Charged Residues at the Intracellular Boundary of Transmembrane Helices 2 and 3 Independently Affect Constitutive Activity of Kaposi’s Sarcoma-associated Herpesvirus G Protein-coupled Receptor*

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Hao H. Ho, Nimalja Ganeshalingam‡, Avia Rosenhouse-Dantsker‡, Roman Osman‡, and Marvin C. Gershengorn§

From the Division of Molecular Medicine, Department of Medicine, Weill Medical College and Graduate School of Medical Sciences of Cornell University, New York, New York 10021 and the §Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, New York 10029

Because charged residues at the intracellular ends of transmembrane helix (TMH) 2 and TMH3 of G protein-coupled receptors (GPCRs) affect signaling, we performed mutational analysis of these residues in the constitutively signaling Kaposi’s sarcoma-associated herpesvirus GPCR (KSHV-GPCR). KSHV-GPCR contains the amino acid sequence Val-Arg-Tyr rather than the Asp/Glu-Arg-Tyr (D/E)RY motif at the intracellular end of TMH3. Mutation of Arg-143 to Ala (R143A) or Gln (R143Q) abolished constitutive signaling whereas R143K exhibited 50% of the basal activity of KSHV-GPCR. R143A was not stimulated by agonist, whereas R143Q was stimulated by growth-related oncogene-α, and R143K, similar to KSHV-GPCR, was stimulated further. These findings show that Arg-143 is critical for signal generation in KSHV-GPCR. In other GPCRs, Arg in this position may act as a signaling switch by movement of its sidechain from a hydrophilic pocket in the TMH bundle to a position outside the bundle. In rhodopsin, the Arg of Glu-Arg-Tyr interacts with the adjacent Asp to constrain Arg outside the TMH bundle. V142D was 70% more active than KSHV-GPCR, suggesting that an Arg residue, which is constrained outside the bundle by interacting with Asp-142, leads to a receptor that signals more actively. Because the usually conserved Asp in the middle of TMH2 is not present in KSHV-GPCR, we tested whether Asp-83 at the intracellular end of TMH2 was involved in signaling. D83N and D83A were 110 and 190% more active than KSHV-GPCR, respectively. The double mutant D83A/V142D was 510% more active than KSHV-GPCR. That is, cosubstitutions of D83A were 110 and 190% more active than KSHV-GPCR, whereas R143Q was stimulated by growth-related oncogene-α, and R143K, similar to KSHV-GPCR, was stimulated further. These findings show that Arg-143 is critical for signal generation in KSHV-GPCR. In other GPCRs, Arg in this position may act as a signaling switch by movement of its sidechain from a hydrophilic pocket in the TMH bundle to a position outside the bundle. In rhodopsin, the Arg of Glu-Arg-Tyr interacts with the adjacent Asp to constrain Arg outside the TMH bundle. V142D was 70% more active than KSHV-GPCR, suggesting that an Arg residue, which is constrained outside the bundle by interacting with Asp-142, leads to a receptor that signals more actively. Because the usually conserved Asp in the middle of TMH2 is not present in KSHV-GPCR, we tested whether Asp-83 at the intracellular end of TMH2 was involved in signaling. D83N and D83A were 110 and 190% more active than KSHV-GPCR, respectively. The double mutant D83A/V142D was 510% more active than KSHV-GPCR. That is, cosubstitutions of D83A by Ala and Val-142 by Asp act synergistically to stimulate further. These findings show that Arg-143 and Asp-83 independently affect the signaling activity of KSHV-GPCR.

