A thesis of the Degree of Doctor of Philosophy

감마 2-헤르페스 바이러스의 GPCR 과 CXCR4 의 상호작용에 의한 T 세포의 활성화

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February 2017

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Major in Biomedical Sciences

Department of Biomedical Sciences

Seoul National University Graduate School

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지도 교수 조 남 혁

이 논문을 의학박사 학위논문으로 제출함
2017 년 2 월

서울대학교 대학원
의과학과 의과학전공
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Constitutive activation of T cells by γ2-herpesviral GPCR through the interaction with CXCR4

by
Eun-Kyung Kwon

A thesis submitted to the Department of Biomedical Sciences in partial fulfillment of the requirement of the Degree of Doctor of Philosophy in Medical Science at Seoul National University

February 2017

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ABSTRACT

Members of the herpesviral family use multiple strategies to hijack infected host cells and exploit cellular signaling to ensure successful infection and propagation. Among the most intriguing weapons in the arsenal of pathogenic herpesviruses are the constitutively active virally-encoded G protein-coupled receptors (vGPCRs). The open reading frame (ORF) 74 of γ2-herpesviruses encodes a G protein-coupled receptor which is highly conserved in members of this subfamily and is homologous to the CXCR2 chemokine receptor. In contrast to host chemokine receptors, which are activated in a ligand dependent manner and predominantly couple to $G\alpha_{i/o}$ proteins, several vGPCRs are constitutively active and can couple to multiple G proteins. Even though vGPCRs modulate various cellular signaling pathways to contribute to viral pathogenesis such as immune evasion and proliferative disorders, the molecular details of how vGPCRs continuously activate cellular signaling are largely unknown. Here, I report that the vGPCR of Herpesvirus *saimiri* (HVS), an oncogenic γ-herepesvirus, constitutively activates T cells via a heteromeric interaction with cellular CXCR4 in the absence of any cognate ligand. Constitutive T cell activation also occurs with expression of the vGPCR of Kaposi's sarcoma-associated herpesvirus (KSHV), but not the vGPCR of Epstein-Barr virus (EBV). Expression of HVS vGPCR down-regulated the surface expression of CXCR4 but did not induce the degradation of the chemokine receptor, suggesting that vGPCR/CXCR4 signaling continues in cytosolic compartments. The physical association of vGPCR with CXCR4 was demonstrated by proximity ligation assay as well as immunoprecipitation. Interestingly, the constitutive activation of T cells

by HVS vGPCR is independent of TCRβ and proximal T cell receptor (TCR) signaling molecules, such as Lck, and ZAP70, whereas CXCR4 silencing by shRNA abolished T cell activation by vGPCRs of HVS and KSHV. Furthermore, previously identified inactive vGPCR mutants failed to interact with CXCR4. These findings on the positive cooperativity of vGPCR with cellular CXCR4 in T cell activation extend our current understanding of the molecular mechanisms of vGPCR function and highlight the importance of heteromerization for GPCR activity.

Keywords: Herpesviruses, vGPCR, Chemokine-receptor, Heterodimerization, T cell activation, Internalization

Student number: 2010-30605

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LIST OF ABBREVIATIONS

AP-1 : Activating Protein-1

BAC : Bacmid clone

BSA : Bovine Serum Albumin

cAMP : Cyclic adenosine monophosphate

CD : Cluster-of-Differentiation

cDNA : complementary Deoxyribonucleic acid

CMV : Cytomegalovirus

CXCR4: CX-chemokine receptor 4

DAG : diacylglycerol

DMEM : Dulbecco's modified Eagle's medium

EBV : Epstein-Barr virus

EDTA: ethylenediaminetetraacetic acid

EF1α : Elongation Factor 1 Alpha

eGFP : enhanced Green Fluorescent Protein

ELISA : Enzyme-Linked ImmunoSorbent Assay

ER : endoplasmic reticulum

FACS : Fluorescence Activated Cell Sorter

FBS : Fetal Bovine Serum

GST : Glutathione *S*-transferase

HEK 293T cells : Human embryonic kidney 293T cells

HVS : Herpesvirus *saimiri*

IL-2 : Interleukin-2

IP_3 : Inositol 1,4,5-triphosphate

KSHV : Kaposi's sarcoma-associated herpesvirus

MACS : Magnetically Activated Cell Sorter

mTOR : mammalian Target Of Rapamycin

NFAT : Nuclear Factor of Activated T cells

NF-κB : Nuclear Factor κB

PBMC : Human Peripheral Blood Mononuclear Cell

PBS: phosphate-buffered saline

PCR : Polymerase Chain Reaction

PI3K : Phosphoinositide 3-kinase

PIP2 : phosphatidylinositol-4,5-bisphosphate

PLA : proximity ligation assay

PLC: Phospholipase C

PMCA : plasma membrane Ca^{2+} ATPase

SERCA : sarco/endoplasmic reticulum Ca^{2+} ATPase

TCR : T cell antigen receptor

vGPCRs : viral G protein-coupled receptors

INTRODUCTION

G-protein-coupled receptors (GPCRs) constitute a diverse family of membrane receptors which can be activated by ligands varying from light (e.g., rhodopsin) to proteins (e.g., chemokine receptors). Despite being activated by such a variety of ligands, the receptors themselves are similar in structure composition. All GPCRs are composed of seven transmembrane domains, and couple to hetero-trimeric G-proteins resulting in a plethora of cellular responses [47]. They participate in a wide variety of physiological functions, including neurotransmission, angiogenesis, chemotaxis, and cellular proliferation [48]. They are also involved in a number of human diseases, which is reflected by the fact that GPCRs are the target of 50 ~ 60% of all present therapeutic agents [49].

Herpesviruses are widespread pathogens that establish lifelong latent infections, possibly via a long evolutionary history of specific interactions with their host. A hallmark of all herpesviruses is their ability to establish a lifelong latent infection in the host. During latent infection, viral gene expression is highly attenuated and the viral genome remains stably associated with the cell. In the lytic phase, viral gene expression and DNA replication ensue, leading to the production of progeny virions and eventual lysis of the cell. Pathogenesis caused by these viruses is usually seen in the context of host immunosuppression or cross-species transmission. Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), and Herpesvirus *saimiri* (HVS) are among the most widely studied gamma-herpesviruses to date. All three viruses have been shown to be associated with a wide variety of cancers. HVS and EBV have also been shown to transform lymphoid cells in culture and to induce lymphoproliferative diseases in the natural or experimental host. The striking correlation between herpesviruses and disease induction in

primates enables a study of the contributions of individual viral genes to cell growth transformation [50].

The members of the herpesviral family use multiple strategies to hijack infected host cells and exploit cellular signaling to ensure successful infection and propagation. One of these strategies includes encoding multiple regulatory viral proteins, such as cytokines, chemokines, and viral G protein-coupled receptors in their genomes. All the viral genomes of β- and γ-herpesviruses encode at least one vGPCR which is homologus to cellular chemokine receptors [1,2]. vGPCRs were probably derived from the host genome during evolution and modified to support various aspects of the viral life cycle, concomitant with viral pathogenesis such as immune evasion and proliferative disorders. In contrast to host chemokine receptors, which are activated in a ligand dependent manner and predominantly couple to $G\alpha_{i/o}$ proteins, several vGPCRs are constitutively active and can couple to multiple G proteins, thereby harnessing GPCR signaling in host cells and organisms [1,2]. Since some naturally occurring, constitutively active cellular GPCR variants have been shown to be associated with diverse pathophysiological conditions in humans [3], the constitutively active vGPCRs might play a crucial role in viral pathogenesis and could be drug targets [2]. Indeed, constitutively active vGPCR of KSHV is causally associated with the initiation and progression of Kaposi's sarcoma [4,5]. KSHV vGPCR is capable of activating various signaling pathways, including the PI3K/AKT/mTOR axis for cellular proliferation [6,7], and leads to the activation of cellular transcription factors such as NF-κB, NFAT, and AP-1 [8,9]. The mTOR inhibitor, rapamycin, has been shown to inhibit the progression [10]. Another human γ-herpesvirus, EBV, also possesses a constitutively active vGPCR, BILF1 [51]. EBV BILF1 can downregulate MHC-I/peptide complexes [52] and block the phosphorylation of RNA-dependent protein kinase (PKR) [53], thereby suppressing host antiviral responses. It also has transforming potential that is dependent on constitutive signaling [54]. Therefore, herpesviral vGPCRs may confer diverse advantages in viral replication and dissemination during life-long infection in a host.

However, the molecular and structural details of how vGPCRs activate specific cellular signaling cascades remain largely unknown [1].

HVS is a T cell lymphotropic virus and the prototype of the oncogenic γ2-herepesviruses (or rhadinoviruses), which also includes the human viral homologue, KSHV [11]. HVS, which is classified into three subgroups A, B, and C [55], is apathogenic in its natural host, the squirrel monkey, whereas experimental infection of HVS, especially subgroup C strains, in various primate species and rabbits causes acute and fatal T cell lymphoma [11]. In addition, HVS subgroup C strains can transform human T cells to continuously proliferate *in vitro* in the absence of antigenic or mitogenic stimulation [12]. Therefore, HVS has been considered a good model system to study the mechanisms for transformation, pathogenic capability as well as apathogenic persistence of γ herpesviruses in the host [11]. Similar to other γ herpesviruses, HVS harbors various host cell modulatory genes, including a vGPCR [13]. Previously, it was reported that HVS vGPCR activates a broad range of signaling pathways through multiple G proteins [14]. However, the functional role of HVS vGPCR in viral pathogenesis and/or natural latency has been poorly defined, especially in T cells.

In this study, I investigated functional phenotypes of T cells expressing HVS vGPCR and found that the vGPCR can constitutively activate T cells in the absence of ligands. Surprisingly, constitutive activation of T cells by the HVS vGPCR is independent of TCRβ chain and proximal TCR signaling molecules, such as, Lck, and ZAP70, but requires a physical interaction with cellular CXCR4. I also showed that HVS vGPCR constitutively down-regulated the chemokine receptor from the surface and sequestered it in intracellular compartments. Although it was shown that

EBV vGPCR physically interacts with CXCR4 and consequently inhibits CXCR4 signaling potentially via scavenging $G\alpha_i$ proteins [15], my unexpected findings on the positive cooperativity of HVS vGPCR with cellular CXCR4 in T cell activation shed light on the function of hetero-dimerization of vGPCR. In addition, the functional phenotypes induced by HVS vGPCR were reproduced by KSHV vGPCR, but not by EBV vGPCR, suggesting that the functional activity of vGPCR is evolutionary conserved solely in γ2-herpesviruses. These results may not only provide new insight into the molecular basis of vGPCR in viral pathogenesis of γ2-herpesviruses but also open new avenues for studying the intermolecular interactions between GPCRs that lead to the activation of cellular signaling.

MATERIALS AND METHODS

1. Ethnics statement

Ethnical approval for using human peripheral blood monocytes from healthy volunteers was granted by the Institutional Review Board of Seoul National University Hospital (IRB No. C-1412-089-634). All the healthy volunteers provided written informed consent prior to blood collection.

2. Cell culture and stimulation

Cultures of human embryonic kidney (HEK) 293T cells and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Jurkat T and SupT-1 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin, respectively. J.RT3-T3.5 (ATCC, TIB-153), J.CaM1.6 (ATCC, CRL-2063), P116 (ATCC, CRL-2676), and J.gamma1 (ATCC, CRL-2678) cells, which have deficient expression of TCRβ, Lck, ZAP70, and PLCγ1, respectively, were obtained from ATCC (www.atcc.org) and maintained in the same manner as Jurkat T cells. Lipofectamine2000 (Invitrogen) or calcium phosphate (Clontech) was used for transient expression of vGPCRs or CXCR4 in HeLa or 293T cells. Electroporation using a Bio-Rad electroporator (260 V, 975 μF) or Neon Transfection System (Invitrogen, 2,150 V, 20 ms) was used for transient expression of vGPCR in Jurkat T and SupT-1 cells. For TCR stimulation, Jurkat T cells were incubated with anti-CD3/CD28 coated Dynabeads (ThermoFisher Scientific) for 18

h at 37°C. In order to examine the effect of AMD3100 (Sigma-Aldrich), a CXCR4 antagonist, on the activation of T cells by vGPCR, Jurkat T cells were pre-incubated with 10 μM of AMD3100 for 30 min, and then cells were transduced with lentiviral vectors encoding vGPCR. Jurkat cells expressing vGPCR were analyzed for cellular activation after 24 h of incubation in the presence of AMD3100. Primary T lymphocytes were isolated from human peripheral blood mononuclear cells (PBMCs) by negative selection using the Miltenyi magnetically activated cell sorter (MACS) with pan T isolation kit (Miltenyi Biotec) and cultured in RPMI supplemented with 20% fetal bovine serum at a density of 1×10^6 cells/ml. Primary T lymphocytes were electroporated using Neon Transfection System (2,150 V, 20 ms).

