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MINIREVIEW

Herpesvirus-Encoded G Protein-Coupled Receptors as Modulators of Cellular Function

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ABSTRACT

Human herpesviruses (HHVs) are widespread pathogens involved in proliferative diseases, inflammatory conditions, and cardiovascular diseases. During evolution, homologs of human chemokine receptors were integrated into the HHV genomes. In addition to binding endogenous chemokines, these viral G protein-coupled receptors (vGPCRs) have acquired the ability to signal in a constitutive manner. Ligand-induced and ligand-independent and autocrine and paracrine signaling properties of vGPCRs modify the functions of the expressing cells and lead to transformation and escape from immune surveillance. Furthermore, cross-talk or heterodimerization with endogenous chemokine receptors represent other ways for vGPCRs to modify intracellular signaling and cellular functions. As such, these viral receptors seem to play a prominent role during viral pathogenesis and life cycle and thus represent innovative antiviral therapies.

G protein-coupled receptors (GPCRs) are essential mediators of cellular communication. Their cell surface expression allows easy access and activation by their extracellular ligands to initiate intracellular signaling cascades. The physiological role of GPCR activation ranges from gene transcription to cellular migration and proliferation. Besides being activated by their cognitive ligands, GPCRs can signal independently from ligand activation. This so-called constitutive activity is the molecular basis of various pathologies (Smit et al., 2007). It is noteworthy that, during evolution, human herpesviruses (HHV) of the β and γ families hijacked human GPCR while coexisting with their host (Brunovskis and Kung, 1995). These viral GPCRs (vGPCRs) show considerable homology to chemokine receptors, which are essential in the development of the hematopoietic system and regulation of cellular homeostasis (Mackay, 2001). In addition, chemokine receptors play a prominent role in cancer development (e.g., by inducing cellular proliferation or by modifying cellular migration patterns), resulting in cancer metastasis (Balkwill, 2004). Although human chemokine receptors require ligand activation to exert a physiological function, most of the vGPCRs show high levels of constitutive activity (Vischer et al., 2006b). As for human GPCRs, the constitutive modulation of signaling pathways by vGPCRs may play a role in the development of HHV-related diseases. As such, these vGPCRs may serve as therapeutic targets for the intervention of viral pathogenesis.

γ-Herpesvirus-Encoded G Protein-Coupled Receptors

The Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) and the Epstein-Barr virus (EBV or HHV-4) are lymphotropic viruses that are involved in proliferative dis-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; vGPCR, viral G protein-coupled receptor; CMV, cytomegalovirus; COX-2, cyclooxygenase-2; CREB, cAMP-responsive element binding protein; EBV, Epstein-Barr virus; FAK, focal adhesion kinase; HCMV, human cytomegalovirus; HHV, human herpesvirus; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma associated virus; VEGF, vascular endothelial growth factor; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PI3K, phosphatidylinositol phosphate 3-kinase; mTOR, mammalian target of rapamycin; TSC, tuberous sclerosis complex; PKR, RNA-dependent protein kinase; WT, wild type; PGE2, prostaglandin E2; PI-103, 3-[4-(4-morpholinyl)pyrido[3′,2′:4,5]furo[3,2-*d*]pyrimidin-2-yl]-phenol; Bay 11-7082, (*E*)-3-(4-methylphenylsulfonyl)-2-propenenitrile; VUF2274, 4-(4-chlorophenyl)-4-hydroxy-*a*,*a*-diphenyl-1-piperidinepentanenitrile hydrochloride.

eases that are especially apparent in immunodeficient patients. KSHV is particularly harmful for HIV-infected patients presenting AIDS because the development of Kaposi's sarcoma is a major health threat in this population (Sullivan et al., 2008). Initially discovered as the causative agent of Kaposi sarcoma (KS), KSHV is also implicated in two other major lymphoproliferative diseases: primary effusion lymphoma, and Castleman's disease, also apparent in AIDS patients (Sullivan et al., 2008). EBV-related diseases are B cell- and epithelial cell-specific diseases, namely Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma. These diseases are especially common in immunosuppressed patients such as HIV-infected patients. In addition, immunosuppressed transplant patients are at risk with the development of EBV-related post-transplant lymphoproliferative diseases.