Kaposi’s sarcoma-associated herpesvirus (KSHV)† (human herpesvirus 8) is a γ-herpesvirus that has been implicated in the pathogenesis of several human diseases including Kaposi’s sarcoma (KS), primary effusion lymphoma, and a subset of multicentric Castleman’s disease (1, 2). A G protein-coupled receptor (KSHV-GPCR) is encoded within the genome of KSHV (3, 4). It has been shown that KSHV-GPCR exhibits constitutive signaling via the phosphoinositide-specific phospholipase C pathway (5). When expressed in mouse fibroblasts, KSHV-GPCR stimulates cell proliferation, causes transformation, and promotes angiogenesis mediated by vascular endothelial growth factor (6). Recently, expression of this viral receptor was shown to induce the formation of angioproliferative lesions in transgenic mice that were similar to the lesions of Kaposi’s sarcoma (7). Because persistently activated GPCRs can function as human tumor genes (8), we suggested that the constitutive signaling activity of KSHV-GPCR may play an important role in KSHV-induced tumorigenesis (9). Understanding the details of KSHV-GPCR structure that cause constitutive signaling, therefore, may allow for the design of drugs to treat KS and primary effusion lymphoma.

In previous reports (10), we tested the hypothesis that the amino-terminal extracellular domain of KSHV-GPCR may serve as a “tethered ligand” that constitutively activates the receptor. Although we found that the amino terminus was important for high affinity binding of chemokines, we showed that the amino terminus was not important for constitutive signaling. This observation was recently confirmed (11). Therefore, other domains within KSHV-GPCR are involved in causing the receptor to signal constitutively. Rosenkilde et al. (11) identified residues at the extracellular loop/fifth transmembrane helix (TMH5) and TMH6 boundaries, which when mutated decreased agonist stimulation and residues in TMH2, which when mutated decreased basal signaling. We sought to identify additional domains within KSHV-GPCR that cause the receptor to signal constitutively. In particular, we were interested in discovering mutations that could lead to increased basal signaling so as to use computer models of these receptors to delineate the structural changes that constitute KSHV-GPCR activation.

The highly conserved amino acid sequence, Asp/Glu-Arg-Tyr ((D/E)RY), at the intracellular end of TMH3 of other GPCRs of the rhodopsin family has been shown to be important in receptor activation. It was initially shown that the charged pair of Glu-Arg was needed for rhodopsin activation because double solution; HBSS, HEPES HBSS; Groα, growth-related oncogene-α; IP, inositol phosphate.

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‡ To whom correspondence should be addressed: Weill Medical College of Cornell University, 1300 York Ave., Rm. A328, New York, NY 10021. Tel.: 212-746-6275; Fax: 212-746-6289; E-mail: mgersho@mail.med.cornell.edu.

† The abbreviations used are: KSHV, Kaposi’s sarcoma-associated herpesvirus; GPCR, G protein-coupled receptor; TMH, transmembrane helix; PCR, polymerase chain reaction; HBSS, Hank’s balanced saline solution; HEPES HBSS; Groα, growth-related oncogene-α; IP, inositol phosphate.
of virions (12). With α1p-adrenergic receptors, mutation of the Arg to some amino acids caused inhibition of stimulated signaling whereas mutation to Lys increased basal signaling, and mutation of Asp to Ala caused a marked increase in basal activity (13, 14). In the receptor for gonadotropin-releasing hormone, which has an Asp-Arg-Ser sequence at the intracellular end of TM6, mutation of Arg markedly decreased agonist-stimulated activation whereas the Asp to Asn mutation was less active as the wild type (Asp→Ala did not express, Ref. 15). In the CB2 cannabino-

noid receptor, mutation of Arg to Ala inhibited agonist-stimulated signaling, Asp to Ala abolished signaling, and Tyr to Ala inhibited signaling; there was no apparent effect on basal signaling (16). Mutation of the Arg of the DRY sequence in the oxytocin receptor caused the receptor mutant to be constitutively active (17). With the histamine H2 receptor, which is constitutively active, mutation of Asp caused increased constitutive signaling whereas mutation of Arg caused a decrease in signaling activity (18). The sequence at the putative intracellular end of TM3 in KSHV-GPCR is Val-Arg-Tyr. It is particularly relevant, therefore, that mutation of the Asp of the DRY sequence in the chemokine receptor CXXCR2 (the human recep-

tor most homologous to KSHV-GPCR) to Val caused constitutive activation of this receptor (19).

