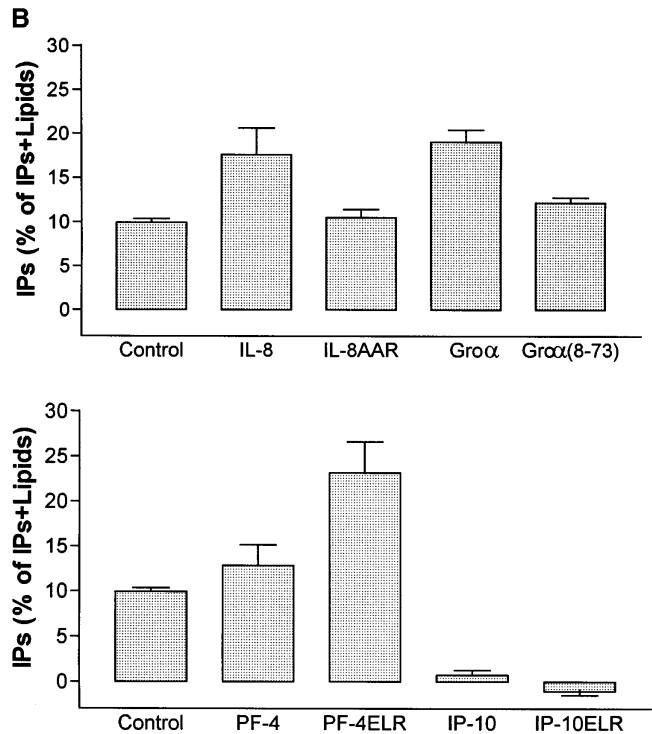
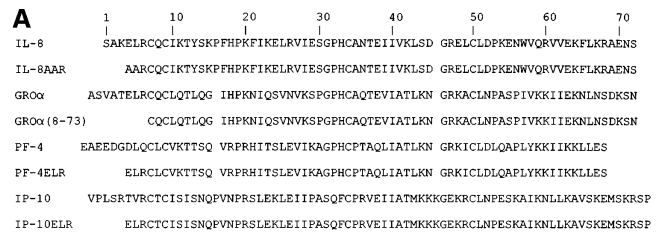


Figure 2. Effects of ELR-containing chemokines and chemokines that do not contain the ELR sequence on KSHV-GPCR signaling. (A) Amino acid sequences of IL-8, IL-8AAR, GRO- α , GRO- α (8-73), PF-4, PF-4ELR, IP-10, and IP-10ELR. (B) The experiments were performed as described in the legend to Fig. 1. The top illustrates native chemokines that contain the ELR sequence and their analogues in which ELR was deleted. The bottom illustrates native chemokines that do not contain the ELR sequence and their analogues in which ELR was added. All chemokines were added at a concentration of 1 μ M. The signaling with IL-8, GRO- α , PF-4ELR, IP-10, and IP-10ELR was significantly different from control. The data represent the mean \pm SD of triplicate determinations in a representative experiment.

these analogues do not activate KSHV-GPCR. Thus, disruption of the ELR motif in IL-8 and GRO- α causes loss of agonist activity. Similar results were found with IL-8 binding to its natural receptors CXCR1 and CXCR2. ELR-modified PF-4 (PF-4ELR) is a potent activator of CXCR2, whereas native PF-4 has neither agonist nor antagonist properties for known receptors (18). We found that PF-4ELR activates KSHV-GPCR, whereas native PF-4 is inactive. Thus, addition of the ELR sequence to PF-4 results in acquisition of agonist activity. However, addition of the ELR sequence did not convert all CXC chemokines into KSHV-GPCR agonists. IP-10ELR does not activate CXCR1 or CXCR2 (18). IP-10ELR, like native IP-10, not only failed to activate KSHV-GPCR but inhibited constitutive signaling. Therefore, both IP-10ELR and IP-10

are inverse agonists of KSHV-GPCR. Thus, the NH₂-terminal region of IP-10 is not involved in its inverse agonist activity and other regions of the IP-10 protein are important for KSHV-GPCR inhibitory activity (14). Furthermore, the structural requirements of IP-10 for activation of CXCR3 and inhibition of KSHV-GPCR differ.