3. Plasmids and lentiviral vector

All the gene constructs for transient and stable expression in mammalian cells were cloned into pDEF3 GST [43], pHJEF IERES eGFP [44], pmCherry-C1 (Clontech), or pEF1α-IRES-Puro (Clontech). cDNAs encoding Human CXCR4 and EBV vGPCR were obtained from Addgene. HVS vGPCR was cloned from a HVS Bacmid clone (BAC43) which was kindly provided by Dr. Armin Ensser. Plasmid constructs encoding KSHV vGPCR were kindly provided by Dr. Pinghui Feng. The cDNA fragments containing full-length or mutant vGPCRs were sub-cloned into pHJEF IRES eGFP vector after PCR-based site-directed mutagenesis. The recombinant lentiviruses were obtained by co-transfection of lentiviral plasmids together with packaging vector psPAX2 and envelope vector pMD2.G into 293T cells. Cell culture supernatants containing the virus were collected at 48 h after

transfection and used for lentiviral transduction.

4. Reporter assays

293T cells were transfected with plasmids encoding vGPCR together with the NFAT, AP-1, or NF-κB luciferase reporter plasmids (Stratagene). Plasmid encoding Renilla luciferase (pRL-CMV, Promega) was co-transfected as an internal control to evaluate transfection efficiency. At 48 h post-transfection, cells were washed once in ice-cold PBS and lysed in 200 μl of 1 x passive lysis buffer. Luciferase activity was assayed using the dual luciferase assay kit (Promega) according to the manufacturer's instructions. The indicated amount of GCP-2 (R&D System) was added at 24 h after transfection, and luminescence was measured as described above. Luminescence was measured with 96 microplate luminometer (DTX880, Beckman Coulter Inc) and normalized with Renilla luciferase activity.

5. Measurements of secreted IL-2

GFP-positive Jurkat cells transduced with lentiviral vector encoding vGPCR were sorted and incubated for 48 h. IL-2 secretion in the cell culture medium was measured with an enzyme-linked immunosorbent assay kit (BD Pharmingen) according to the manufacturer's instruction.

6. Flow cytometric analysis

Cells were washed with ice-cold fluorescence activated cell sorter (FACS) buffer (PBS containing 1% bovine serum albumin (BSA) and 1 mM EDTA), fixed with 4% paraformaldehyde, and subsequently stained with the following antibodies

for 30 min at 4 °C: APC/Cy7-conjugated anti-CD69 (clone FN50, eBiosciences), PE/Cy7-conjugated anti-TCRαβ (clone IP26, eBiosciences), PerCP-Cy5.5-conjugated anti-CD4 (clone OKT4, eBiosciences), APC-conjugated anti-CXCR4 (clone 12G5, eBioscience), PE-conjugated anti-CD45 (clone HI30, BD Pharmingen). The cells were analyzed using a FACS Canto II flow cytometer (BD Biosciences). Data were analyzed by FlowJo software version 8.8.6 (FlowJo).

7. Immunofluorescence analysis and *In situ* Proximity ligation assay (PLA)

The cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized with 0.2% Triton X-100 for 15 min. After blocking with 5% BSA in PBS, the cells were incubated with mouse anti-Flag M2 (for vGPCR, Sigma) and rabbit anti-CXCR4 (clone 12G5, Abcam) antibody at 4°C for 16 h in a humidifying chamber. Alexa488- or Alexa594-conjugated anti-mouse and anti-rabbit IgG antibodies (Molecular Probes) were used as secondary antibodies.

A proximity ligation assay kit (Olink Bioscience Ltd) was used to detect co-localization of vGPCR and endogenous CXCR4 *in situ* according to the manufacturer's instruction (46). Briefly, Jurkat cells were electroporated with HVS vGPCR and incubated for 48 h., fixed with 4% paraformaldehyde for 15 min- at room temperature, permeabilized with 0.2% Triton X-100 and blocked with DuoLink blocking buffer for 30 min at 37°C. Cells were then incubated with primary antibodies against Flag (mouse monoclonal, for vGPCR) and CXCR4 (rabbit) diluted in DuoLink antibody diluents for 60 min, washed and then further incubated for another 1 h at 37°C with species-specific PLA probes (Plus and Minus probes) under hybridization conditions and in the presence of two additional

oligonucleotides to facilitate the hybridization only in close proximity (< 40 nm). A ligase was then added to join the two hybridized oligonucleotides to form a closed circle and initiate a rolling-circle amplification using the ligated circle as a template after adding an amplification solution to generate a concatemeric product extending from the oligonucleotide arm of the PLA probe. Lastly, a detection solution consisting of fluorescently labeled oligonucleotides was added, and the labeled oligonucleotides were hybridized to the concatemeric products. The signal was detected as a distinct fluorescent dot in the Alexa Fluor 488 or Alexa Fluor 594 channel and analyzed by confocal microscopy (Olympus). All images were analyzed and processed using the Olympus Fluoview or Adobe Photoshop software.

8. CXCR4 Knock-Down in Jurkat Cells

CXCR4-knockdown (K/D) in Jurkat cells was performed using a lentivirus system encoding shCXCR4 (clone ID., V2LHS_172391, Open Biosystems). The target sequence (ACAGCAACTAAGAACTTGG) of human CXCR4 was inserted into pGipz lentiviral vector. Lentiviruses were produced by transient transfection using packaging plasmids (psPAX2 and pMD2.VSV-G purchased from Addgene) after calcium phosphate-mediated transfection into 293T cells.

Virus containing media were collected at 72 h post-transfection and used for transduction after passing the supernatants through a 0.45 μm filter. Nonsilencing-pGipz lentiviral shRNAmir control (RHS4346, Open Biosystems) construct was used as a negative control. Viral transduction was assessed by GFP expression and the transduced cells were selected by 2 μg/ml puromycin (Sigma-Aldrich). Stable knockdown of CXCR4 was confirmed by FACS analysis and immunoblotting for

CXCR4.

9. Pull-down assay, co-immunoprecipitation, and immunoblotting

The Glutathione *S*-transferase (GST) pull-down assay was performed using lysate of Jurkat cells electroporated with pDEF3/vGPCR-GST construct [45]. In brief, 1×10^8 cells were harvested and lysed in NP-40 buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 5 mM EDTA) supplemented with a protease inhibitor cocktail (Roche). Post-centrifuged supernatants were precleared with protein A/G beads (Santa cruz) at 4°C for 2 h and then incubated with 50% slurry of glutathione-conjugated sepharose beads (Amersham Biosciences) at 4°C for 4 h. Precipitates were washed extensively with lysis buffer. Proteins bound to glutathione beads were eluted with SDS-PAGE sample buffer after boiling at 70°C for 5 min.

For immunoprecipitation assay, 293T cells transfected with the indicated plasmids were harvested and lysed in 1 ml of RIPA buffer (50 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). The whole cell lysates were pre-cleared by centrifugation (15,000 × *g*, 4°C for 10 min) and then incubated with the indicated antibody and protein A/G-agarose beads at 4°C overnight with gentle rotation. The immunocomplex was collected by centrifugation, washed 3 times with a buffer (0.1% Triton X-100, 50 mM Tris–Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA), eluted with 2 × Laemmli sample buffer, and subjected to SDS-PAGE.

Antibodies used in immunoblot analysis were as follows: mouse anti-FLAG M2 (Sigma), rabbit anti-CXCR4 polyclonal antibody (Abcam), goat anti-AU1-

polyclonal antibody (Bethyl Laboratories), mouse anti-β-actin monoclonal antibody (Santa cruz), mouse anti-GAPDH monoclonal antibody (Calbiochem), rabbit anti-GST polyclonal antibody (Santa cruz), mouse anti-p38 monoclonal antibody (Santa cruz), mouse anti-phospo-p38 monoclonal antibody (Santa cruz), rabbit anti-PLCβ3 polyclonal antibody (Santa cruz), rabbit anti-phopho-PLCβ3 polyclonal antibody (Santa cruz), rabbit anti-Akt polyclonal antibody (Cell Signaling), rabbit anti-phospho-Akt polyclonal antibody (Cell Signaling), mouse anti-p44/42 MAPK(Erk1/2) monoclonal antibody (Cell Signaling), and mouse anti-phospho-p44/42 MAPK(Erk1/2) monoclonal antibody (Cell Signaling). Detection of protein bands was performed by using an enhanced chemiluminescence system (Pierce).

10. Measurement of Intracellular Calcium

Calcium mobilization was evaluated using Fluo-4-Direct kit (Invitrogen) in accordance to manufacturer's specifications. In brief, Jurkat cells expressing vGPCR were labeled for 60 min (30 min at room temperature and 30 min at 37°C) with the Fluo-4-Direct calcium assay buffer containing Probenecid (Invitrogen). Cells were then washed and further incubated with fresh medium for 20 min. Live cells were analyzed with a FACS Canto II flow cytometer (BD Biosciences) and the fluorescence signal was quantified via FlowJo software version 8.8.6 (FlowJo).

16. Statistical analysis

The data was analyzed using the GraphPad Prism 6.0 software (Graph Pad software Inc.). Data are presented as the mean ± standard deviation.

RESULTS

Constitutive activation of cellular signaling by γ2-herpesiviral GPCRs.

In order to confirm HVS vGPCR activation of signaling without any ligands, I performed reporter assays driven by NFAT, AP-1, and NF-κB transcription factors in 293T cells (Figure 1). HVS vGPCR actively enhanced NFAT and AP-1-dependent gene expression in 293T cells in dose-dependent manner, but failed to induce the NF-κB-dependent reporter. It activated NFAT-mediated transcription as efficiently as KSHV vGPCR, which is highly and constitutively active [8,17], whereas its effect on AP-1-dependent transcription was relatively weaker than KSHV vGPCR. I also examined whether HVS vGPCR could further elevate transcriptional activity in response to its cognate agonist, GCP-2/CXCL6 [14] (Figure 2). Upon addition of GCP-2 (up to 2.5 x 10^{-7}M) to HVS vGPCR-expressing cells, none of the transcription factors showed enhanced activity. Therefore, HVS vGPCR constitutively activates NFAT and AP-1-dependent gene expression but failed to respond to a previously reported agonist, GCP-2, in my experimental setting.

I further confirmed the activation of cellular signaling by HVS vGPCR in 293T cells and Jurkat cells by immunoblot (Figure 3). Transient overexpression of HVS vGPCR in both 293T and Jurkat cells induced phosphorylation of PLC-β, p38 MAP kinase, and AKT as efficiently as KSHV vGPCR did. Phosphorylation of ERK by HVS vGPCR expression was observed in Jurkat cells but not in 293T cells. Differential activation of ERK by KSHV vGPCR in a cell-type dependent manner has been previously reported [18].

Since activation of NFAT transcription factor is primarily mediated by the increased intracellular calcium, I also assessed cytosolic calcium with a fluorescent probe in Jurkat cells expressing HVS vGPCR. As expected, vGPCR expression increased cytosolic calcium level by 1.6 fold, compared to control cells (Figure 4). These results clearly show that HVS vGPCR, like KSHV vGPCR, is capable of constitutively activating cellular signaling.