In this study, we have performed mutational analysis of the residues at the intracellular end of TM2 and of nonconserved Asp residues in TM2 and TM3 in KSHV-GPCR. We have focused primarily on the effects of these residues on constitutive signaling, because it is easier to understand the intramolecular interactions that lead to conformational changes involved in receptor activation in the absence of agonist binding. Arg-143 in KSHV-GPCR was determined to be critical for sig-

naling. Moreover, mutation of Asp-53 caused increased basal signaling, and mutations at these two positions acted synergis-

tically to increase constitutive signaling. A model is presented that predicts these site-specific substitutions act independently to activate KSHV-GPCR.

**EXPERIMENTAL PROCEDURES**

**Construction of KSHV-GPCR Mutants—All KSHV-GPCR mutants were constructed by PCR using plasmid 3.1-KSHV-GPCR as template unless otherwise stated. V142N, R143A, R143K, R143Q, Y144A, and Y144F were constructed using the following oligonucleotides: (a) 5′-TACCTGGTATTTATCGCTCAGACCCAGGAGCAGTATAGCTCAGACCCAGCACAACACTGAGCT-3′; (b) 5′-CAGGGAGTACCTCCAGCTAAGCTAAGCTTACGGCAGCAGGGAAGTACTGACCTGAACTGACAG-3′; and (e) 5′-ACTTGCTTCTGGGCCCCGAACTGAGT-3′. The two overlapping primers were as follows: (a) 5′-CGGGAGGAGGAGGATAGCTTACGCTTCTGGGTTCTGCTGAACTGACAG-3′ and (b) 5′-ACCAGGAAGGATAGCTTACGCTTCTGGGTTCTGCTGAACTGACAG-3′ as the sense primer. **HindIII** and **KpnI** restriction sites were used to clone the PCR products into pcDNA 3.1-KSHV-GPCR. All the mutants were confirmed by sequencing.**

**For mutants V142D, D132A, and D132N, the following antise-

sense oligonucleotides were used: (b) 5′-ATTCGAGGAGGATAGCTTACGCTTCTGGGTTCTGCTGAACTGACAG-3′ and (e) 5′-ACTTGCTTCTGGGCCCCGAACTGAGT-3′. The two overlapping primers were as follows: (a) 5′-CGGGAGGAGGAGGATAGCTTACGCTTCTGGGTTCTGCTGAACTGACAG-3′ and (b) 5′-ACCAGGAAGGATAGCTTACGCTTCTGGGTTCTGCTGAACTGACAG-3′ as the sense primer. **HindIII** and **KpnI** restriction sites were used to clone the PCR products into pcDNA 3.1-KSHV-GPCR, and the mutants were confirmed by sequencing.**

**Overlapping PCRs were performed to generate DS8A and DS8N KSHV-GPCRs. In both constructions the same sense and antise-

nsens primers were used: (d) 5′-CAATGT-GAGTAC-TCCGGA-AAGTCT-AATT-GTAAAAC-ACC-ATGGCCGGCCGAGATTTCTACACATGTC-3′ and (m) 5′-ACTTGCTTCTGGGCCCCGAACTGAGT-3′. The two overlapping primers were as follows: (a) 5′-CGGGAGGAGGAGGATAGCTTACGCTTCTGGGTTCTGCTGAACTGACAG-3′ and (b) 5′-ACCAGGAAGGATAGCTTACGCTTCTGGGTTCTGCTGAACTGACAG-3′ as the sense primer. **HindIII** and **KpnI** restriction sites were used to clone the PCR products into pcDNA 3.1-KSHV-GPCR, and the mutants were confirmed by sequencing.**

**The double mutant DS8A/D132A was generated using primer k and i as sense and antisense primer, respectively, using DS8A KSHV-GPCR as the template. **HindIII** and **KpnI** restriction sites were used to clone the PCR products into pcDNA 3.1-KSHV-GPCR, and the mutants were confirmed by sequencing.**