Both KSHV and EBV genomes contain a single vGPCR, referred to as ORF74 and BILF1, respectively. ORF74 was originally presented as a viral homolog of the CXCL8 receptor CXCR2. ORF74 binds to a broad range of human chemokines from the CXC family (CXCL1, 2, 3, 4, 5, 6, 7, 8, 10, and 12) and CC family (CCL1 and CCL5), as well as the KSHV-encoded chemokine vCXCL2 (Fig. 1). Studies revealed that CXCL10, CXCL12, and vCXCL2 act as full inverse agonists; CXCL6 is a partial inverse agonist; CXCL4, 5, 7, and 8 act as neutral antagonists; CXCL2 is a partial agonist; and CXCL1 and CXCL3 are full agonists (Geras-Raaka et al., 1998a,b; Gershengorn et al., 1998; Rosenkilde et al., 1999, 2000). Besides presenting ligand-induced signaling, ORF74 also exhibits strong constitutive signaling to a multitude of signaling pathways (Sodhi et al., 2000, 2006; Marinissen and Gutkind, 2001; Montaner et al., 2001; Shepard et al., 2001; Smit et al., 2002; Cannon and Cesarman, 2004; Liu et al., 2004). The EBV-encoded BILF1 receptor presents limited homology to chemokine receptors, with highest homology to CXCR4. Until now, attempts to deorphanize this receptor have proven unsuccessful, and BILF1 ligands still remain unknown. Like other vGPCRs, BILF1 signals in a constitutive manner (Beisser et al., 2005; Paulsen et al., 2005).

Oncogenic Function of the KSHV-Encoded Chemokine Receptor ORF74

KSHV was initially identified as the etiological agent of KS in patients infected with HIV (Chang et al., 1994). The proliferation of KSHV-infected spindle-shaped endothelial cells seems to be the driving force in KS tumors, which present aberrant angiogenesis and lymphangiogenesis (Colman and Blackbourn, 2008). Although it was originally believed that the latent genes expressed in cells surviving KSHV infection may predominantly contribute to the development of KS, expression of candidate viral latent genes in endothelial cells failed to initiate Kaposi's sarcomagenesis in transgenic mice in vivo (Montaner et al., 2003). Instead, attention has long been paid to the lytic gene ORF74 that is expressed in KS lesions in humans (Cesarman et al., 1996). Shortly after its identification, this viral chemokine receptor was promptly identified as a constitutively active transforming viral gene in transfected cells in vitro and in xenograft models (Arvanitakis et al., 1997; Bais et al., 1998). Furthermore, numerous in vivo transgenic models confirmed the implication of ORF74 in the development of KS, and light has been shed on several mechanisms activated by this receptor for pathogenesis (Yang et al., 2000; Montaner et al., 2003; Sodhi et al., 2004; Jensen et al., 2005; Grisotto et al., 2006). ORF74 is able to activate various signaling pathways, mediated by a broad range of kinases and transcription factors, which results in the induction of proinflammatory and angiogenic genes. In particular, ORF74 constitutively couples to $G\alpha_{q/11}$ and $G\beta\gamma$ subunits to activate the p44/42 mitogen-activated protein kinases (MAPKs) (Smit et al., 2002). In concert with p38 and Jun N-terminal kinase MAPK activation, the hypoxia-inducible transcription factor hypoxia-inducible factor-1α is subsequently triggered and mediates the transcription of the vascular endothelial growth factor (VEGF) gene, giving rise to an angiogenic phenotype (Sodhi et al., 2000). In addition, ORF74 expression in KSHV-negative KS-derived cells induces the constitutive activation of the NF-κB transcription factor and the expression and release of proangiogenic and