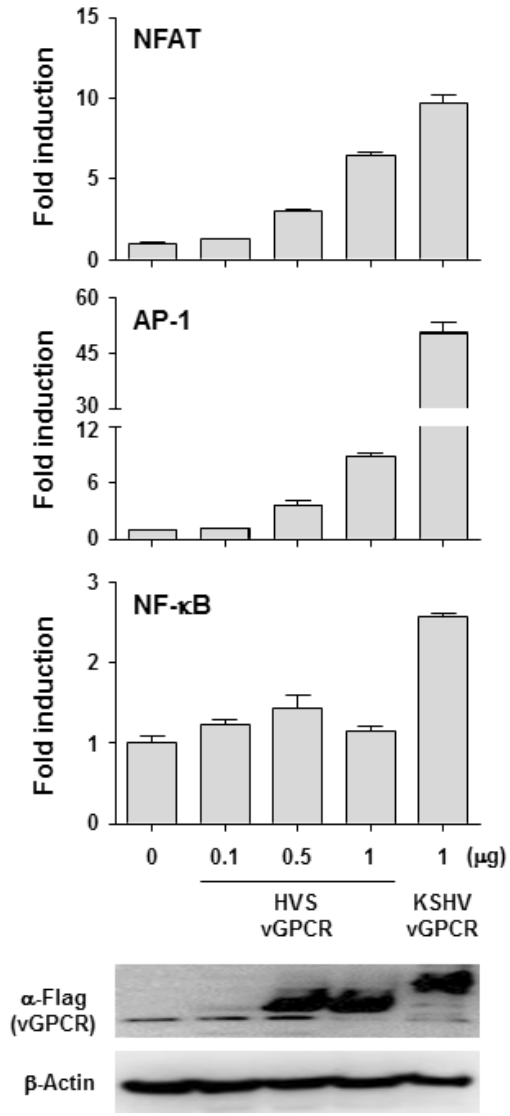


Figure 1. Activation of NFAT and AP-1 transcription factor activity by HVS vGPCR. Human embryonic kidney (HEK) 293T cells were transfected with the indicated reporter plasmid and increasing amounts (0.1 μg to 1 μg) of plasmid encoding HVS vGPCR. Activation of transcription factors was assessed by luciferase reporter assays. KSHV vGPCR was used as the positive control. Representative immunoblots showing vGPCR expression and β-actin, as a loading control, are presented in the bottom panels.

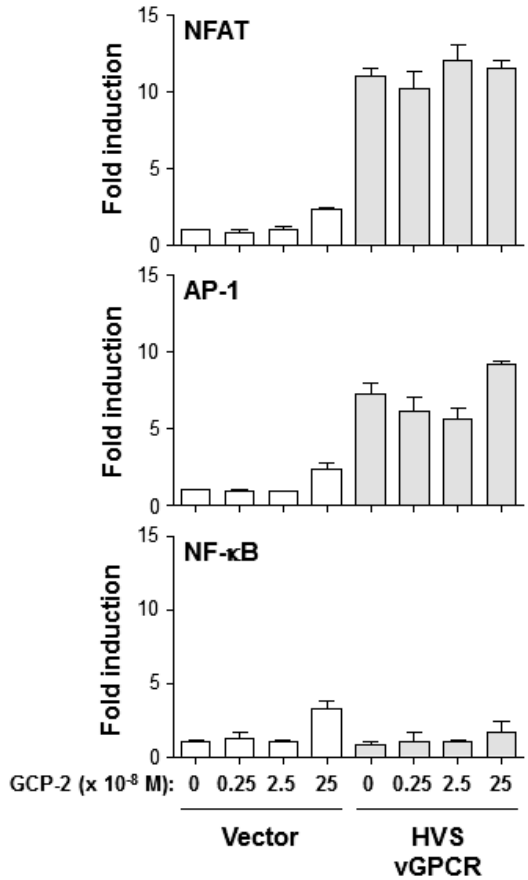


Figure 2. Effect of GCP-2 on HVS vGPCR induced activation of transcription factors. HEK 293T cells were transfected with the indicated reporter plasmid and plasmid encoding HVS vGPCR (1 μg/well). Activation of transcription factors was assessed by luciferase reporter assay after treating cells with the indicated amount of GCP-2 for 24 h. Each assay was performed in triplicate and data are expressed as the mean + standard deviation.

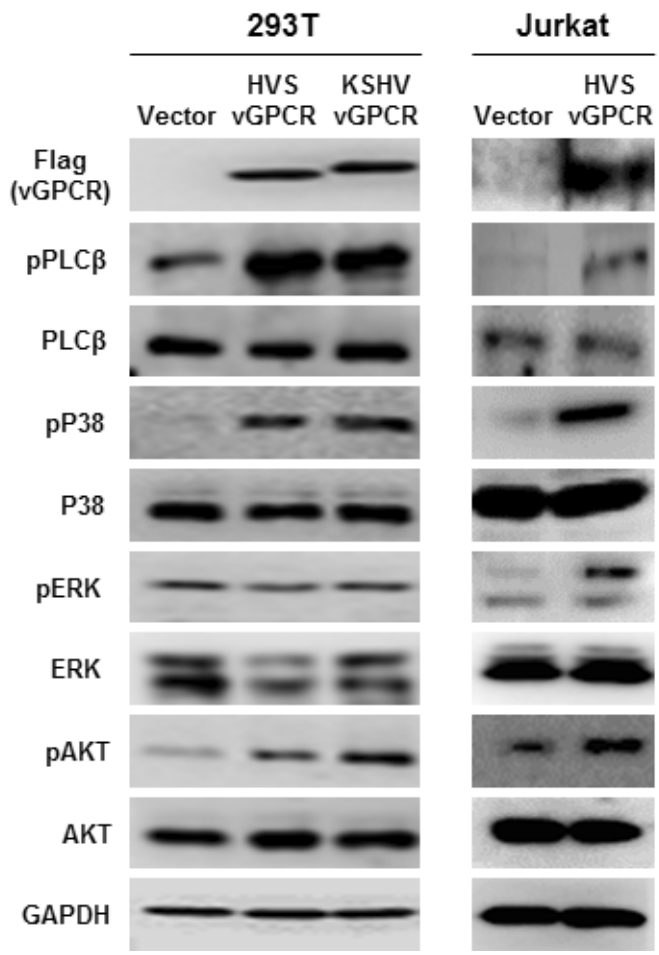


Figure 3. Activation of cellular signaling by HVS vGPCR expression. Whole cell lysates of 293T cells or Jurkat cells expressing vGPCR were analyzed by immunoblotting with antibodies against the indicated signaling proteins.

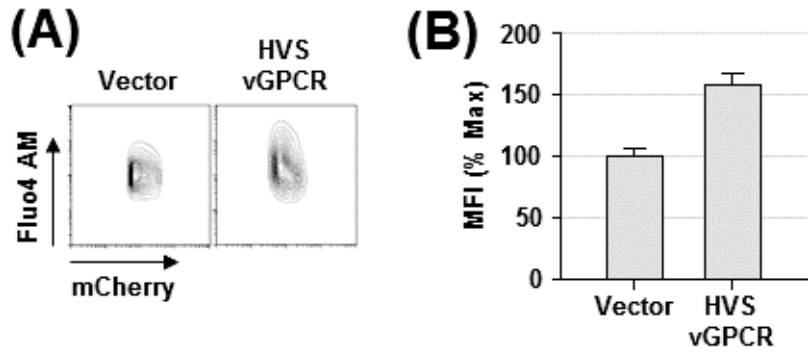


Figure 4. Constitutive elevation of intracellular calcium level by HVS vGPCR expression. (A) Jurkat cells electroporated with HVS vGPCR fused with mCherry were loaded with Fluo-4-AM and fluorescent intensities were quantified by flow cytomery analysis at 48 h after electroporation. (B) MFI is relative mean fluorescence intensity of Fluo-4-AM (mCherry gated) from three independent experiments (mean + S.D.).

Activation of T cells by γ2-herpesiviral GPCRs.

To investigate and compare the effect of γ-herpesviral vGPCRs on T cells, Jurkat cells were electroporated or lentivirally-transduced with plasmids encoding vGPCRs from HVS, KSHV, or EBV and CD69 surface expression was measured at 48 h after electroporation as an indicator of T cell activation (Figure 5A-C). Expression of vGPCRs from HVS or KSHV, but not EBV, significantly enhanced CD69 expression. Activation of Jurkat cells was further confirmed by detection of IL-2 secretion by increased with HVS or KSHV vGPCR expression, but not with EBV vGPCR (Figure 5D).

In addition to the T cell activation marker, CD69, I also examined other T cell receptors including TCR and CD4 and a chemokine receptor, CXCR4. Surface expression of TCR, CD4 as well as CXCR4 were significantly down-regulated in cells expressing vGPCR of HVS or KSHV, whereas expression of EBV vGPCR induced downregulation of CXCR4 but not TCR and CD4 (Figure 5A-C and Figure 6A-B). Marginal changes in surface expression of CD45 were observed in cells expressing vGPCRs.

In order to confirm the effect of vGPCRs on T cell activation, $CD3^+$ primary T cells from human PBMCs were electroporated with empty vector or plasmid encoding HVS vGPCR (Figure 7). Interestingly, activation of primary T cells, as measured by CD69 surface expression, was observed only in a fraction of cells where surface expression of CXCR4 was down-regulated (Figure 7A, left panel). This negative correlation of CXCR4 down-regulation and CD69 up-regulation was also observed in Jurkat cells expressing vGPCRs of HVS or KSHV (Figure 5B). Cellular activation was reproducibly observed in another T cell line, SupT1 cells,

exogenously expression of HVS or KSHV vGPCRs, but not EBV vGPCR (Figure 6C and D). Surface expression of CXCR4 was also down-regulated in SupT1 cells expressing vGPCRs. These results clearly indicate that expression of HVS or KSHV vGPCR can induce cellular activation of T cells without any ligand, but EBV vGPCR failed to do so. In addition, changes in surface expression of CXCR4 or TCR/CD4 molecules in T cells suggest a potential link of those receptors with cellular activation by vGPCRs.

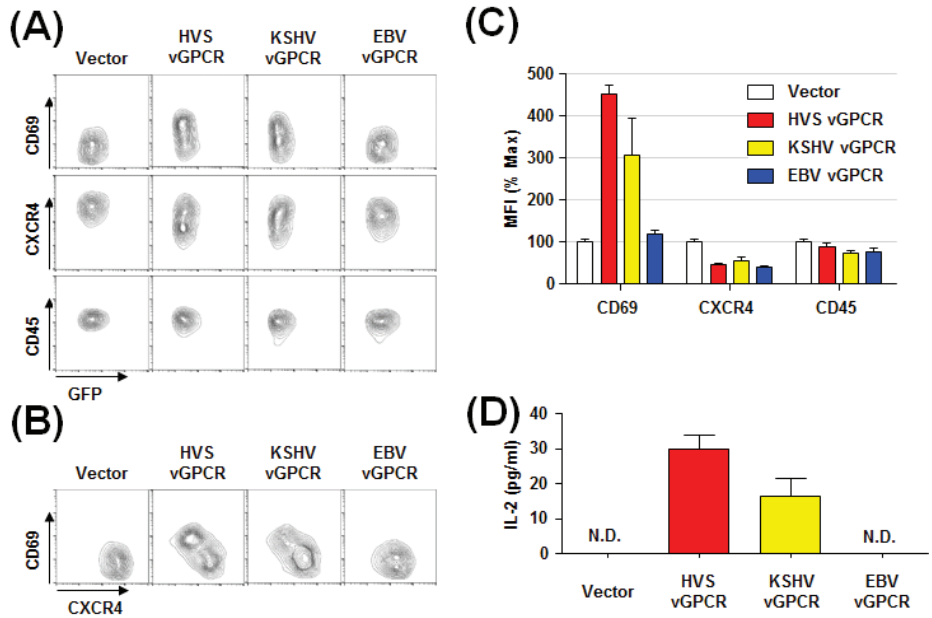


Figure 5. Constitutive activation of T cells by γ2-herpesiviral GPCRs. (A) and **(B)** Jurkat cells were electroporated with pEF1α-IRES-eGFP plasmid encoding the indicated vGPCR and the surface level of cellular markers were analyzed in GFP-positive cells by flow cytometry at 48 h after electroporation. **(C)** Surface expression of the indicated cellular markers was quantitated from triplicate experiments in Jurkat cells expressing vGPCRs. **(D)** Jurkat cells were electroporated with pEF1α-IRES-eGFP plasmid encoding the indicated vGPCR. At 36 h post-electroporation, the supernatants were used to assay IL-2 production by ELISA.

