The N Terminus of Kaposi’s Sarcoma-associated Herpesvirus G Protein-coupled Receptor Is Necessary for High Affinity Chemokine Binding but Not for Constitutive Activity*

Hao H. Ho, Dongyi Du, and Marvin C. Gershengorn‡

From the Program in Molecular Medicine, Weill Graduate School of Medical Sciences and the Division of Molecular Medicine, Department of Medicine, Weill Medical College of Cornell University, New York, New York 10021

Kaposi’s sarcoma-associated herpesvirus (KSHV) contains a gene encoding a G protein-coupled receptor (KSHV-GPCR) that is homologous to mammalian chemokine receptors. KSHV-GPCR signals constitutively (in an agonist-independent manner) via the phosphoinositide-inositol 1,4,5-trisphosphate pathway. Because it has been proposed that the N terminus (N-TERM) of other GPCRs may act as tethered agonists, we determined whether the N-TERM of KSHV-GPCR is necessary for constitutive signaling activity. The N-TERM of KSHV-GPCR with those of two other GPCRs, deletion of residues within the N-TERM, and disruption of a putative disulfide bond that may hold the N-TERM in close proximity to extracellular loop 3 do not affect constitutive signaling activity but decrease chemokine binding. There were differences in the effects of mutation of the N-TERM on binding of the chemokines growth-related oncogene α, which is an agonist, and interferon-γ-inducible protein-10, which is an inverse agonist. The effects of chemokine binding were accompanied by changes in chemokine regulation of KSHV-GPCR signaling. We conclude that the N-TERM is not necessary for constitutive KSHV-GPCR signaling, i.e. the N-TERM is not a tethered agonist, but plays a crucial role in binding of chemokine ligands and of chemokine regulation of KSHV-GPCR signaling.

Kaposi’s sarcoma is a multifocal angioproliferative tumor that is often first apparent in the skin or mucous membranes but may involve the viscera as well. Currently, it is the most common AIDS-related tumor. The causative agent for this disease appears to be a member of the herpesvirus family known as Kaposi’s sarcoma-associated herpesvirus (KSHV)1 or human herpesvirus 8, which is also likely involved in the pathogenesis of primary effusion lymphoma and Castleman’s disease (1). One of the genes encoded by KSHV, ORF-74, is a chemokine-like G protein-coupled receptor (KSHV-GPCR). KSHV-GPCR has been found to be a constitutively (or basally) active receptor that signals through the phosphoinositide-inositol 1,4,5-trisphosphate pathway, stimulates cell proliferation, and transforms rodent fibroblasts (2, 3). KSHV-GPCR binds growth-related oncogene α (Groα), interferon-γ-inducible protein-10 (IP-10) and other chemokines (2, 4–6). Based on its signaling properties and stimulation of cell proliferation, we suggested that KSHV-GPCR may play an important role in KSHV-induced tumorigenesis (2, 3). Thus, delineation of the mechanism of regulation of KSHV-GPCR signaling may lead to insights into the pathogenesis of Kaposi’s sarcoma and to drugs that may treat Kaposi’s sarcoma.

Based on the mechanism of activation of protease-activated receptors (7) in which the N terminus (N-TERM) of the GPCR functions as a “tethered ligand,” we considered whether the N-TERM of KSHV-GPCR acts as a tethered agonist to constitutively activate the receptor. Protease-activated receptors are not constitutively active but undergo proteolysis of their extreme N-TERM to uncover the activating domain within the N-TERM. We reasoned that changes within the N-TERM of KSHV-GPCR during viral evolution could have led to its serving as a tethered agonist without requiring protease cleavage. It is noteworthy that mutations within the melanocortin-1 receptor have been suggested to cause constitutive activation by mimicking the effect of agonist (8) and that the binding of the chemokine monocyte chemoattractant protein-1 to the N-TERM of its cognate GPCR, CC chemokine receptor 2 (CCR2), may produce a pseudo-tethered ligand (9). Because the N-TERMS of other chemokine receptors are involved in ligand binding (10), we also determined whether the N-TERM was involved in regulation of KSHV-GPCR signaling by chemokines (4–6). Out data show that the N-TERM of KSHV-GPCR, although involved an important role in ligand binding and chemokine regulation of signaling, is not necessary for the constitutive activity of the receptor.