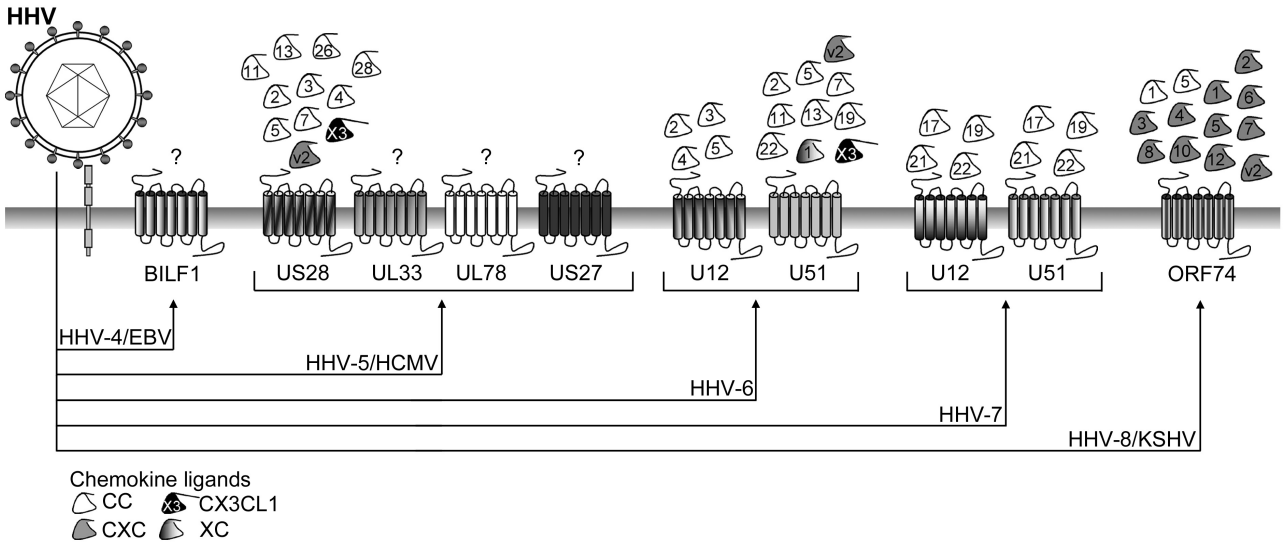

Fig. 1. HHV-encoded vGPCRs. HHVs infect cells by binding to specific cellular receptors. Upon transcription of the viral genome, vGPCRs are expressed and can bind various chemokines. Human chemokine ligands from the CC (open), CXC (gray), CX3C (filled) and XC (shadow) classes and the KSHV-encoded CXCL2 chemokine (gray, v2) bind to various vGPCRs. For some receptors, ligands still need to be determined.

proinflammatory factors such as interleukin-6, CXCL8, CCL5, granulocyte macrophage colony-stimulating factor, E-selectin, and vascular and endothelial adhesion molecules vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (Pati et al., 2001). Secretion of soluble factors by ORF74-expressing cells is able to subsequently induce NF-κB transcriptional activation in neighboring cells, confirming a paracrine mechanism of transformation for ORF74 (Martin et al., 2008).

In vivo models have greatly contributed to the understanding of ORF74-mediated transformation and the identification of molecular signaling pathways as potential therapeutic treatments. Several transgenic model systems expressing ORF74 in hematopoietic or endothelial cells resulted in the formation of tumors resembling those observed in patients with KS (Yang et al., 2000; Montaner et al., 2003). Although the constitutive activity of this vGPCR was suspected to be the main driving force of tumorigenesis, transgenic mice expressing different mutants of ORF74 showed that both ligand-independent and ligand-dependent signaling were important for angioproliferation (Holst et al., 2001). Neither the ORF74-L^{91}D mutant, which did not present any constitutive activity but could be activated with chemokines, nor the Δ2-22-ORF74 mutant, which presents constitutive activity but cannot bind chemokines, led to the development of tumors in transgenic mice (Holst et al., 2001). However, the ORF74-R^{208}H;R^{212}H, which presents constitutive activity but cannot be further stimulated with agonist ligands, presented a reduced severity of disease (Fig. 2). This confirmed that ligand stimulation is necessary for the full penetrance of the disease (Holst et al., 2001).