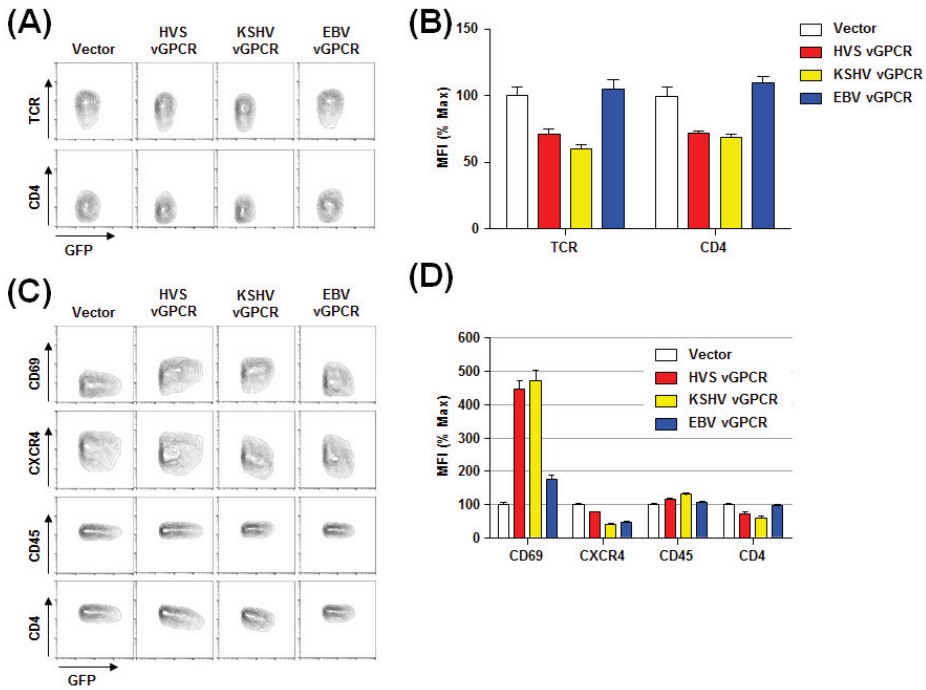


Figure 6. Surface expression of cellular markers in Jurkat cells expressing vGPCRs. (A) Surface expression of TCR and CD4 in GFP-positive Jurkat cells expressing vGPCRs. **(B)** Surface expressions of TCR and CD4 were quantitated from triplicate experiments in Jurkat cells expressing vGPCRs. **(C)** Surface expressions of the indicated cellular molecules in GFP-positive SupT1 cells expressing vGPCRs. **(D)** Surface expressions of the indicated cellular markers were quantitated from triplicate experiments in SupT1 cells expressing vGPCRs.

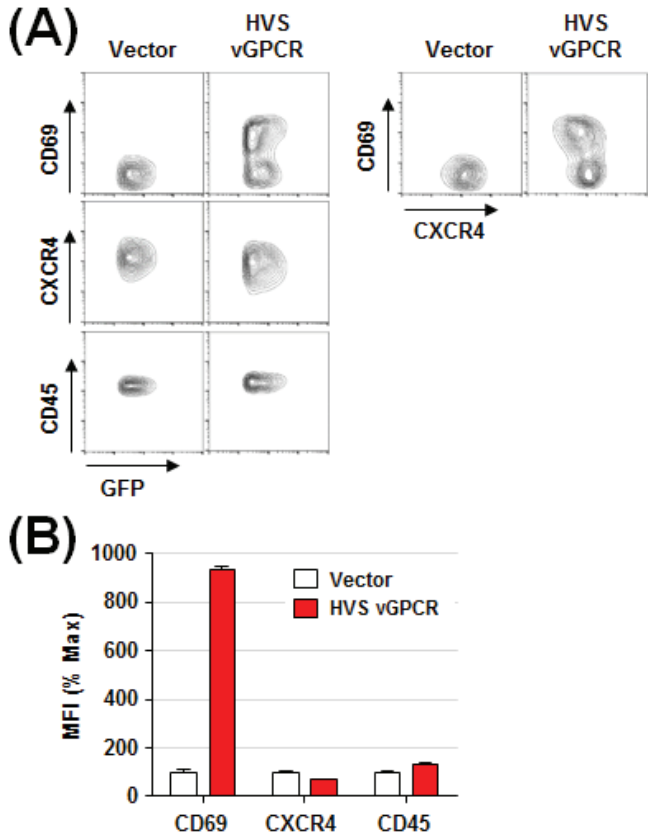
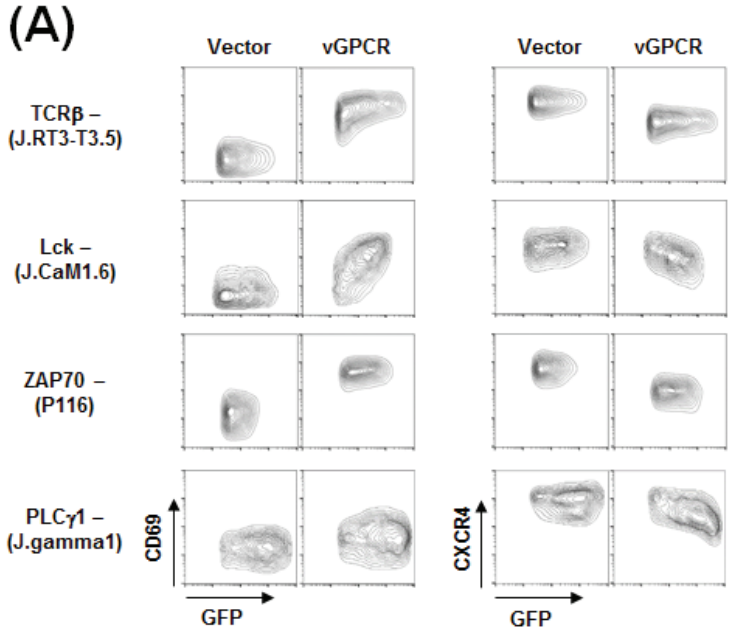


Figure 7. Constitutive activation of primary T cells by HVS vGPCR. (A) Primary T cells isolated from human PBMCs were electroporated with pEF1α-IRES-eGFP plasmid encoding HVS vGPCR and the surface levels of cellular markers were analyzed in GFP-positive cells by flow cytometry at 48 h after electroporation. **(B)** Surface expression of the indicated cellular markers was quantitated from triplicate experiments in primary T cells expressing HVS vGPCR.

Upstream T cell receptor signaling complex is not required for cellular activation by HVS vGPCR

Since I observed concurrent downregulation of TCR and CD4 molecules in T cells by vGPCR expression, I next assessed the role of upstream TCR signaling components in T cell activation by using mutant Jurkat cell lines. Jurkat cell lines harboring mutations in TCRβ chain (J.RT3-T3.5), Lck (J.CaM1.6), ZAP70 (P116), or PLCγ1 (J.gamma1), were electroporated with empty vector or plasmid encoding HVS vGPCR and cellular activation was examined by measuring CD69 surface expression. As seen in Figure 8, expression of HVS vGPCR in the mutant cell lines lacking TCRβ, Lck, or ZAP70 could still induce significant cellular activation regardless of the mutations, indicating that the proximal components of early TCR signaling are not required for T cell activation by HVS vGPCR. Interestingly, surface expression of CXCR4 was drastically down-regulated in the mutant cell lines upon vGPCR expression. In the absence of PLCγ1, however, the level of CD69 surface expression is lower than that of the other mutant cell lines, suggesting that PLCγ1 might be partially involved in the activation signaling mediated by vGPCR expression.


(A)
Vector
vGPCR
Vector
vGPCR
TCRβ –
(J.RT3-T3.5)
Lck –
(J.CaM1.6)
ZAP70 –
(P116)
PLCγ1 –
(J.gamma1)
CD69
GFP
CXCR4
GFP

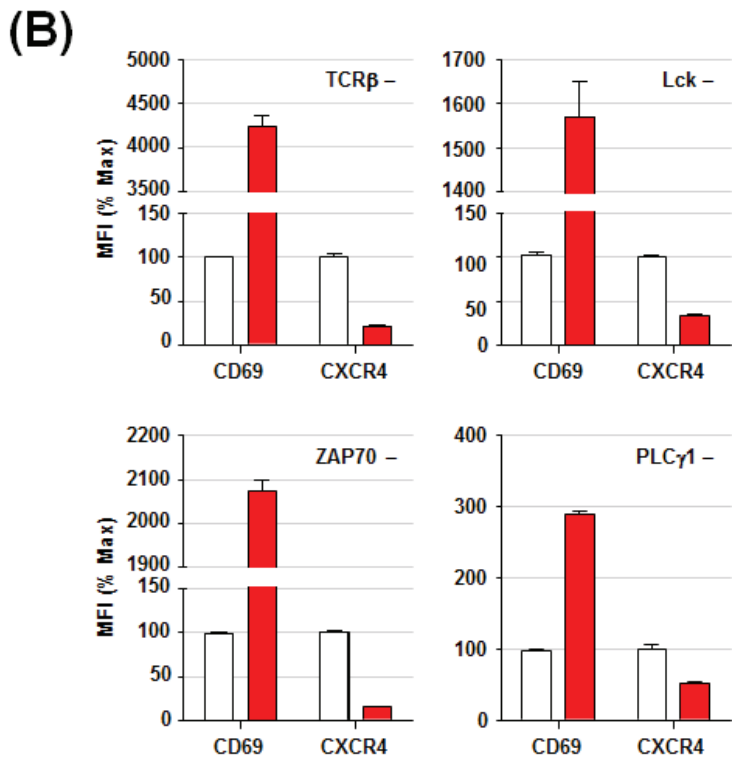

(B)
TCRβ –
Lck –
ZAP70 –
PLCγ1 –
MFI (% Max)
CD69
CXCR4

Figure 8. Activation of T cells by HVS vGPCR is independent of proximal T cell signaling components. (A) Mutant Jurkat cell lines deficient in the indicated signaling molecules were electroporated with pEF1α-IRES-eGFP plasmid encoding HVS vGPCR and the surface levels of cellular markers were analyzed in GFP-positive cells by flow cytometry at 48 h after electroporation. **(B)** Surface expression of the indicated cellular markers was quantitated from triplicate experiments in the cell lines expressing HVS vGPCR.

Involvement of CXCR4 in T cell activation by HVS vGPCR

Expression of vGPCRs in T cells not only induced T cell activation but also down-regulated surface expression of CXCR4. In addition, it was reported that herpesviral vGPCRs physically interact with cellular chemokine receptors including CXCR4 [1]. Therefore, I analyzed the potential role of cellular CXCR4 in T cell activation by HVS vGPCR. First, I examined whether HVS vGPCR physically interacts with CXCR4. As seen in Figure 9A, vGPCR specifically interacted with cellular CXCR4 in GST-pull down assays. In addition, overexpressed HVS vGPCR co-localized with cellular CXCR4 in both HeLa and Jurkat cells in intracellular compartments (Figure 10B), indicating that they interact *in vivo*. The specific interaction of vGPCR and endogenous CXCR4 was further confirmed by an *in situ* proximity ligation assay [19] (Figure 9C). Based on the microscopic imaging assay, most CXCR4 complexed with vGPCR is localized in the cytoplasm. When I examined the total protein level of CXCR4 in vGPCR-expressing Jurkat cells, there was no significant change in the amount CXCR4 with vGPCR expression (Figure 10). Therefore, expression of vGPCR in T cells may affect intracellular trafficking of CXCR4 via a physical interaction and thereby reduce its surface expression without significantly changing the protein level of the chemokine receptor. Also, this indicates the involvement of a receptor recycling pathway.