EXPERIMENTAL PROCEDURES

Construction of KSHV-GPCR Mutants—All KSHV-GPCR mutants were constructed by PCR using pCNA 3.1-KSHV-GPCR as template (2, 1–2–11), 1–2–11/4A, 1–1–22, and 1–2–29 were constructed using the following oligonucleotides: (a) 5′-CAAGAATTCACCATGGAATTCC-CAATGGGAAACTTCAGGGGAAATGTGGGATTTCC-3′, (b) 5′-CCGGAGATCTCCGCGGACCCCACTGGGCAATCTGGAATCTGAATGAACTTCAGG-3′, (c) 5′-CAAGAATTCACCATGGAATTCC-CAATGGGAAACTTCAGGGGAAATGTGGGATTTCC-3′, and (d) 5′-CAAGAATTCACCATGGAATTCC-CAATGGGAAACTTCAGGGGAAATGTGGGATTTCC-3′, respectively, as sense primers, and (e) 5′-ATGCTTGTGTGCGGACCCCACTGGGCAATCTGGAATCTGAATGAACTTCAGG-3′ as the antisense primer. EcoRI and EcoRV restriction sites were used to clone the PCR fragments into pCNA 3.1-KSHV-GPCR.

Overlapping PCRs were performed to generate C39A and C286A KSHV-GPCRs. In both constructions, the same sense primer was used: (f) 5′-CAAGGAAATTCCTCGAAGGCGAATGGAAGTAGAGTTTG-3′. For antisense primers, C39A used primer e, and
C286A used primer g (5’-GTGTTAAGGCGGCTCTAGACTGCGAGC-3’). The two overlapping primers were as follows: (h) 5’-CTGAAAGTGACCGGCTGCAGATGCGACGCTGCGTTCCTACG-3’ and (i) 5’-CACAGGGTCTACTGCAAGACCTGCTTACCTGCAGACGGAAGTCCTGTGGGCAGGGCGG-3’ were used on template plDNA-CXCR3, and primers n (5’-CATCTGAGGAACTTGCGGCTTTGCTAGCAGGTAAGTCATCTAGTGTTGCAGGCTGCAGGG-3’ and e) were used on plDNA 3.1-KSHV-GPCR. Then, the two PCR fragments were combined, and primers q and e were used to amplify them. In the making of hCTR3-NT, primers o (5’-CAAGAATTCCACCATGGACTACAAGGACGACGACGAGATGACAAAG-3’ and p) 5’-GGTCTCATCTCACACATGATGTGGACAGGTCGTCATT-3’ were used to amplify them. The amino acid sequences of the N-TERMs of WT and deletion mutants of KSHV-GPCR.

RESULTS AND DISCUSSION

We studied WT KSHV-GPCR and the following KSHV-GPCR mutants (Fig. 1): CXCR3-NT, in which the first 50 residues of the N-TERM were substituted by the first 50 residues of CXC chemokine receptor 3 (CXCR3), the cognate receptor for IP-10 and monokine-induced by interferon-γ (13); hCTR3-NT, in which the first 50 residues of the N-TERM were substituted by the first 86 residues of human calcitonin receptor type 3 (14); ∆T(2–11), lacking amino acid residues 2–11; ∆T(2–11)/4A, lacking residues 2–11 and having Asp-12, Asp-13, Asp-14, and Glu-15 substituted by Ala; ∆T(1–22), lacking residues 1–22; ∆T(2–29), lacking residues 2–29; C39A, in which Cys-39 was substituted by Ala; C286A, in which Cys-286 was substituted by Ala; and C39A/C286A, in which Cys-39 and Cys-286 were substituted by Ala.