The Akt pathway is crucial in the development of human KS and was shown to play a prominent role in ORF74-induced Kaposi's sarcomagenesis. ORF74 constitutively couples to Gα_i and G$\beta\gamma$ subunits, leading to the activation of the phosphatidylinositol phosphate 3-kinase (PI3K) and the downstream Akt kinase (Montaner et al., 2001; Smit et al., 2002; Sodhi et al., 2004). Mutation of the arginine residue 143 in transmembrane domain 2 into an alanine, R^{143}A, resulted in an inactive mutant that induced neither Akt activation nor tumor formation in an endothelial-specific expression animal model for KS (Ho et al., 2001; Sodhi et al., 2004) (Fig. 2). ORF74-mediated constitutive activation of Akt is also further potentiated by agonist chemokines (Bais et al., 2003). In addition, ORF74 expression constitutively up-regulates the expression of the VEGF receptor VEGFR-2/KDR. When stimulated with VEGF, KDR in turn also activates Akt (Bais et al., 2003) (Fig. 3). This indicates that Akt can be activated in an autocrine and paracrine manner. It is noteworthy that Akt has been recently shown as a central point in the activation of the PI3K/Akt/mTOR pathway (Manning et al., 2002). The ORF74-mediated constitutive activation of Akt protein kinase induces the phosphorylation of the tuberous sclerosis complex (TSC), leading to its degradation. Be-

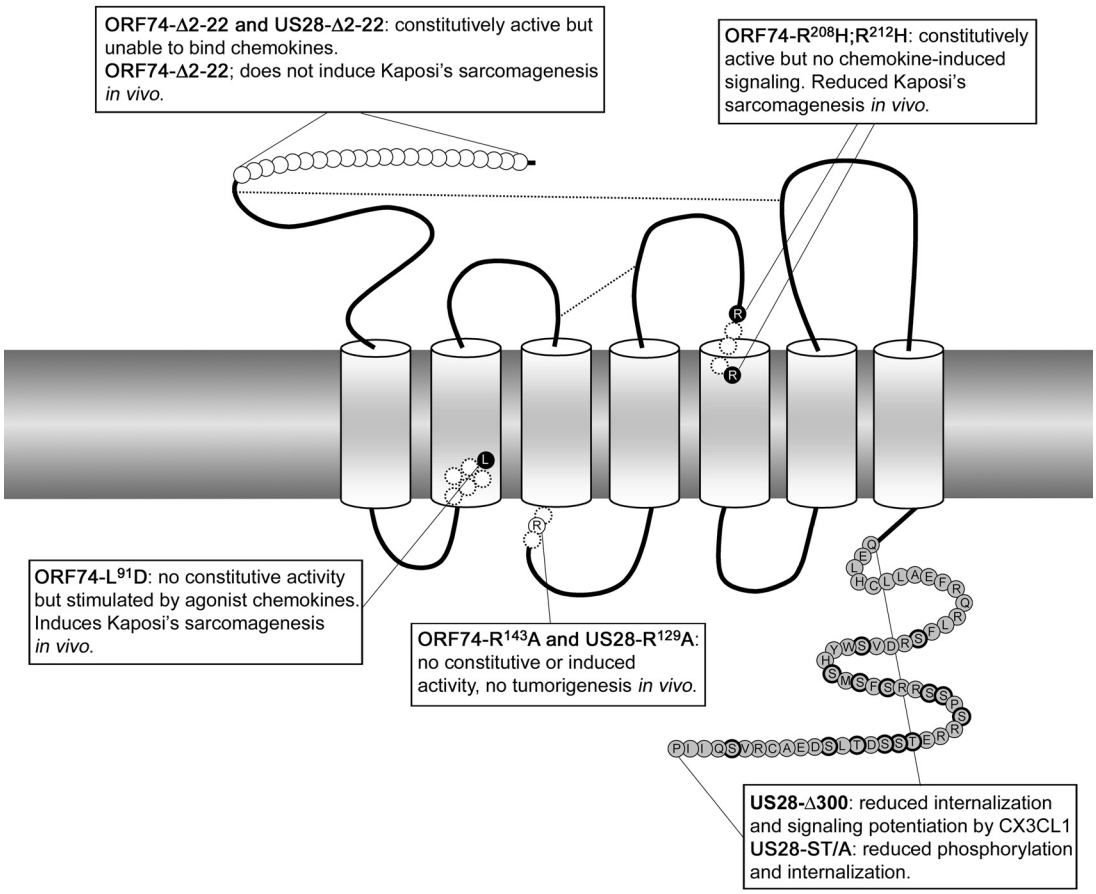

Fig. 2. Phenotypes of the vGPCRs ORF74 and US28 mutants. Residues mutated only in ORF74 are highlighted in filled circles, whereas gray circles indicate US28-specific mutant residues. Mutated amino acids generated for both ORF74 and US28 are indicated with open circles. Dotted circles represent undefined amino acids and are used to visualize the position of mutated amino acids in transmembrane domains or intra/extracellular loops. Dotted lines represent the conserved disulfide bridges between the extracellular loops 2 and 3 in ORF74 and US28 vGPCRs.

cause TSC acts as a negative regulator of the mammalian target of rapamycin (mTOR), its degradation results in the activation of mTOR (Sodhi et al., 2006). Downstream kinases and transcription factors modulate gene expression and result in cell growth (Yap et al., 2008).