The stimulatory effects of the ELR-containing chemokines are not peculiar to NIH 3T3 cells because PF-4ELR stimulates KSHV-GPCR signaling in COS-1 cells when they are grown under the same conditions as NIH 3T3 cells. In COS-1 cells expressing KSHV-GPCRs grown in medium containing 1% calf serum rather than 5% Nu-SerumTM as in our previous study (7), PF-4ELR increased basal signaling by KSHV-GPCR by 2.4±0.12-fold whereas PF-4 did not increase basal signaling (1.3±0.18-fold); untransfected COS-1 cells did not respond to PF-4ELR.

IL-8 is the best studied chemokine with regard to its structure-activity relationships (19). Experiments with hybrids identified features of IL-8 that are required for function. Apart from the NH₂-terminal ELR motif, the two disulfide bridges, residues 10-15 and residues 30-35, which make up an atypical turn, were important for IL-8 activity in neutrophils. In the three-dimensional structure of IL-8 (20, 21), the ELR sequence and the residue 30-35 sequence are connected through the 7-34 disulfide and the conformation of the turn was proposed to be critical for receptor interaction (22). We have used hybrids between IL-8 and IP-10 that had been generated previously for studies of IL-8 structure and function (22) (Fig. 3), to begin to assess the domains within IL-8 that are necessary for KSHV-GPCR activation. For our studies it is fortuitous that these hybrids were constructed between IL-8 and IP-10 as we can use them to examine the structural requirements for both the agonist activity of IL-8 and potentially the inverse agonist activity of IP-10. We studied the effects of several IL-8/IP-10 hybrids (Fig. 3). H1 inhibited KSHV-GPCR signaling and, therefore, is an inverse agonist. H4 exhibited modest activation of KSHV-GPCR and, therefore, may be a partial agonist. In contrast, H15 and H25 markedly activated KSHV-GPCR signaling and were, therefore, equivalent to IL-8 in their ability to activate KSHV-GPCR. Based on the sequences of these four hybrid molecules, we suggest that the ELR sequence, residues 10-15, and the turn comprising residues 30-35 of IL-8 are important for KSHV-GPCR activation. Although the importance of the ELR sequence is clear, we cannot conclude that the other two regions are needed because it is possible that the increased efficacies of H4, H15, and H25 were due to loss of inhibitory sequences from IP-10 rather than acquisition of stimulatory sequences from IL-8. We think, however, that this is unlikely.

Although we had shown previously that IP-10 is an inverse agonist at KSHV-GPCR, that is, it inhibits constitutive signaling in the COS-1 cell system (14), we were not able to determine whether IP-10 was as an antagonist of agonist-stimulated KSHV-GPCR signaling. Fig. 4 illustrates that IP-10 caused concentration-dependent inhibition of KSHV-GPCR signaling stimulated by IL-8 and PF-4ELR. The potencies of IP-10 inhibition of PF-4ELR-stimulated signaling, IL-8-stimulated signaling, and constitutive signaling were similar; half-maximally effective concentrations were 1.1 nM (0.39–3.3), 0.48 nM (0.17–1.4), and 1.9 nM (0.44–7.9), respectively. Thus, as with other inverse agonists (23), IP-10 exhibits antagonism of agonist-stimulated signaling also. Moreover, because the poten-

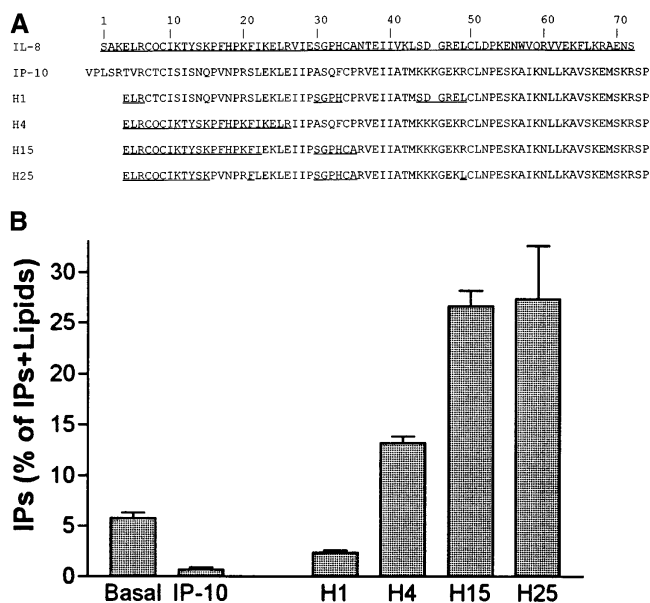


Figure 3. Stimulation of KSHV-GPCR signaling by IL-8/IP-10 hybrid molecules. (A) Amino acid sequences of IL-8, IP-10, and hybrids. IL-8 and the sequences of IL-8 present in the hybrid molecules are underlined. (B) The experiments were performed as described in the legend to Fig. 1. All chemokines were added at a concentration of 1 μ M. The signaling with IP-10, H1, H4, H15, and H25 were significantly different from control. The data represent the mean \pm SD of triplicate determinations in a representative experiment.