All mutant KSHV-GPCRs exhibited constitutive signaling activity when expressed in COS-1 cells (Fig. 2). Groc is a CXC chemokine that contains the sequence ELR and is a high affinity agonist at KSHV-GPCR (6, 15). We found that CXCR3-NT, hCTR3-NT, ∆T(2–11)/4A, ∆T(1–22), and ∆T(2–29) did not bind 125I-Groc with sufficiently high affinity to be characterized in a binding assay. Indeed, in addition to WT, only ∆T(2–11) exhibited high affinity binding of 125I-Groc. Binding of 125I-Groc to ∆T(2–11) was only 10% of that to WT. These data show that the N-TERM of KSHV-GPCR is important for binding Groc. To estimate the level of expression of receptors that did not bind 125I-Groc with high affinity, we developed a chemiluminescent enzyme-linked immunosorbent assay using H/F-tagged receptors. The His residues allowed the solubilized receptors to be chelated by Ni²⁺ bound to polyacrylamide at the bottom of wells in a 96-well plate. The FLAG epitopes of the Ni²⁺-adsorbed receptors were bound with the monoclonal antibody M2 and measured with a secondary antibody conjugated to α-galactosidase. Fig. 3 illustrates the direct relationship between the luminescent signal and the binding of 125I-Groc to WT KSHV-GPCR-H/F. These data validate this assay for quantitation of receptor expression. There was no difference in constitutive signaling between untagged and H/F-tagged WT and KSHV-GPCR mutants (data not shown).

Fig. 4 illustrates an experiment in which H/F-tagged, mutant KSHV-GPCRs were expressed in HEK 293EM cells. As shown in Fig. 3 in COS-1 cells, CXCR3-NT and hCTR3-NT exhibited constitutive activity. The basal levels of signaling...
Experimental Procedures" and is defined as the increase in inositol phosphate (IP) second messenger molecules accumulated during a 2-h incubation minus that accumulated in untransfected or mock-transfected cells. 125I-Gro binding was calculated as the difference between total and nonspecific binding and is presented as cpm bound. The data represent the mean ± S.D. of three determinations in one representative experiment of two performed.

Basal signaling was measured in COS-1 cells as described under "Experimental Procedures" and is defined as the increase in inositol phosphate (IP) second messenger molecules accumulated during a 2-h incubation minus that accumulated in untransfected or mock-transfected cells. 125I-Gro binding was calculated as the difference between total and nonspecific binding and is presented as cpm bound. The data represent the mean ± S.D. of three determinations in one representative experiment of two performed.

were 28 and 39% of WT for CXC3-NT and hCTR3-NT, respectively. The level of expression of CXC3-NT was 18% of WT, and that of hCTR3-NT was 44% of WT. Thus, the lowered basal levels of signaling of these two KSHV-GPCR mutant receptors appeared to be due to their lower levels of expression (see below). These findings show that the N-TERM of KSHV-GPCR is not needed for basal signaling activity.

To confirm that the N-TERM is not needed for basal signaling and to begin to delineate the domains within the N-TERM that are necessary for binding and regulation of KSHV-GPCR signaling by chemokines, we used KSHV-GPCR mutant receptors that had deletions within their N-TERMs. Fig. 4 illustrates the levels of basal signaling and expression of these mutant receptors in HEK 293EM cells. We consistently observed lower levels of basal signaling and of receptors in cells expressing these mutant receptors than in cells expressing WT KSHV-GPCR. The levels of basal signaling were 20, 64, 33, and 42% of WT, and the levels of expression were 43, 57, 11, and 17% of WT for Δ(2–11), Δ(2–11)/4A, Δ(1–22), and Δ(2–29), respectively. These N-TERM deletion mutant receptors, therefore, appear to exhibit a lowered basal signaling that is proportional to their lowered levels of expression (see below). These data further support the conclusion that the N-TERM is not involved in constitutive signaling of KSHV-GPCR.

Fig. 5 illustrates that the N-TERM of KSHV-GPCR is important for Groa binding. As shown in Fig. 2, only WT and Δ(2–11) bound 125I-Gro with high affinity. The loss of binding in Δ(2–11)/4A shows that the negatively charged sequence Asp-Asp-Glu at positions 12–15 is important for binding Groa, which, like other chemokines (16), contains a number of Lys and Arg residues in its C terminus. The decreased binding of 125I-Gro to Δ(2–11) compared with WT is not accounted for by a decrease in the affinity of 125I-Gro binding (Fig. 5) but represented a lower level of expression of Δ(2–11) of approximately 40% of WT (Fig. 4). The binding affinities for Groa were indistinguishable for WT and Δ(2–11) with equilibrium inhibitory constants (Ki) values of 2.8 (2.1–3.7 nm) and 5.3 nm (3.4–8.3 nm), respectively. By contrast, Δ(2–11) exhibited a 10-fold decrease in binding affinity for IP-10, which is a CXC chemokine that does not contain the ELR sequence and is an inverse agonist of KSHV-GPCR (4, 6), compared with WT; Ki for IP-10...
of 120 nM (72–190 nM) for Δ(2–11) and 12 nM (10–15 nM) for WT. These data suggested that although both Groα and IP-10 bind to KSHV-GPCR, at least in part, via interacting with the N-TERM, there are different domains within the N-TERM that interact with Groα and IP-10.