ORF74 as a Promising Drug Target against KS

The high constitutive activation of ORF74 and its implication in the development of KS in various animal models highlights the potential of this vGPCR as drug target. Although only a few cells express ORF74 in human and experimental KS lesions (Chiou et al., 2002; Grisotto et al., 2006), the down-regulation of ORF74 in the course of KS development may represent an effective therapeutic approach. In a transgenic mouse model, the continuous expression of ORF74 in endothelial cells was required for the development of KS and down-regulation of receptor expression lowered the production of angiogenic factors, leading to disease regression (Jensen et al., 2005). In addition, in a xenograft tumor model, coinjection of ORF74-expressing cells with cells transfected with other latent KSHV genes potentiated neoplasia development (Montaner et al., 2006), confirming the suspected cooperative role between the lytic ORF74 gene and other latent genes (Montaner et al., 2003). It is noteworthy that although only a few ORF74-expressing cells were present in the tumors, their targeted pharmacological elimination effectively reduced tumor progression and induced the apoptosis of cells expressing latent genes (Montaner et al., 2006). Furthermore, the pharmacological targeting of signaling pathways activated by this vGPCR in the course of KS development also demonstrates promising results. The recent use of a dual inhibitor of both PI3K and mTOR, PI-103, reduced the proliferation of ORF74-expressing cells to a better extent than a specific inhibitor of either PI3K or mTOR and subsequently reduced tumor formation in a xenograft model (Chaisuparat et al., 2008). As such, the pharmacological inhibition of either ORF74 or its downstream signaling pathways may be of therapeutic interest in the treatment of KS.