**Transfected cells were re-seeded into 24-well plates 24 h after transfection in Dulbecco’s modified Eagle’s medium with 5% Nu-Serum (Collaborative Research) and 1 μCi/ml myo-[3H]inositol (PerkinElmer Life Sciences).** 48 or 72 h after transfection, the medium was removed, and cells were rinsed with Hank’s balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4 (HBBSH). The cells were then incubated in HBBSH containing 10 mM LiCl in the absence or presence of growth-related oncogene α (Gros), which is a chemokine agonist for KSHV-

GPCR (20, 21), for 24 h at 37 °C. Accumulated inositol phosphates (IPs) were measured using ion-exchange chromatography as described (22). **IP accumulation was analyzed as [3H]IPs/[3H]lipids + [3H]IPs.**

**Competition Binding—Cells were transfected and seeded as described above except that no myo-[3H]inositol was added.** 48 h after reseeding, cells were rinsed once with cold HBBSH and then incubated in cold HBBSH supplemented with 0.5% bovine serum albumin and 10 mM HEPES and 50 μM 4-(Gros) (PerkinElmer Life Sciences) in the absence or presence of various concentrations of Gros for 4 h at 4 °C. At the end of the incubation, the cells were washed twice with 1 ml of cold HBBSH containing 0.5 mM NaCl and lysed with 0.5 ml of 0.4 N NaOH. Cell lysates were transferred to glass test tubes and counted in a gamma counter.

**Computer Modeling—Construction of the model of KSHV-GPCR was as follows. The helical boundaries of KSHV-GPCR were determined from a multiple alignment of the sequence of the receptor with those of the other chemokine receptor sequences. The core domain of extracellular loop 1 (Asn-65), TM3 (Arg-143), TM5 (Pro-233), and TM7 (Pro-311) were designed as a turn. Because extracellular loop 3 is too long to design as a turn, it was constructed by homology to a sequence identified in a loop from the top of TMH4 to Cys-196, which putatively forms a disulfide bridge. The loop has a short loop, i.e., a turn, it was constructed by homology to a sequence identified in a loop of extracellular loop 2 spanned the sequence from the disulfide bridge to the ends of the loop agrees well with the distance between TMH6 and TMH3 of KSHV-GPCR.**

**The short loops, i.e., intracellular loops 1, 2, and 3 and extracellular loop 1 were designed as turns such that the distance between the ends of the loops was approximately equal to the distance between the helices that the loops were connecting. Extracellular loop 2 was divided into two segments. The first extracellular loop 2 segment spanned the sequence from the top of TMH4 to Cys-196, which putatively forms a disulfide bridge with Cys-118 at the top of TMH6. The second segment of extracellular loop 2 spanned the sequence from the disulfide bridge to the point the loop connected with TMH5. Each of the segments was designed as a turn. Because extracellular loop 3 is too long to design as a turn, it was constructed by homology to a sequence identified in a known structure of the protein 1EMS (Nit-Flag Histidine Triad Fusion Protein) with 77% similarity and 41% identity. The loop has a distinct helical segment followed by a turn, and the distance between the ends of the loop agrees well with the distance between TMH6 and TMH7. The loops were connected manually and the entire system was minimized with a distance-dependent dielectric constant. The construction of the receptor was done with Insight and the structure optimization with CHARMM 26. The chemokine receptor CCR2 was constructed in a similar way using the aligned sequence from the multiple alignment as above.**

**Statistical Analysis—Statistical analyses were performed by analys-

is of variance.**
RESULTS AND DISCUSSION

To show that Arg-143 at the intracellular end of TMH3 is involved in signaling in KSHV-GPCR, Arg-143 was mutated to Ala, Gln, or Lys. The three mutant receptors exhibited binding affinities for $^{125}$I-labeled Gro$\alpha$ that were within 2-fold of the affinity of KSHV-GPCR (Figs. 1A and 2B). We measured constitutive signaling activity of these receptors as ligand-independent signaling that is directly related to the level of receptors expressed transiently in COS-1 cells. Fig. 1B illustrates the results from experiments in which the basal production of inositol phosphate second messenger molecules was measured in populations of cells expressing different levels of KSHV-GPCR and mutants of Arg-143. Basal signaling was measured as the slope of the regression line of inositol phosphate production versus $^{125}$I-Gro$\alpha$ binding. The dotted line is basal signaling of KSHV-GPCR. B, equilibrium inhibitory constants ($K_i$).