To investigate the functional role of CXCR4 in vGPCR-mediated T cell activation, I established a CXCR4-knockdown (K/D) Jurkat T cell line by using lentiviral shRNA targeting cellular CXCR4 and characterized it. Knockdown of surface expression as well as protein expression of CXCR4 was confirmed in the CXCR4 K/D Jurkat cells (Figure 11). CXCR4 K/D in Jurkat cells slightly enhances

surface expression of TCR/CD3 molecules but does not significantly affect activation status as measured by CD69 expression (Figure 12) nor did it affect viability (data not shown). Although slightly decreased when compared to that of control cells, CXCR4 K/D Jurkat cells were also efficiently activated by stimulation with anti-CD3/CD28 coated beads, suggesting that their TCR signaling pathway remains intact, (Figure 13). However, overexpression of HVS vGPCR tagged with mCherry severely impaired cellular activation in CXCR4 K/D cells compared to control cells (Figure 14A and B). Similar results were reproduced in CXCR4 K/D cells expressing KSHV vGPCR (Figure 15).

In order to evaluate the effect of AMD3100, an antagonist of CXCR4, on the activation of T cells by vGPCR. Jurkat cells were pretreated with AMD3100 and then transduced with lentiviral vector encoding HVS vGPCR. HVS vGPCR expression activated T cells, but surface expression of CXCR4 was further downregulated (Figure 16). Therefore, only the physical interaction with CXCR4 might be required for the constitutive activity of HVS vGPCR.

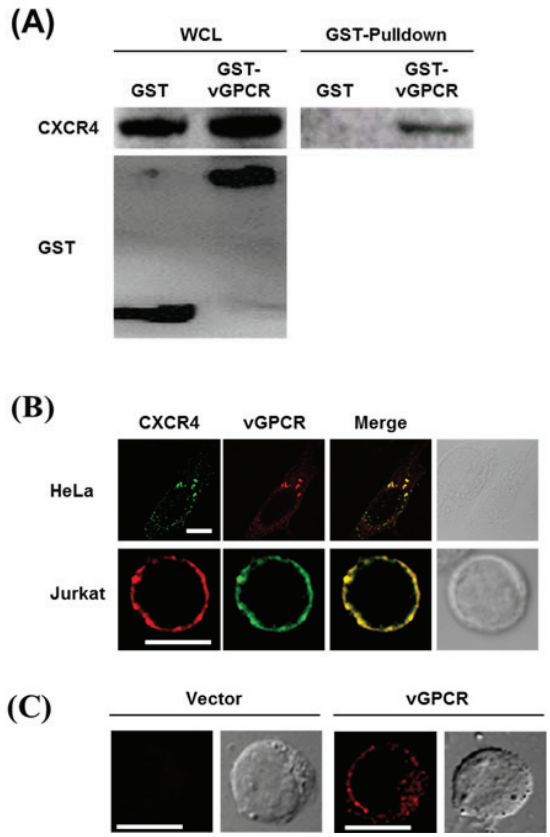


Figure 9. HVS vGPCR interacts with cellular CXCR4. (A) Jurkat cells were electroporated with a plasmid encoding GST or GST-vGPCR and cellular lysates were applied for GST-pulldown assay. Interaction of vGPCR with cellular CXCR4 was assessed by immunoblot using an anti-CXCR4 antibody. **(B)** HeLa and Jurkat cells expressing flag-tagged vGPCR were co-stained with antibodies against flag-tag (red) and cellular CXCR4 (green) and observed under confocal microscopy. White bar, 10 μm. **(C)** Jurkat cells expressing HVS vGPCR were analyzed by proximity ligation assay (red). Cells were visualized by confocal microscopy. White bar, 10 μm.

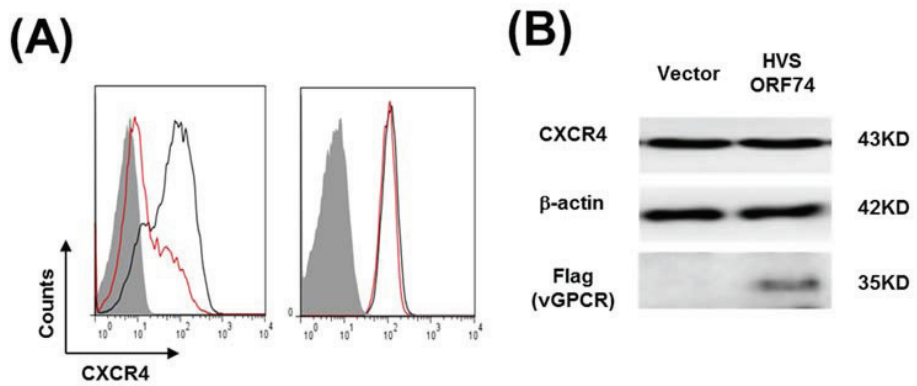


Figure 10. Expression of CXCR4 in Jurkat cells expressing HVS vGPCR. (A) Jurkat cells were transduced with a lentiviral vector encoding HVS vGPCR and analyzed for CXCR4 level by flow cytometry before and after permeabilization. Gray-filled: unstained, black line: vector-control, red-line: vGPCR-positive. **(B)** Cellular lysates of Jurkat cells expressing vGPCR were applied for immunoblot with the indicated antibodies.

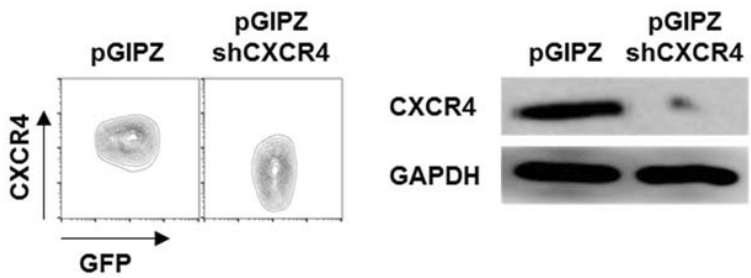


Figure 11. Establishment of stable CXCR4 knockdown Jurkat cells. CXCR4 knockdown was performed using lentiviral vector encoding shRNA targeting CXCR4 and the surface expression (left panels) and protein level (right panels) of CXCR4 in the established cell lines were analyzed by flow cytometry and immunoblot respectively. GAPDH was used as a loading control.

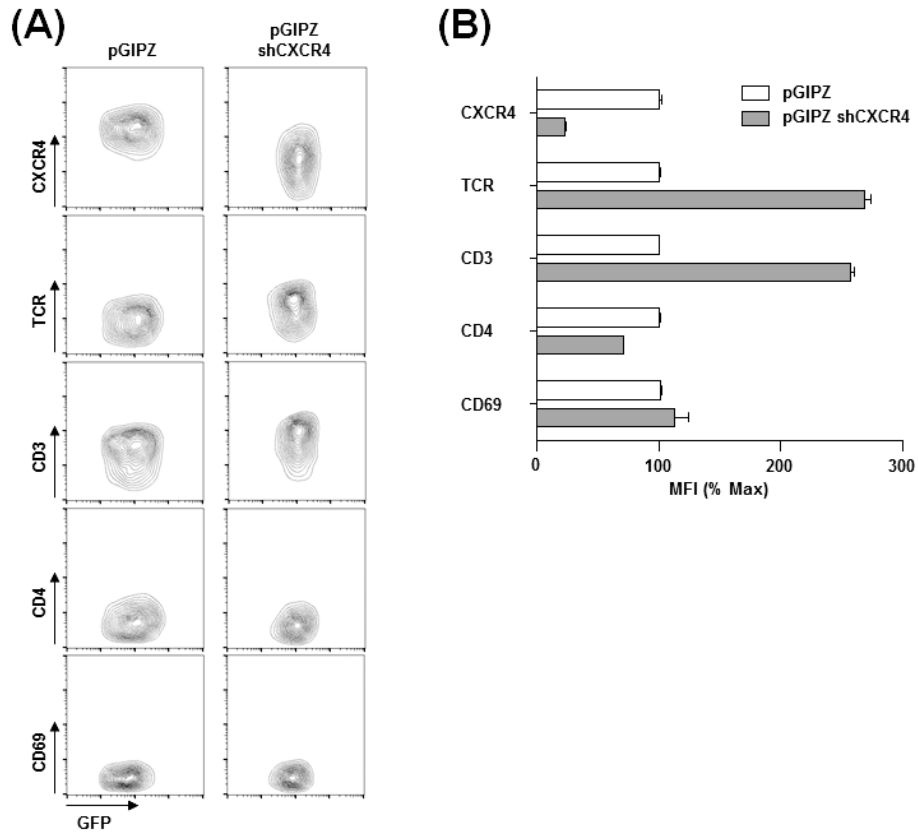


Figure 12. Surface expressions of cellular markers in CXCR4-knockdown Jurkat cells. (A) CXCR4 was silenced in Jurkat cells by lentiviral shRNA and surface expressions of the indicated molecules were analyzed by flow cytometry. (B) Surface expressions of the indicated cellular markers were quantitated from triplicate experiments.

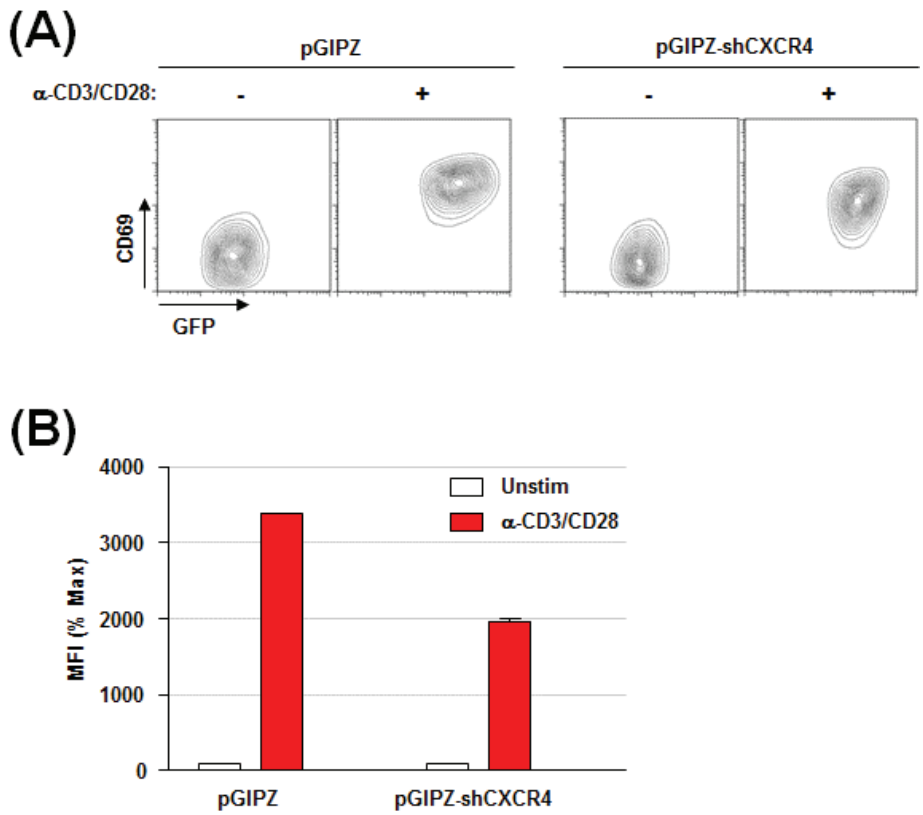


Figure 13. Activation of CXCR4-knockdown Jurkat cells by TCR stimulation. **(A)** T cell activation was assessed by surface expression of CD69 in Jurkat cells at 18 h of stimulation with anti-CD3/CD28 antibody-coated beads. (B) Surface expression of CD69 was quantitated from triplicate experiments.