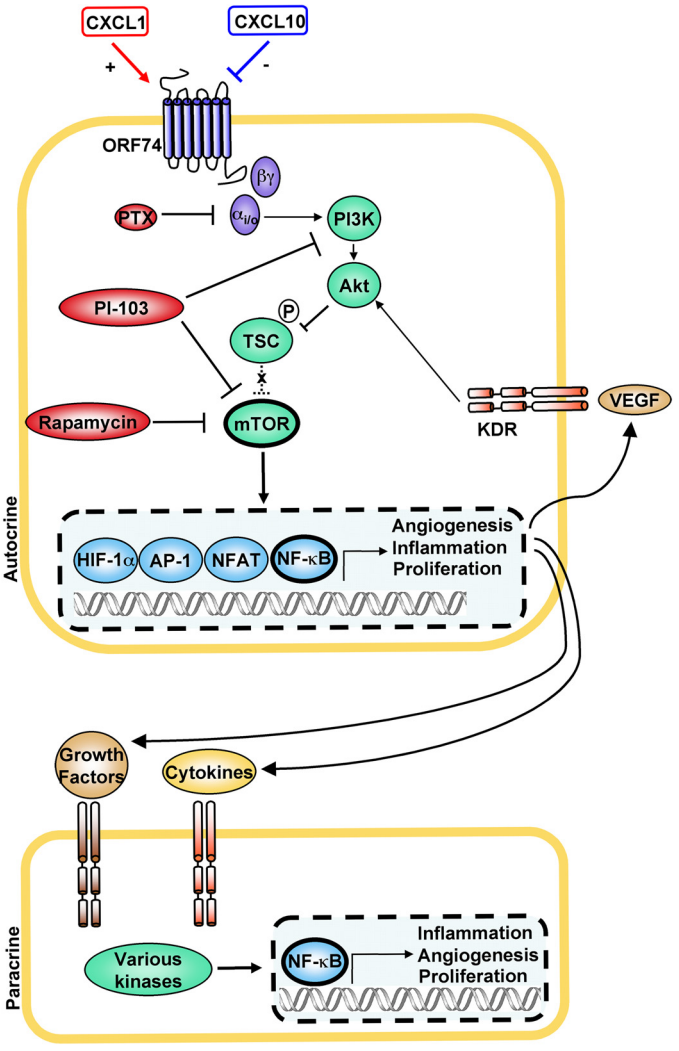

Fig. 3. ORF74-induced autocrine and paracrine signaling during cellular transformation. ORF74 constitutively activates G protein subunits and the downstream PI3K/Akt kinases. In turn, the TSC protein is inactivated by phosphorylation and leads to the accumulation of mTOR. Various transcription factors are activated and promote angiogenesis, inflammation and proliferation. ORF74-expressing cells secrete various growth factors and cytokines that bind to their respective receptors and promote transformation in a paracrine manner. The single inhibition of mTOR with rapamycin reduces ORF74-induced KS development but is less effective than the dual inhibition of both PI3K and mTOR with PI-103 (Chaisuparat et al., 2008).

EBV-Encoded BILF1 Contributes to Immune Escape

The EBV-encoded BILF1 receptor is a lytic gene that seems to present a function different from the KSHV-encoded ORF74 (Beisser et al., 2005). BILF1 physiological function has been examined in the context of immune evasion. Expression of BILF1 constitutively inhibits the phosphorylation of RNA-dependent protein kinase (PKR) (Beisser et al., 2005). Upon viral infection, double-stranded RNA binds to PKR, leading to its phosphorylation and activation. As a result, the overall cellular translational machinery is stopped, prohibiting viral replication, and only a few specific apoptotic genes are transcribed (García et al., 2006). This mechanism serves to prevent viral spreading by elimination of the infected cells. Thus, the inhibition of PKR by BILF1 may serve EBV to prevent cellular antiviral response. In addition, it was demonstrated recently that BILF1 expression also serves in immune escape by down-regulating antigen-presenting MHC class I cells of epithelial and melanoma cells (Zuo et al., 2009). BILF1 protein physically associated with MHC class I complexes, increasing their lysosomal degradation and down-regulating their surface expression. This mechanism was independent of constitutive G protein coupling because the BILF1 mutant $K^{122}A$, unable to constitutively activate NF-κB, down-modulated MHC I surface expression to a similar extent than the WT receptor (Zuo et al., 2009). As such, BILF1 expression was shown to evade CD8+ T-cell response, preventing recognition by the host immune system. The generation of transgenic mice expressing BILF1 is required to understand how BILF1 influences cellular fate and which mechanisms are activated during this process.

β-Herpesvirus-Encoded GPCRs

The β-HHV family consists of the human cytomegalovirus (HCMV also known as HHV-5), HHV-6, and HHV-7. All of these viruses present a broad cellular tropism and are involved in various pathologies. HCMV infects a broad range of cell types and is a known risk factor in immunocompromised patients. Immunosuppressed HCMV-infected transplantation patients are prone to graft rejection because of the de-

velopment of cardiac allograft vasculopathy, resulting in cardiac allograft loss. Furthermore, ongoing HCMV infection is increasingly linked to the development of proliferative and cardiovascular pathologies. Several studies have shown the presence of HCMV in atherosclerotic lesions (Horváth et al., 2000; Chen et al., 2003) as well as in several neoplastic conditions such as colon, prostate, and breast cancers and in glioblastoma (Cobbs et al., 2002; Harkins et al., 2002; Samanta et al., 2003; Söderberg-Nauclér, 2008). As such, HCMV infection seems to be particularly detrimental in patients with an impaired immune system. HCMV encodes four GPCRs referred to as US27, US28, UL33, and UL78. Like other virus-encoded GPCRs, US28 and UL33 possess constitutive signaling abilities, whereas US27 and UL78 do not (Vischer et al., 2006a). Although US27, UL33, and UL78 have been shown to present sequence homology to chemokine receptors, so far no ligands were shown to bind these vGPCRs. As such, these receptors are still considered orphan receptors. So far, the most studied vGPCR is US28 (Vischer et al., 2006a). It presents homology to the CC and CX3C chemokine receptors and was shown to bind CC (CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL13, CCL26, and CCL28), CX3C chemokines (CX3CL1), and the viral chemokine vCXCL2 (Gao and Murphy, 1994; Billstrom et al., 1998; Kledal et al., 1998; Penfold et al., 2003) (Fig. 1). The CCL2, CCL5, CCL7, and CX3CL1 chemokines exhibit agonistic activity on the receptor (Gao and Murphy, 1994; Billstrom et al., 1998; Vomaske et al., 2009), whereas in other assays, CX3CL1 has also shown inverse agonistic activity (Casarosa et al., 2001). The chemokines CCL3 and CCL4, although binding US28 with high affinity, have not yet shown signaling abilities.