We next mutated Tyr-144 to Phe or Ala. Y144F and Y144A exhibited binding affinities for $^{125}$I-labeled Gro$\alpha$ (Fig. 2B), basal signaling activities (Fig. 2A), and Groa-stimulated signaling activities (data not shown) that were not different from KSHV-GPCR. Thus, Tyr-144 is not important for KSHV-GPCR binding and signaling.

In contrast to most GPCRs of the rhodopsin family, there is a Val before Arg-Tyr at the intracellular end of TMH3 in KSHV-GPCR. As noted above, mutation of the Asp in the DRY sequence of chemokine receptor CXCR2 to Val caused the receptor to become more constitutively active (19). With mutation of Val-142 to Asn and Asp in KSHV-GPCR, V142N exhibited an affinity that was indistinguishable from KSHV-GPCR, and V142D exhibited binding affinity for $^{125}$I-labeled Gro$\alpha$ that was within 2-fold of KSHV-GPCR (Fig. 2B). V142N exhibited a basal level of signaling that was indistinguishable from KSHV-GPCR ($p > 0.1$, Fig. 2A). Figs. 2A and 3 illustrate that V142D
that V142D exhibited the same basal signaling activity as a each experiment to measure basal signaling accurately. We, therefore, determined receptor expression in GPCR. Moreover, these levels varied from experiment to experiment. We have found that several of the more active mutants were expressed at lower levels than KSHV-GPCR. Moreover, these levels varied from experiment to experiment. We, therefore, determined receptor expression in each experiment to measure basal signaling accurately.

Based on data from experiments with other GPCRs, for example, the α1-adrenergic receptor (13, 14) and the receptor for oxytocin (17), it has been suggested that the positive side chain of the Arg of the (D/E)RY sequence may interact with a highly conserved Asp residue in the mid-portion of TMH2. There is no Asp in the usual position in TMH2 in KSHV-GPCR, but there is an Asp near the intracellular end of TMH2 that could be part of a putative hydrophilic pocket in the TMH bundle. It is highly conserved Asp residue in the mid-portion of TMH2. For oxytocin (17), it has been suggested that the positive side chain of the Arg of the (D/E)RY sequence may interact with a highly conserved Asp residue in the mid-portion of TMH2. There is no Asp in the usual position in TMH2 in KSHV-GPCR, but there is an Asp near the intracellular end of TMH2 that could be part of a putative hydrophilic pocket in the TMH bundle. It is highly conserved Asp residue in the mid-portion of TMH2. For oxytocin (17), it has been suggested that the positive side chain of the Arg of the (D/E)RY sequence may interact with a highly conserved Asp residue in the mid-portion of TMH2. There is no Asp in the usual position in TMH2 in KSHV-GPCR, but there is an Asp near the intracellular end of TMH2 that could be part of a putative hydrophilic pocket in the TMH bundle. It is highly conserved Asp residue in the mid-portion of TMH2. For oxytocin (17), it has been suggested that the positive side chain of the Arg of the (D/E)RY sequence may interact with a highly conserved Asp residue in the mid-portion of TMH2. There is no Asp in the usual position in TMH2 in KSHV-GPCR, but there is an Asp near the intracellular end of TMH2.