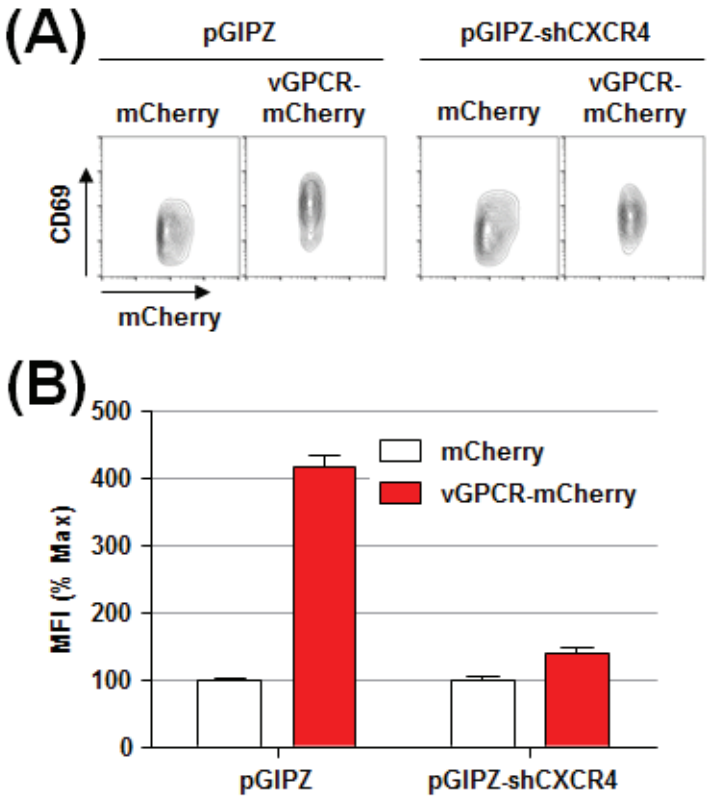


Figure 14. CXCR4 silencing inhibits T cell activation by HVS vGPCR. (A) CXCR4 knockdown cells were electroporated with a plasmid encoding vGPCR fused with mCherry and the surface expression level of CD69 was assessed in mCherry-positive cells by flow cytometry at 48 h after the electroporation. **(B)** Surface expression of CD69 was quantitated from triplicate experiments in cells used in **(A)**.

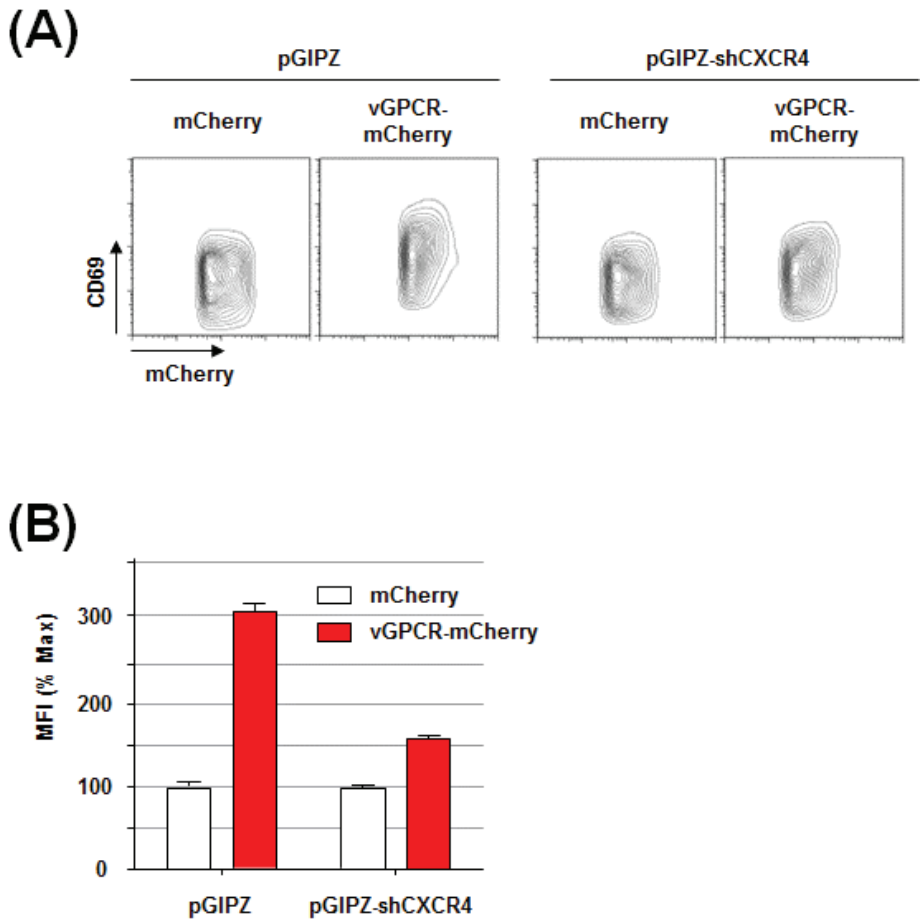


Figure 15. Knockdown of CXCR4 abrogates activation of T cells by KSHV vGPCR. (A) CXCR4 knockdown cells were electroporated with a plasmid encoding vGPCR fused with mCherry and the surface expression level of CD69 was assessed in mCherry-positive cells by flow cytometry at 48 h after the electroporation. **(B)** Surface expression of CD69 was quantitated from triplicate experiments in cells used in **(A)**.

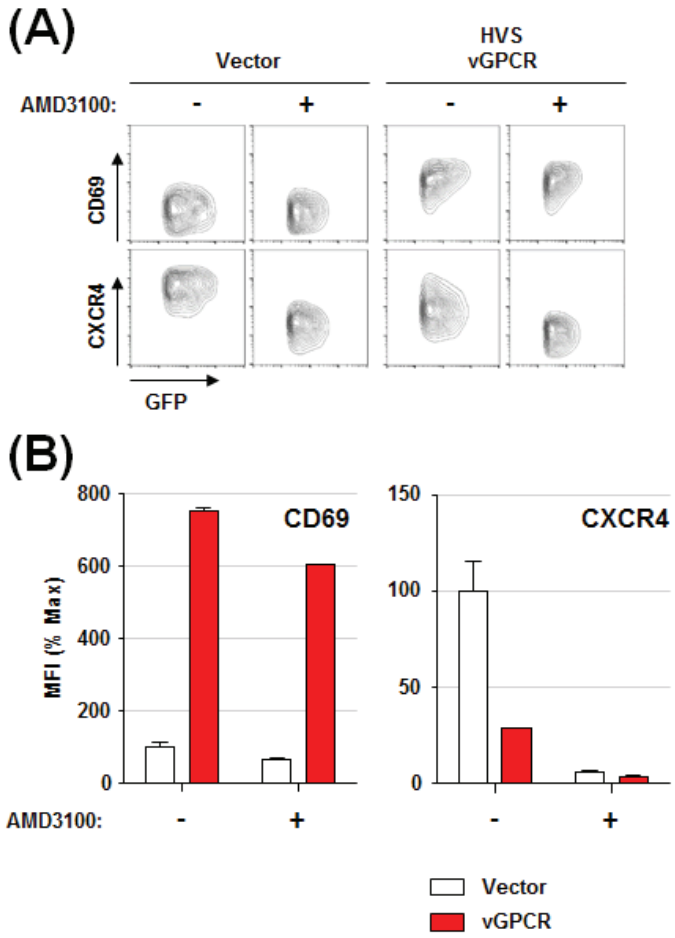


Figure 16. Effect of AMD3100 on the activation of T cells by HVS vGPCR. (A) Jurkat T cells were pre-incubated with 10 μM of AMD3100 for 30 min, and then cells were transduced with lentiviral vectors encoding vGPCR. Surface expressions of CD69 and CXCR4 were assessed in GFP-positive cells by flow cytometry after 24 h of incubation with AMD3100. (B) Surface expressions of CD69 and CXCR4 were quantitated from triplicate experiments.

Functional correlation of CXCR4 interaction with T cell activation by HVS vGPCR mutants

Constitutive activation of cellular signaling by vGPCR of KSHV is affected by several mutations at specific amino acid residues near or within the transmembrane domains as well as in its cytoplasmic C-terminus [16,20,21]. In order to further confirm whether constitutive activation of T cells by HVS vGPCR in the absence of ligand correlates with its interaction with CXCR4, I generated several specific point mutations at amino acid residues conserved in both HVS and KSHV vGPCRs, which have been shown to abolish constitutive activity of KSHV vGPCR without influencing vGPCR expression (Figure 17) [16,20,21]. The level of expression and localization of the mutant vGPCRs in Jurkat cells were not significantly different from wild type vGPCR (Data not shown). Two mutations in transmembrane II of HVS vGPCR, L83D and L86D (which are equivalent to L91D and L94D mutations in KSHV vGPCR, respectively [20]), failed to activate T cells and downregulate CXCR4 from the surface (Figure 18A). In addition, the two mutant proteins did not interact with CXCR4 (Figure 18B). L91D and L94D substitutions in KSHV vGPCR were shown to abolish its constitutive activity, while preserving its ability to be stimulated by an agonist, GROα [20]. One more mutant, R134A of HVS vGPCR (which is the equivalent of R143A in KSHV vGPCR (Figure 17) [21]), showed a phenotype similar to the L83D and L86D mutants when expressed in T cells (Figure 18). The R143A substitution in KSHV vGPCR was shown to abolish its constitutive activity and responsiveness upon stimulation with GROα [21]. Another mutant, D75A of HVS vGPCR, the equivalent of D83A in KSHV vGPCR (Figure 17) [21], showed a similar level of T cell activation and CXCR4 downregulation as in wild

type, and retained its ability to interact with CXCR4 (Figure 18). It was shown that the D83A substitution in KSHV vGPCR could stimulate signaling activity 190% above wild type vGPCR, but poorly was stimulated by GROα [21].

A string of amino acids in the C-terminus of vGPCR is highly conserved in γ2-herpesviruses and is also involved in constitutive activation of G-protein mediated cellular signaling [16]. Therefore, I also generated mutations in the conserved C-terminus and investigate their effects on T cell activation as well as CXCR4 interaction. Deletion of the cytoplasmic C-terminus (Δ309-321) in HVS vGPCR abolished constitutive activation of T cells (Figure 19A-C). The conserved QRM residues might be critical for T cell activation by vGPCR since partial deletion of the QRM sequence from the C-terminus (Δ309-314) failed to activate T cells, while partial deletion of the sequence beyond the conserved motif (Δ315-321) retained the ability for constitutive activation. Therefore, I next generated QRM/AAA or M311G mutations to further define the specific role of the amino acid residues in T cell activation (Figure 19D and E). As reported in a previous study using KSHV vGPCR [16], mutations in cytoplasmic QRM or M311 residues in HVS vGPCR abrogated or dramatically reduced the constitutive activity of vGPCR. Moreover, the substitutions also abolished or diminished its interaction with CXCR4 (Figure 19F). Taken together, these results showing that all point mutations that failed to activate T cells were also unable to interact with CXCR4 strongly suggest that HVS vGPCR interaction with cellular CXCR4 is functionally linked to its constitutive activation of T cells.

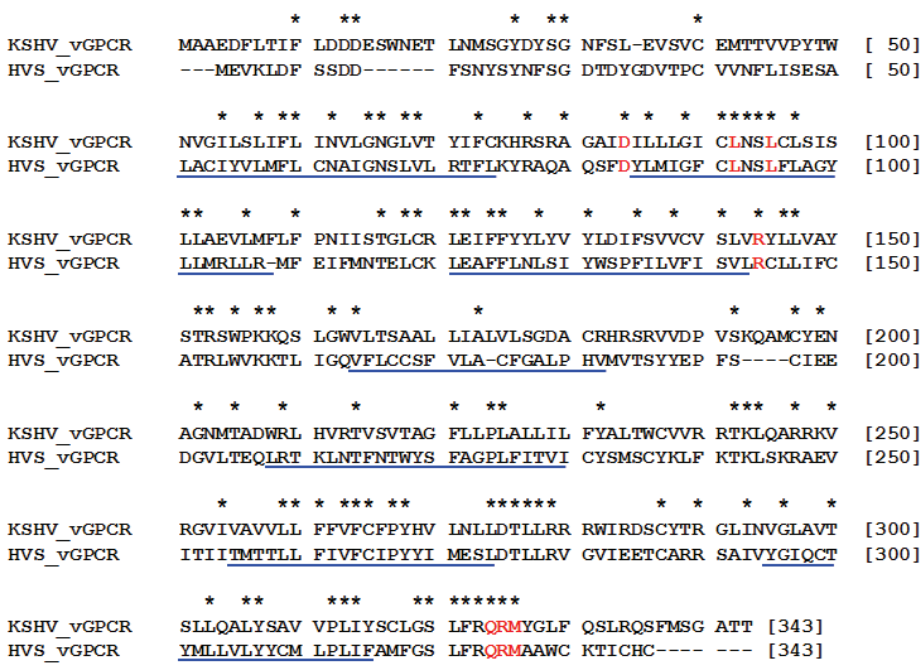


Figure 17. Comparison of amino acid sequences of HVS and KSHV vGPCRs.