US28 Scavenges Chemokines

Because of its chemokine binding ability, the role of US28 during immune evasion has been postulated. Shortly after the discovery of the US28 gene as a potential GPCR in the HCMV genome (Chee et al., 1990), several CC chemokines were found to bind and be scavenged by this receptor (Kuhn et al., 1995; Bodaghi et al., 1998; Kledal et al., 1998). US28 binds the CC chemokines CCL2 and CCL5, which seemed to be depleted in HCMV-infected cells. It is noteworthy that the down-regulation was not mediated by a transcriptional repression of these genes but was due to the expression of US28 in HCMV-infected cells (Bodaghi et al., 1998). Using radiolabeled chemokines, Bodaghi et al. (1998) demonstrated that US28 was constitutively internalizing these chemokines during HCMV infection. As such, it was postulated that US28 may provide a way for the virus to escape immune surveillance by avoiding clearance by immune cells. Using a static monocytes adhesion assay, Boomker et al. (2006) investigated whether US28 expression impaired the attachment of leukocytes to endothelial surfaces expressing chemokines. To rule out the effect of constitutive activity, they used the G protein-uncoupled mutant, US28-R^{129}A, which presents an expression pattern similar to that of US28-WT but does not induce any signaling activity. Although the US28-R^{129}A mutant could scavenge chemokines in vitro, it was unable to inhibit leukocyte recruitment, refuting a role for US28 during viral immune escape (Boomker et al., 2006).

The mechanism of chemokine depletion induced by US28 was attributed to its constitutive internalization ability (Fraile-Ramos et al., 2001). In transiently and stably transfected cells, US28 expression is restricted to the perinuclear region and is associated with endocytosis markers. Antibody-feeding experiments indicated that cell surface-expressed US28 undergoes constitutive internalization independently from ligand stimulation (Fraile-Ramos et al., 2001). In addition, the coupling of G proteins was not important for constitutive internalization because the G protein-uncoupled mutant US28-R^{129}A internalized in a manner similar to that of the WT receptor. In contrast, the C terminus of US28 showed to be the crucial determinant responsible for the ligand-independent endocytosis of the receptor (Fig. 2). By removing the C-terminal tail of US28 or replacing it with another human or viral GPCR, the mutant receptor presented a signaling activity higher than the WT receptor, which could be further potentiated by CX3CL1 (Waldhoer et al., 2003). Mutation of all serine and threonine residues from the C-terminal tail of US28 into alanine, US28-ST/A mutant (Fig. 2), abolished the phosphorylation and internalization of the receptor (Mokros et al., 2002). Thus, these studies highlighted that US28 C-terminal is an important structural motif for the constitutive internalization of the receptor, which is responsible for the sequestration of chemokines.

HCMV-Encoded US28 Function in Tumorigenesis

HCMV gene products and proteins are found in several types of proliferative malignancies such as colon, prostate, and breast cancers, and in malignant glioblastoma (Söderberg-Nauclér, 2006, 2008). Although the presence of viral proteins does not demonstrate a causative link between viral infection and human diseases, an oncomodulatory role for HCMV has been hypothesized (Cinatl et al., 2004; Michaelis et al., 2009). We initially demonstrated that the expression of US28 induces the constitutive activation of $G\alpha_q$ and $G\beta\gamma$ proteins, leading to the activation of phospholipase C and the transcription factor NF-κB (Casarosa et al., 2001). Pretreatment of the cells with pertussis toxin confirmed that these cellular processes were not mediated by $G\alpha_{i/o}$ proteins. Point and deletion mutations identified residues important in the constitutive activity of the receptor. By mutating arginine residue 129 into an alanine, US28-R^{129}A, and by deleting US28 N terminus to impair ligand binding, US28-Δ2-22, we demonstrated that G protein activation, but not chemokine stimulation, is important for the constitutive formation of inositol phosphates by US28 (Casarosa et al., 2003; Waldhoer et al., 2003) (Fig. 2). In addition, US28 is responsible for the activation of the cAMP-responsive element binding protein (CREB) and the nuclear factor activated in T-cells transcription factors through p38 MAPK- and p42/p44 MAPK-dependent pathways (McLean et al., 2004).