Asn, D132A exhibited an affinity that was within 2-fold of KSHV-GPCR (p < 0.001)(Figs. 2A and 4). We next constructed the double mutant D83A/D132A that exhibited basal signaling activity that was 510% above KSHV-GPCR (p < 0.01). The basal signaling activity of V142D, however, was only minimally stimulated by Groa (data not shown). These findings are different from those reported by Rosenkilde et al. (11) who concluded that V142D exhibited the same basal signaling activity as KSHV-GPCR. In their analysis, however, Rosenkilde et al. (11) did not compare basal signaling to receptor expression but compared it instead to the amount of plasmid used in the transfection and assumed that the levels of receptor expression were similar. We have found that several of the more active mutant receptors were expressed at lower levels than KSHV-GPCR. Moreover, these levels varied from experiment to experiment. We, therefore, determined receptor expression in each experiment to measure basal signaling accurately.

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D83N exhibited basal signaling activity that was 510% above KSHV-GPCR (p < 0.01)(Figs. 2A and 4). We next constructed the double mutant D83A/D132A that exhibited basal signaling activity that was 510% above KSHV-GPCR (p < 0.01)(Figs. 2A and 4). That is, the effect of having both mutations in the same receptor was synergistic with regard to basal signaling compared with D83A and V142D with individual mutations. This finding is consistent with the idea that these substitutions affected basal signaling via similar mechanisms (see below).

We next constructed the double mutant D83A/V142D. Although D83A/V142D exhibited the same high affinity for 125I-labeled Groa as KSHV-GPCR, the level of D83A/V142D expression was always lower than KSHV-GPCR when the same amounts of plasmid DNA were used for transfection. D83A/V142D exhibited basal signaling activity that was 510% greater than KSHV-GPCR (p < 0.001, Figs. 2A and 4). That is, the effect of having both mutations in the same receptor was synergistic with regard to basal signaling compared with D83A and V142D with individual mutations. This finding is consistent with the idea that these substitutions affected basal signaling via similar mechanisms (see below).

Another negatively charged residue that could be part of a putative hydrophilic pocket in the TMH bundle is Asp-132. Although D83A/V142D exhibited the same high affinity for 125I-labeled Groa as KSHV-GPCR, V142D was the most active KSHV-GPCR receptor mutant we have found. Because other constitutively active GPCR mutants have been shown to be less stable than native, basally inactive receptors (18, 24), it is possible that the low level of expression of D83A/V142D is caused by its decreased stability.

Lastly, to determine whether these most constitutively active receptors could be inhibited by the three chemokines found to be inverse agonists of KSHV-GPCR (25–27), we measured the effects of interferon γ-inducible protein-10, stromal-derived factor-1 and viral monocyte inflammatory protein-II. All three chemokines inhibited V142D and D83A to an extent similar to inhibition of KSHV-GPCR but none of them had a consistent effect on basal signaling by D83A/V142D (Fig. 5). We cannot provide an explanation for these differences because the mechanism by which inverse agonists inhibit KSHV-GPCR signaling is not known. It seems, however, that the ability of
the inverse agonists to inhibit signaling by these receptors was inversely related to the level of constitutive activity of the receptor. Other KSHV-GPCR mutants have been found to be unresponsive to inverse agonist inhibition but these were receptors with mutations in their amino-terminal domains that caused loss of binding (10, 11).

We constructed a computer model of KSHV-GPCR to help explain our findings (Fig. 6). The model predicts that Val-142 and Arg-143 in KSHV-GPCR are at the intracellular end of TMH3. In rhodopsin (28), in which the sequence at the intracellular end of TMH3 is ERY, as well as in our model of CXCR2 (not shown) in which the sequence is DRY, the side chain of Arg-143 interacts with the negatively charged residue proximal to it. In KSHV-GPCR such an interaction is impossible because Val-142 replaces the negatively charged residue. Thus, the side chain of Arg-143 has no partner to stabilize its positive charge. Asp-83, which is predicted to be positioned at the intracellular end of TMH2 in KSHV-GPCR, is highly conserved in the chemokine receptor family. In other receptors of the rhodopsin family, this residue is an Asn. An examination of the model of KSHV-GPCR (Fig. 6) shows that this residue is directed between TMH1 and TMH7 and is stabilized by a set of interactions with backbone N–H groups. It is also positioned close to Arg-78 in intracellular loop 1. The position and orientation of Asp-83 place it on the opposite side of TMH2 pointing away from TMH3. Thus, this residue shows no interaction with Arg-143 in TMH3. This is in full agreement with the position of the conserved Asn in TMH2 of rhodopsin as seen in its recent crystal structure (28). The model predicts that Arg-143 and Asp-83 do not interact. As described above, our experimental findings provide functional evidence that these residues do not interact also.