cies of these effects are indistinguishable, it is likely that the mechanism of inhibition of agonist-stimulated signaling is similar to that of inhibition of constitutive signaling.

Human chemokine receptors are activated by specific chemokines that contain the appropriate complementary binding sites (11, 24). KSHV-GPCR is a receptor that is capable of signaling and binds a wide range of chemokines. KSHV-

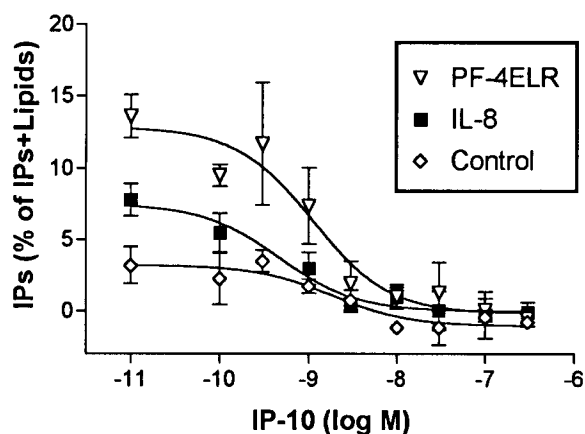


Figure 4. Inhibition of IL-8 and PF-4ELR stimulation of KSHV-GPCR signaling by IP-10. The experiments were performed as described in the legend to Fig. 1 except that IP-10, at the concentration shown, was added 15 min before LiCl. PF-4ELR (30 nM) or IL-8 (300 nM) was added immediately after LiCl. The data represent the mean \pm SD of triplicate determinations in a representative experiment.

GPCR binds both CXC and CC chemokines, whereas native chemokine receptors are, in general, specific for either CXC or CC chemokines. However, even though KSHV-GPCR appears to bind many chemokines, we find that only ELR-containing CXC chemokines further activate its constitutive signaling. The Duffy antigen receptor for chemokines (25) is a chemokine receptor-like protein that has no measurable signaling properties and is found on erythrocytes, endothelial cells, and Purkinje cells of the cerebellum. The function of the Duffy antigen receptor for chemokines is not known but it has been proposed to serve as a binding protein that clears chemokines from the circulation. In addition, glycosaminoglycans bind CXC and CC chemokines (26) but do not appear to be involved in signaling. The KSHV-GPCR is unique because it binds a wide range of chemokines, but only IL-8-related chemokines, which have the ELR motif and other conserved domains, stimulate signaling. This is consistent with the hypothesis that a KSHV precursor originally acquired either CXCR1 or CXCR2 and mutation and selection resulted in KSHV-GPCR, a receptor that is constitutively activated and can bind other chemokines, features that are presumably advantageous to the virus. Future experiments will test the role of the chemokine-KSHV interaction in infectivity, pathology, and tumorigenesis.

Data support the idea that KSHV infection is associated with KS (4) and primary effusion lymphomas (3). However, direct evidence that KSHV infection causes either of these tumors has not yet been presented. To demonstrate that KSHV-GPCR plays a pathogenic role in these diseases it will be necessary to develop a model system in which KSHV infection leads to tumorigenesis and then show that KSHV-GPCR signaling is important. Our previous discovery of an inhibitor (14) and in this report of activators of KSHV-GPCR signaling should allow direct testing in an animal model of the hypothesis that KSHV infection results in expression of KSHV-GPCR and that its signaling leads to tumorigenesis. Lastly, if KSHV-GPCR is involved in tumorigenesis in humans, for example by stimulating angiogenesis (27), then it is likely that activation of KSHV-GPCR by endogenous chemokines would affect the pathobiology of KSHV infection. For example, tissue-specific expression of endogenous chemokines could account for the localization of KS tumors.

Acknowledgments

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