Fig. 6 illustrates that mutations of the N-TERM that decrease binding of Groα and IP-10 decrease the activities of these chemokines to stimulate and inhibit KSHV-GPCR signaling, respectively. CXCR3-NT, hCTR3-NT, Δ(2–11)/4A, Δ(2–22), and Δ(2–29) did not bind Groα, and Groα did not stimulate signaling of these mutant receptors, nor did IP-10 inhibit it. It is not surprising that IP-10 does not inhibit signaling by, for example, hCTR3-NT, because the N-TERM of hCTR3, which is important in binding its cognate ligand calcitonin (17, 18), is very different from that of KSHV-GPCR. It was perhaps surprising that basal signaling of CXCR3-NT was not inhibited by IP-10, because the N-TERM of CXCR3 is likely involved in binding IP-10 to wild-type CXCR3 (10). We cannot determine whether IP-10 does not bind to CXCR3-NT or whether it binds but does not inhibit signaling because radioiodinated IP-10, which is used to measure binding to CXCR3 (19), does not exhibit high affinity binding to KSHV-GPCR (data not shown). In contrast to the lack of effects of both Groα and IP-10 on the other mutant receptors, Groα stimulated signaling by Δ(2–11), but 1000 nM IP-10 did not inhibit it. The lack of inhibition of Δ(2–11) signaling by IP-10 is not due to its reduced affinity because the concentration of IP-10 used in the experiment is estimated to occupy more than 95% of the available receptors. Therefore, the deletion of residues 2–11 must inhibit some postbinding step that is necessary for IP-10 to inactivate the receptor. That is, there must be a consequence of the binding of IP-10 to residues 2–11 in the N-TERM of KSHV-GPCR, perhaps by mediating a change in IP-10 conformation that allows IP-10 to act as an inverse agonist. Thus, the N-TERM is important in binding chemokines and in allowing a secondary effect that allows chemokines to regulate KSHV-GPCR signaling. We have previously proposed that a conformational change occurs in another ligand, thyrotropin-releasing hormone, upon binding to its cognate GPCR and that the induced conformation is biologically more active than the predominant conformation found in solution (20).

There is a conserved Cys residue in the N-TERM and another in the third extracellular loop in chemokine receptors that appear to form a disulfide bond and were shown to be important for ligand binding in other chemokine GPCRs (9, 21, 22). To determine whether Cys residues in the N-TERM and extracellular loop-3 of KSHV-GPCR may form a disulfide bond, we constructed C39A, C286A, and C39A/C286A receptors. All three Cys-to-Ala mutant receptors were expressed at the cell surface and exhibited constitutive signaling activity (Fig. 7). The levels of basal signaling were 61, 68, and 68% of WT, and the levels of expression were 79, 80, and 29% of WT for C39A, C286A, and C39A/C286A, respectively. Thus, as with the other N-TERM mutant KSHV-GPCRs (see above), the lowered basal signaling of the Cys-to-Ala mutant receptors appeared to be due to their lowered levels of expression (see below). We did not observe any high affinity binding of 125I-Groα to these mutants (data not shown). To support the idea that the decrease in binding was caused by loss of a disulfide bond, we pretreated cells expressing WT receptors with dithiothreitol and N-ethylmaleimide. As expected, we found a marked decrease (>95%) in high affinity binding of 125I-Groα to cells in which WT KSHV-GPCR was reduced and alkylated (data not shown). Fig. 8 illustrates that 100 nM Groα stimulated but 1000 nM IP-10 did not inhibit signaling of C39A, C286A, or C39A/C286A receptors. To further delineate the effects of disruption of this disulfide, we measured the concentration dependences of Groα stimulation. Fig. 9 illustrates that there were similar rightward shifts of the Groα concentration-response curves for all three Cys-to-Ala mutant receptors compared with WT. The half-maximally effective concentration (EC50) of Groα was 7.1 nM (4.9–10 nM) in cells expressing WT and 71–79 nM (19–340 nM) in those expressing the Cys to Ala substitution mutant receptors. Thus, the lack of high affinity binding appears to be caused by 10-fold decreases in affinities. It is noteworthy that the double mutant, C39A/C286A, did not show a greater increase in EC50 for Groα than C39A or C286A. This lack of additivity of effects of two mutations has been taken as evidence that the two mutations affect the same aspect of GPCR structure (23) and, therefore, further support the idea that there is a disulfide bond between Cys-39 and Cys-286 in WT KSHV-GPCR. This disulfide bond is important for proper interaction between the N-TERM of KSHV-GPCR and chemokine ligands.