Because basal signaling by KSHV-GPCR is very active (5), we hypothesize that its structure is a model for the active states of GPCRs of the rhodopsin family including agonist-occupied receptors. Study of the structure-function relationship of constitutively active GPCRs (8) has the advantage of elucidating the three-dimensional structure of the active state of the receptor in the absence of a complicating ligand. This approach has been taken in a small number of instances. For example, we showed that models of the three-dimensional structure of constitutively active receptors for thyrotropin-releasing hormone (TRH) predict conformations similar to that of the TRH-occupied receptor (29). Lin et al. (30) predicted from a model of the luteinizing hormone receptor the changes in receptor structure that cause activation and used functional analysis of naturally occurring, constitutively active receptors to support their predictions. We suggest that further structure/function studies of basally active GPCRs will provide important insights into the mechanism of receptor activation.

In the study reported here, we constructed and characterized a number of mutant receptors in which charged residues in TMH2 and TMH3 were substituted to gain insight into the role of these residues in signaling by KSHV-GPCR. As discussed in the Introduction, charged residues within the (D/E)RY sequence at the intracellular end of TMH3 have been shown to play important roles in signaling by a number of GPCRs (12–19). Charged residues in other domains within GPCRs have been shown to be important for signaling also. For example,
Perez and coworkers (31, 32) presented evidence that disruption of a salt-bridge between an Asp in TMH3 and a Lys in TMH7 may be involved in cAMP-adrenergic receptor activation. Donohue et al. (33) developed support for the idea that a salt-bridge between an Asp at the extracellular loop 1/TMH2 boundary and an Arg at the extracellular loop 3/TMH7 boundary constrained the gastrin-releasing peptide receptor in conformation that is needed for coupling to G proteins. We showed that an Arg residue in TMH6 of the TRH receptor was involved in receptor activation (34). Here we found several different phenotypes with regard to signaling properties of KSHV-GPCR mutants substituted at charged residues even though all the mutant receptors exhibited high binding affinity for Groo. D132N exhibited basal and Groo-stimulated signaling activity indistinguishable from KSHV-GPCR. R143Q had little basal activity but exhibited-fold stimulation by Groo that was similar to KSHV-GPCR whereas R143K had a 50% reduced basal activity but was stimulated by Groo to the same level as KSHV-GPCR. In contrast, R143A did not signal basally and could not be stimulated by Groo. A number of mutant receptors such as D83A, D83N, and D132A exhibited higher basal activity than KSHV-GPCR but were only minimally stimulated by Groo. Thus, some single site-specific substitutions of charged residues led to receptors that were more basally active than KSHV-GPCR. The double mutants D83A/R143K and D83A/R143Q exhibited basal signaling activity that appeared to be equal to the additive effects of the single mutations. That is, these receptors exhibited basal activities that were intermediate between those of D83A and R143K or R143Q. Perhaps the most interesting receptor phenotype was that exhibited by the double mutant D83A/V142D. This mutant had basal signaling activity that was markedly greater than either single mutant D83A or V142D (see below). Mutations in other domains of KSHV-GPCR have previously been shown to generate receptors with different phenotypes including receptors that do not bind ligands with high affinity (10, 11), receptors that exhibit higher basal signaling than KSHV-GPCR but are not stimulated by Groo (11) such as D83A, D83N, and D132A, and receptors that exhibit lowered basal activity but could be stimulated by agonist (11), such as R143Q and R143K. Thus, selective mutation of KSHV-GPCR can lead to different phenotypes, similar to what has been reported with other GPCRs.