Amino acid residues that are critical for the constitutive activation of vGPCRs are indicated in red. Predicted transmembrane regions are underlined.

*: conserved amino acids.

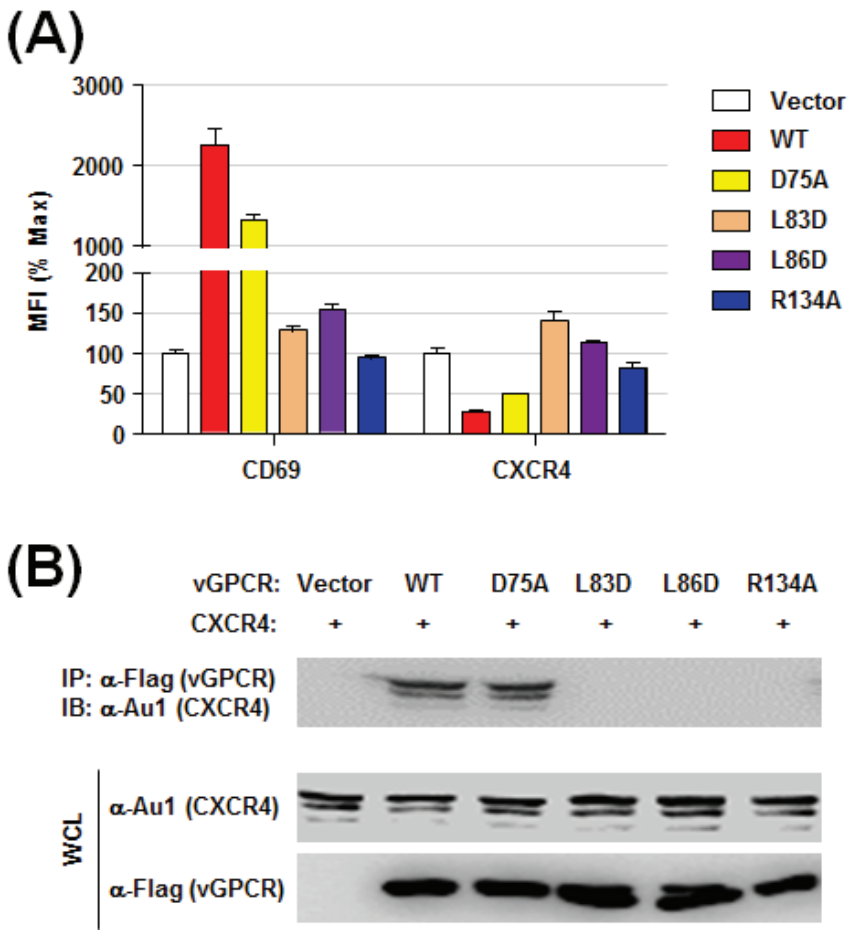
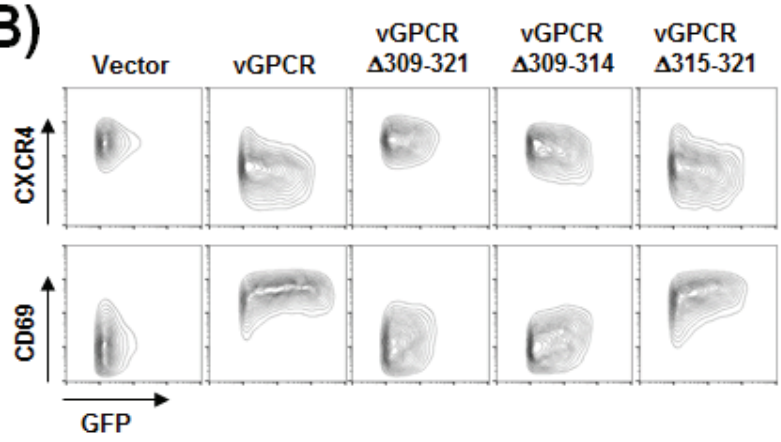


Figure 18. Inactive mutant vGPCRs fail to interact with CXCR4. (A) Jurkat cells were electroporated with plasmids encoding wild type vGPCR or inactive mutants and examined for the surface expressions of cellular markers in triplicate experiments. **(B)** 293T cells were transfected with plasmid encoding wild type vGPCR or the indicated mutants, together with a plasmid encoding CXCR4. Cellular lyasates were precipitated with anti-flag antibody (vGPCRs) and applied for immunoblot with the indicated antibodies.

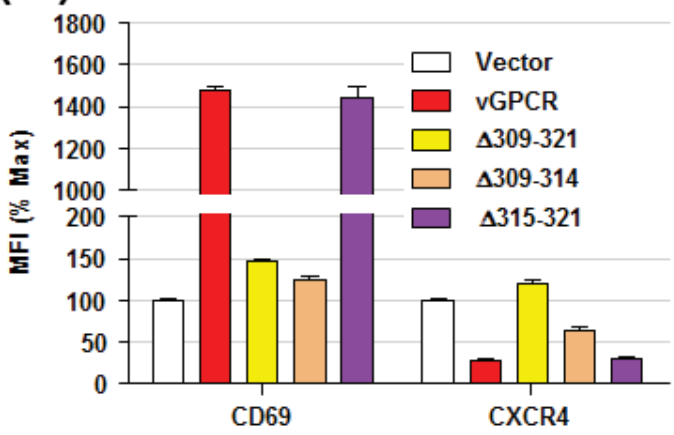
(A)

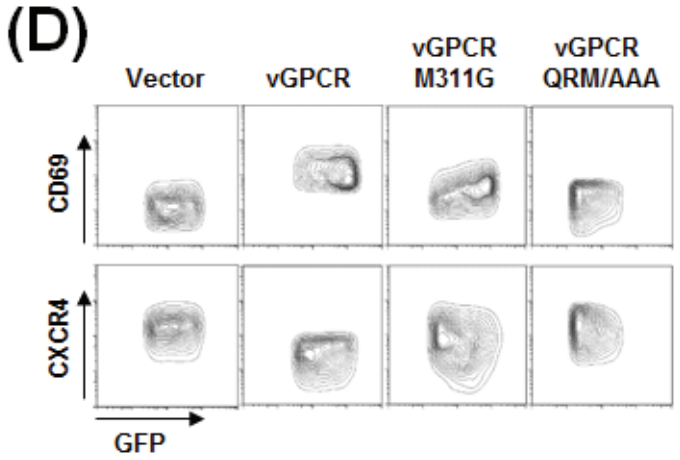
F_{303} G S L F R Q_{309} R M A A W_{314} C K T I C H C_{321}

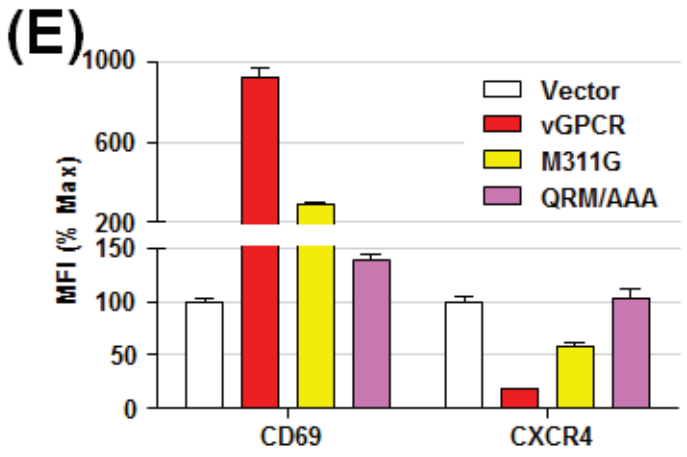
(B)



(C)




(D)
Vector
vGPCR
vGPCR M311G
vGPCR QRM/AAA
CD69
CXCR4
GFP


(E)
MFI (% Max)
1000
600
200
150
100
50
0
Vector
vGPCR
M311G
QRM/AAA
CD69
CXCR4

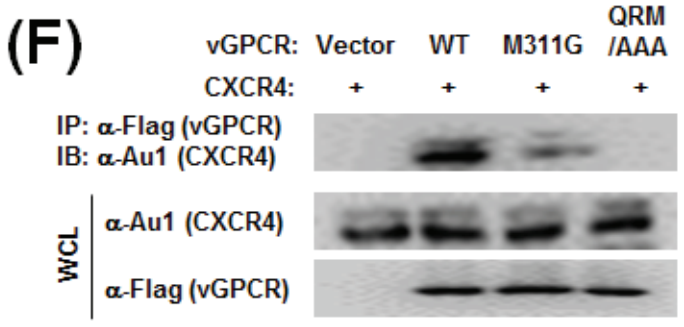

(F)
vGPCR: Vector WT M311G QRM /AAA
CXCR4: + + + +
IP: α-Flag (vGPCR)
IB: α-Au1 (CXCR4)
WCL
α-Au1 (CXCR4)
α-Flag (vGPCR)

Figure 19. Inactive mutations in cytoplasmic domain of vGPCR abrogate its interaction with CXCR4. (A) Amino acid sequence of the cytoplasmic tail of HVS vGPCR. **(B)** Jurkat cells were electroporated with plasmids encoding wild type vGPCR or the indicated mutants and examined for the surface expression of cellular markers in GFP-positive cells. **(C)** Surface expressions of the indicated cellular markers were quantitated from triplicate experiments in Jurkat cells used in **(B). (D)** Jurkat cells were electroporated with plasmids encoding wild type vGPCR or the indicated mutants and examined for the surface expression of cellular markers in GFP-positive cells. **(E)** Surface expressions of the indicated cellular markers were quantitated from triplicate experiments in Jurkat cells used in **(D). (F)** 293T cells were transfected with plasmid encoding wild type vGPCR or the indicated mutants, together with a plasmid encoding CXCR4. Cellular lysates were precipitated with anti-flag antibody (vGPCRs) and applied for immunoblot with indicated antibodies.

DISCUSSION

Since the discovery of the presence of three viral genes encoding homologs of cellular GPCRs in the genome of human cytomegalovirus (HCMV) in 1990 [22], vGPCRs have been identified in a number of β- and γ-herpesviruses as well as poxviruses [23]. vGPCRs are most homologous to chemokine receptors among host GPCR family members and appear to be exploited by the viruses for various pathogenic functions such as viral spread, immune evasion, and oncogenesis [23,24].

Orf74 of HVS is a homolog of host CXCR2 [25] and the first viral vGPCR identified in oncogenic γ-herpesviruses, which includes EBV and KSHV [13]. Even though EBV and KSHV vGPCRs have been extensively characterized for their pathogenic roles in viral immune evasion and oncogenesis [2,24], the role of HVS vGPCR in viral pathogenesis remains poorly defined [14,25]. In addition, the effect of HVS vGPCR on cellular signaling in T cells has never been assessed even though HVS is a lymphotropic rhadinovirus), like KSHV, and can cause fulminant T-cell lymphoma in non-natural host primates and transform human T cells *in vitro* [11]. Therefore, in the current study, I investigated the potential mechanisms exploited by HVS vGPCR in host T cells.