In this study, we probed the role of the N-TERM in KSHV-GPCR signaling. We find that the N-TERM is not required for basal (or constitutive) signaling by KSHV-GPCR. That is, the N-TERM does not serve as a tethered agonist. This is, perhaps, most easily appreciated in Fig. 10, which shows a direct correlation between the levels of constitutive signaling and of expression for 9 of the 10 KSHV-GPCRs studied herein. We do not understand why Δ(2–11) appeared to exhibit a lower basal signaling activity relative to its expression than the other receptors. We conclude, therefore, that there is a domain(s) in KSHV-GPCR other than the N-TERM that causes the receptor to signal constitutively. A related conclusion regarding the involvement of a domain in addition to the N-TERM for chemokine activation of a mammalian GPCR has been drawn. Pease et al. (24) found that chimeras of CCR1 and CCR3 that

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**Fig. 6.** Effects of Groα and IP-10 on signaling by WT and N-TERM deletion mutant KSHV-GPCRs. Basal signaling in COS-1 cells was measured and defined as described in the legend to Fig. 2. Groα or IP-10 was added at the start of the experimental incubation with LiCl. The data represent the mean ± S.D. of triplicate determinations in two experiments.

**Fig. 7.** Basal (constitutive) signaling activities and receptor expression of WT and Cys-to-Ala mutant KSHV-GPCRs. Basal signaling and receptor expression in HEK 293EM cells were measured as described in the legend to Fig. 4. The data represent the mean ± S.D. of triplicate determinations in one representative experiment of two performed.
conserved NPXXY sequence in the intracellular region of transmembrane helix 7 of rhodopsin subfamily members that have been shown to be involved in signaling (26). Both the D/E of the (D/E)RY sequence and the N of the NPXXY are Val residues in KSHV-GPCR. However, KSHV-GPCR mutants in which either of these residues was substituted by the conserved residue exhibited unchanged basal signaling activities.2

Mammalian chemokine receptors generally bind chemokines of the CXC or CC family, but not both (27). For most chemokine receptors, important interactions between ligand and receptor involve the receptor N-TERM (10). KSHV-GPCR binds CXC and CC chemokines (2), and we have now shown that important interactions involve the KSHV-GPCR N-TERM. It is, therefore, interesting that a mammalian seven-transmembrane spanning protein, Duffy antigen/receptor for chemokines, which is a protein with a topology similar to GPCRs but which has not been shown to exhibit signaling activity (28), binds CXC and CC chemokines with primary interactions involving its N-TERM (29). There is, however, no sequence homology between the N-TERMs of KSHV-GPCR and Duffy antigen/receptor for chemokines.

We provide evidence for a disulfide bond between Cys-39 in the N-TERM and Cys-286 in extracellular loop-3 of KSHV-GPCR and found that it was important in binding chemokines. A disulfide bond between Cys residues in the same positions of two mammalian chemokine receptors, CCR2 (9) and Duffy antigen/receptor for chemokines (21), have been described, and this pair of Cys residues is highly conserved in chemokine receptors in general (16). A pairs of Cys residues, which appears to form a disulfide bond between the N-TERM and third extracellular loop, is present in the bradykinin B2 receptor, another rhodopsin subfamily member (30). This pair of Cys residues, however, is not found in most GPCRs.

Our findings that show that the N-TERM of KSHV-GPCR is important for binding and regulation of signaling by chemokine agonists and inverse agonists is similar to the findings with mammalian chemokine GPCRs (10). These data are consistent with the idea that KSHV-GPCR is a receptor that was “pirated” by KSHV (31) that retains important aspects of mammalian chemokine receptor biology.

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