The phenotype of the double mutant D83A/V142D was unexpected. Before the construction of our model, we believed that Arg-143 and Asp-83 may interact, as has been proposed for the Arg of (D/E)RY and a highly conserved Asp in TMH2 of other GPCRs (13, 15), even though Asp-83 was not in the same position in TMH2 as the highly conserved Asp. Our findings, however, are consistent with the idea that Asp-83 at the intracellular end of TMH2 and Arg-143 at the intracellular end of TMH3 are important for KSHV-GPCR signaling but act independently of one another. We draw this conclusion based in particular on the observation that the double mutant D83A/V142D exhibits a higher level of constitutive signaling than would be expected by the addition of the increased signaling exhibited by the two single mutants D83A and V142D. Indeed, if Arg-143 and Asp-83 interact in KSHV-GPCR, we would expect that the double mutant would exhibit basal signaling activity equal to that of one of the individual mutant receptors. Moreover, our model is consistent with independent effects of substituting these two residues because the model of KSHV-GPCR predicts that the sidechain of Arg-143 resides in a hydrophilic pocket formed by helices 1, 2, and 7 with the TMH bundle and the sidechain of Asp-83 faces outside the bundle in a position on the side of TMH2 away from TMH3 near intracellular loop 1. That is, these two residues are predicted to be in the wrong orientation relative to one another and too far apart to interact. We can speculate on a mechanism to explain this phenotype that is consistent with the predictions of our model and with our data. It is possible that Arg-143 resides in the TMH bundle in a partially activated receptor and that movement out of the bundle caused by agonist stimulation in KSHV-GPCR or by interaction with Asp substituted at position 142 in V142D leads to further activation. This may occur because Arg-143 out of the bundle interacts with residues in intracellular loop 3 to promote higher affinity coupling to G protein. Asp-83 interacts with residues in intracellular loop 1 in a partially activated receptor and upon agonist stimulation in KSHV-GPCR or substitution by Ala in D83A this interaction is lost, and the loss of this constraint allows intracellular loop 1 to couple to G protein more effectively. The synergism occurs because when both of these changes occur in the same receptor, the coupling between receptor and G protein is markedly enhanced. These observations support the concept that a receptor can exist in multiple conformational states along a continuum from completely inactive to fully active (35).

In conclusion, we have found that charged residues, Arg-143 and Asp-83, at the intracellular ends of TMH2 and TMH3, respectively, upon mutation affect signaling by KSHV-GPCR. We suggest that these residues are directly involved in KSHV-GPCR signaling perhaps by mediating changes in receptor conformation or by interacting with the G protein. It is possible, however, that the signaling changes observed upon their mutation may be caused by indirect effects. Arg at this position, which is highly conserved in all GPCRs of the rhodopsin family, has been shown to be important in signaling in all receptors in which the residue has been studied. Asp at this position, which is found in all chemokine receptors but not in most members of the rhodopsin family, has not been studied in mammalian chemokine receptors. Substitutions that we predict affect both of these residues in D83A/V142D lead to a receptor with more than 6 times the basal signaling activity of KSHV-GPCR but which no longer responds to stimulation by agonist or to inhibition by inverse agonist. KSHV-GPCR is thought to be a pirated chemokine receptor that has mutated to serve an important, but unknown function(s) in the viral life cycle. Our findings show that additional mutation of KSHV-GPCR can create a receptor that is more basally active and not regulatable by chemokines. We suggest, therefore, that the phenotype of high basal but chemokine-regulated signaling activity are attributes of KSHV-GPCR that are important to KSHV survival.

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Charged Residues at the Intracellular Boundary of Transmembrane Helices 2 and 3 Independently Affect Constitutive Activity of Kaposi's Sarcoma-associated Herpesvirus G Protein-coupled Receptor
Hao H. Ho, Nimalja Ganeshalingam, Avia Rosenhouse-Dantsker, Roman Osman and Marvin C. Gershengorn

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