In a previous study, HVS vGPCR was shown to constitutively activate cellular signaling through $G\alpha_i$ and/or $G\alpha_{12/13}$, but not through $G\alpha_q$ [14]. The preferential activation of G protein subtypes by HVS vGPCR was validated by use of specific inhibitors such as pertussis toxin and C3 exoenzyme, which inhibit $G\alpha_i$- and $G\alpha_{12/13}$-dependent signaling, respectively. The vGPCR also constitutively increases

transcriptional activation of serum response element in 293T cells, but failed to induce the activity of cAMP response element-binding protein, NFAT, and NF-κB, which were only up-regulated in a ligand-dependent manner [14]. However, constitutive activation of NFAT-dependent transcription by HVS vGPCR was reproducibly observed in my experiments. In addition, I could not observe inducible activation of the transcription factors by GCP-2, an agonist of HVS vGPCR[14], in cells expressing vGPCR (Figure 2). The levels of constitutive transcriptional activation by HVS vGPCR in the absence of ligand might be exceed the saturating points of our experimental setting and cellular unresponsiveness to GCP-2 might be due to limitations of intracellular signaling mediators[14]. In a previous study, Rosenkilde *et al*., used up to 50 ng (per 35,000 cells/96 well) of plasmid encoding vGPCR to show inducible activation of transcription factors by several vGPCR agonists[14], whereas we used up to 1000 ng (per 200,000 cells/24 well) of DNA since we could not observe significant activation of the reporter system when lower concentration of DNA was used. It is also possible that potential contamination of bovine chemokines including GCP-2, which is as potent as human GCP-2 in chemotactic activity [56], in fetal bovine sera used for cell culture might stimulate HVS vGPCR. The potential effects of bovine chemokines on HVS vGPCR-mediated signaling remains to be determined. Despite the discrepancy in the activation of NFAT transcription factors by HVS vGPCR in 293T cells, its potent activation of T cells, as measured by CD69 upregulation as well as by IL-2 secretion, strongly suggests that it can stimulate cellular signaling mediated by NFAT in cooperation with AP-1, the primary transcriptional regulators of T cell activation [26]. Upstream signaling for NFAT activation includes the stimulation of PLC, an enzyme that

catalyzes the formation of IP_3 and DAG from PIP2. IP_3 in turn activates the IP_3 receptor on the ER to increase intracellular Ca^{2+} levels, resulting in activation of calcineurin phosphatase that dephosphorylates multiple phosphoserines on NFAT, leading to its nuclear translocation and activation [26]. GPCRs trigger a Ca^{2+} signal by activating PLCβ via the active $G\alpha_q$ subunit or Gβγ complex released by receptors that activate $G\alpha_i$ [27]. Previously, it was reported that activation of NFAT-dependent transcription by KSHV vGPCR is partially inhibited by the PLC inhibitor, U-73122, in 293T cells [28]. In addition, $G\alpha_i$ and $G\alpha_q$-coupled ERK1/2 pathways partially contribute to NFAT activation, as measured by specific inhibitors, in primary effusion lymphoma cells expressing KSHV vGPCR [18]. However, Zhang et al. recently showed that KSHV vGPCR-induced NFAT activation is resistant to another PLC inhibitor, adelfosine, and an IP_3R inhibitor, 2-aminoethoxydiphenyl borate, in 293T cells [9].

Instead, the vGPCR physically interacts with SERCA and inhibits its ATPase activity, thereby increasing cytosolic Ca^{2+} concentration and potentiating NFAT activation [9]. They also showed that cyclosporine A, a calcineurin inhibitor, treatment diminished NFAT-dependent gene expression and tumor formation induced by viral GPCRs *in vivo*, indicating that NFAT activation is essential in vGPCR-mediated tumorigenesis [9]. Consistent with this, in the current study, I observed PLC-β activation as well as elevation of cytosolic Ca^{2+} levels in cells expressing HVS vGPCR (Figure 4). Although, I did not characterize the exact mechanism of how HVS vGPCR persistently activates the NFAT pathway, this might result from the direct activation of PLC-β or by inhibition of SERCA as with KSHV vGPCR [9].

The persistent activation of cellular signaling as well as transcriptional

activation of NFAT and AP-1 by HVS vGPCR results in activation of T cells as measured by enhanced surface expression of CD69 and IL-2 secretion. In a previous study that used a transgenic mouse model, CD2 promoter-driven expression of KSHV vGPCR in T cells induced angioproliferative lesions similar to human KS, potentially via an indirect and paracrine mechanism although the phenotypes of cells expressing vGPCR were poorly defined [5]. In this study, I found that HVS vGPCR can activate Jurkat cells as well as primary human T cells. Interestingly, cellular activation is independent of TCRβ and cognate TCR signaling molecules, such as Lck and ZAP70, but dependent on a chemokine receptor, CXCR4. The potential role of CXCR4 in T cell activation and modulation has been proposed by several studies [29-32]. Upon SDF-1, a cognate ligand of CXCR4, stimulation, CXCR4 physically interacts with TCR and utilizes the ZAP70-dependent pathway as well as the $G\alpha_{13}$-RhoA pathway for costimulatory signaling or cellular trafficking [31,33]. SDF-1 signaling via the CXCR4-TCR heterodimer uses PLCβ3 to activate the Ras-ERK pathway and increase intracellular calcium ion concentrations, whereas PLCγ1 is required for SDF-1-mediated migration via a mechanism independent of LAT [32]. In addition, it was demonstrated that simultaneous expression and cooperation between CCR5 and CXCR4 are required for chemokine-induced T cell costimulation at the immunological synapse [29]. Therefore, it seems clear that CXCR4 contributes to T cell activation upon stimulation. In this study, however, I found that T cell activation by HVS vGPCR is mediated by a physical interaction, i.e. heteromeric complex formation, with cellular CXCR4 in the absence of SDF-1 stimulation and is independent of upstream TCR signaling molecules. Then, how does the vGPCR and CXCR4 heteromeric complex stimulate cellular signaling to induce T cell

activation? Crosstalk between GPCRs has been widely recognized to contribute to GPCR-mediated signaling events and functions [30,34-36]. The formation of homo- and/or hetero-oligomeric complexes among GPCRs opens a new dimension of possible molecular and functional GPCR interactions [30,34]. In addition to ligand-binding properties, unique allosteric interactions in GPCR homo- and/or hetero-oligomers may correlate with intrinsic or signaling efficacy [34]. For example, the functional interaction between EBV vGPCR, BILF1, with cellular GPCRs such as CXCR4 and histamine H4 receptor, have been reported and constitutively active vGPCR was shown to impair CXCR4 signaling upon SDF-1 stimulation by scavenging $G\alpha_i$ proteins [15]. One study also showed that KSHV vGPCR inhibits calcium mobilization induced by the thyrotropin-releasing hormone receptor and the muscarinic acetylcholine M1 receptor [37]. Although positive and negative regulation of cellular GPCRs, especially chemokine receptors, by other herpesviral vGPCRs have been reported in multiple studies [24], most studies mainly focused on the changes in signaling efficacy of cellular GPCRs upon ligand stimulation when co-expressed together with a vGPCR. In this study, I found that constitutive activation of T cells by HVS and KSHV vGPCRs is significantly abolished by knock-down of CXCR4, indicating that the interaction of the vGPCRs with cellular CXCR4 is functionally linked to the constitutive activity of vGPCR. In addition, functionally inactive vGPCR mutants are also unable to interact with CXCR4, suggesting that vGPCR function in T cells might be controlled through the formation of heteromeric vGPCR-CXCR4 complexes. Recently, Tripathi A. et al., reported that CXCR4 contributes to the function of the α_1-adrenergic receptor, another GPCR, via heteromeric complex formation [30]. Disruption of the α_1-AR and CXCR4

heteromer by a peptide derived from the transmembrane helix 2 of CXCR4 or by CXCR4 knockdown abolished phenylephrine, an agonist of α_1-AR, induced calcium mobilization and myosin light chain phosphorylation, thereby inhibiting contraction of vascular smooth muscle cells upon α_1-AR activation [30]. Interestingly, a CXCR4 antagonist, AMD3100, did not affect phenylephrine-induced α_1-AR function, suggesting that heteromeric complex formation controls α_1-AR function independent of ligand occupation or the activation status of CXCR4 [30]. Dopamine receptor subtype-2 was shown to undergo similar phenomenon in a study that revealed that its function could be modulated by unoccupied ghrelin receptor via formation of heteromeric complexes [38]. When Jurkat cells were pretreated with AMD3100, HVS vGPCR expression activated T cells, but surface expression of CXCR4 was further downregulated (Figure 16). Therefore, the physical interaction with CXCR4 might be a primary requisite for the constitutive activity of HVS vGPCR regardless of ligand occupation or the activation status of CXCR4. This hypothesis could be further supported by the results showing that all inactive vGPCR mutants examined failed to interact with CXCR4 (Figure 18 and 19). Further analysis on the structural basis of the heteromeric complex needs to be conducted in order to explain how the specific amino acid residues mutated in the inactive forms contribute to heteromeric complex formation with CXCR4 as well as vGPCR activity. In addition, β-arrestin-mediated signaling, which can activate MAP kinases, such as ERK1/2 and p38, and AKT pathways [39,40], may play a role in continuous CXCR4 down-regulation from the cellular surface as well as concomitant signaling activation by vGPCR expression. However, the role of β-arrestin vGPCR-mediated signaling remains unclear [24]. Considering that CXCR4 is the chemokine receptor most

widely expressed in malignant tumors and plays various roles in cellular proliferation, angiogenesis, and metastasis [40-42], exploitation of this chemokine receptor by vGPCR through heteromeric complex formation could be a novel strategy utilized by oncogenic γ2-herpesviruses such as KSHV and HVS.

In summary, herpesviruses encode constitutively active GPCRs which are homologous to chemokine receptors and implicated in viral pathogenesis such as immune evasion and proliferative disorders. Although vGPCRs transmit diverse signaling, molecular details how vGPCRs continuously activate cellular signaling is largely unknown. Here, I demonstrate that vGPCR of HVS, belonging to oncogenic γ2-herepesviruses, constitutively activate T cells via a heteromeric interaction with cellular CXCR4 in the absence of any cognate ligand. The constitutive activity of vGPCR in T cell activation is independent on TCR signaling molecules, whereas CXCR4 silencing abolished T cell activation by vGPCR. These findings on the positive cooperativity of vGPCR with CXCR4 in T cell activation may provide a valuable insight into the functional heteromerization for GPCR function.

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국문 초록

헤르페스 바이러스는 감염과 전파를 위해 숙주의 면역체계와 생체기능을 제어할 수 있는 다양한 바이러스 유전자들을 보유하고 있다. 병원성 헤르페스 바이러스의 가장 매력적인 전략 중 하나는 지속적인 활성을 보이는 G-단백 결합 수용체(GPCR)를 발현한다는 것이다. 감마 2-헤르페스 바이러스의 유전자인 ORF74 는 인간 케모카인 수용체인 CXCR2 와 상동성을 갖는 바이러스성 G-단백 결합 수용체(vGPCR)이다. vGPCRs 은 리간드 없이도 지속적인 활성을 보이며, 바이러스에 대한 숙주면역 회피 또는 세포증식과 관련된 기능을 보유하고 있는 것으로 보고되고 있다. 현재까지 여러 연구들을 통하여 vGPCRs 이 다양한 숙주세포 신호전달 경로들을 조절하는 것이 알려졌지만, 이 바이러스 단백이 어떻게 세포의 신호 전달을 연속적으로 활성화 시키는지에 대한 정확한 분자기전은 아직도 알려진 바가 적다. 본 연구에서는 림프암을 유발하는 감마 2-헤르페스 바이러스 중 하나인 Herpesvirus *saimiri* (HVS)의 vGPCR 이 숙주세포의 케모카인 수용체인 CXCR4 와의 상호 작용을 통하여 지속적으로 T 세포 활성화를 유도하는 것을 확인하였다. CXCR4 및 vGPCR 의 물리적 상호작용은 근접결합분석과 면역 침강법으로 확인하였다. shRNA 에 의한 CXCR4 의 Knock-down 은 HVS vGPCR 에 의한 T 세포의 활성화를 억제하였다. 흥미롭게도, HVS vGPCR 에 의한 T 세포의

활성화는 T 세포 수용체(TCR) 신호전달과정에 관여하는 TCRβ, Lck, 그리고 ZAP70 가 발현되지 않는 돌연변이 세포주에서도 관찰되었는데, 이를 통해 vGPCR 의 T 세포 신호활성화는 TCR 관련 신호전달체계에는 비 의존적인 것으로 확인되었다. 또한, T 세포를 활성화 시키지 못하는 다양한 vGPCR 돌연변이들은 CXCR4 와 상호 작용하지 않았다. 결과를 정리해보면, vGPCR 은 CXCR4 와의 물리적 상호작용을 통해 T 세포를 활성화시키고 있음을 알 수 있으며, 이러한 결과는 이형 GPCR 간의 기능적 상호작용을 통한 신호전달체계의 활성 기전을 이해하는 데 중요한 실마리를 제공할 수 있을 것으로 생각한다.

주요어 : 감마 헤르페스 바이러스, 바이러스 G단백 결합 수용체, 케모카인 수용체, 이형 이합체, T 세포 활성화, 세포 속 유입

학 번 : 2010-30605