Based on the constitutive activation of the proinflammatory NF-κB transcription factor, US28 was suggested to promote inflammation and to potentially act as a viral oncogene (Maussang et al., 2006). Stable transfection of NIH-3T3 cells with US28 resulted in increased cellular proliferation without requiring any ligand stimulation (Maussang et al., 2006). US28-expressing cells presented an increased cell growth, because of the increased expression of the cell cycle protein regulator Cyclin D1. US28 signaling also allowed cells to overcome cell contact inhibition. In a foci formation assay, cells expressing the US28-WT receptor, but not the G protein-uncoupled US28-R^{129}A mutant, led to the formation of

cellular foci, highlighting the role of constitutive G protein activation in the transformation of NIH-3T3 cells in vitro. It is important to note that injection of these US28-WT-expressing cells into nude mice resulted in the tumor formation, highlighting a tumorigenic potential for this HCMV-encoded GPCR (Maussang et al., 2006). To determine molecular effectors of US28 oncogenic potential in NIH-3T3 cells, a microarray study was performed and showed that the expression of several cancer-related genes and NF-κB transcriptional targets was modulated. Besides the activation of oncogenes (e.g., Fus, Akt1, and Mdm2), US28 expression down-modulated anti-inflammatory genes (e.g., Del-1, Timp3) and tumor suppressor genes (e.g., Fs, Rgs4, Hivep3). It is noteworthy that US28 constitutive activity was responsible for the increased expression of the proinflammatory enzyme cyclooxygenase-2 (COX-2) (Maussang et al., 2009). The US28-R^{129}A mutant receptor showed no up-regulation of COX-2. In addition, chemical inhibition of the NF-κB signaling pathway with the IκB inhibitor Bay 11-7082 almost completely inhibited US28-induced COX-2 promoter activation. Because COX-2 protein is up-regulated in a variety of cancers and was shown to be an interesting drug target (Turini and DuBois, 2002), the importance of COX-2 in US28-mediated proangiogenic and tumorigenic phenotype was assessed using the COX-2-specific inhibitor celecoxib. In vitro treatment of US28-expressing NIH-3T3 cells and in vivo treatment of mice xenograft with celecoxib effectively reduced the secretion of VEGF (in vitro) and the resulting blood vessel formation (in vivo). Consequently, celecoxib-treated mice developed tumors at a slower rate compared with control animals when injected with US28-expressing NIH-3T3 cells (Maussang et al., 2009). In addition, detailed molecular analysis of signaling pathways using chemical inhibitors indicated that US28 activates $G\alpha_q$ and $G\beta\gamma$ subunits as well as p38 and p44/42 MAPK to induce VEGF promoter activation (Maussang et al., 2006). Furthermore, the NF-κB transcription factor was important for both the VEGF and COX-2 promoter activation. Because COX-2 inhibition with celecoxib greatly impaired VEGF protein release in the supernatant of US28-expressing NIH-3T3 cells, this indicates that the US28-mediated constitutive coupling to G proteins activates downstream protein kinases and transcription factors. This in turn induces COX-2 gene and protein up-regulation, resulting in the release of prostaglandin E2 (PGE2). By interacting with its putative receptors EP1–4, COX-2-derived PGE2 activates transcription of various genes (e.g., VEGF and cyclin D1) (Maussang et al., 2009) (Fig. 4A). It is important to note that the deletion of US28 gene from HCMV genome impaired the activation of VEGF and COX-2 gene promoter in infected cells, confirming the important role of US28 during viral pathogenesis (Maussang et al., 2006, 2009). Together, these results indicate that US28 constitutive activity induces inflammation and cellular proliferation.

US28 Ligand-Dependent Signaling as a Link to Atherosclerosis

In addition to neoplastic conditions, HCMV infection is also increasingly recognized as a risk factor for the development of cardiovascular diseases such as atherosclerosis (Söderberg-Nauclér, 2006). Infection of smooth muscle cells with HCMV results in cellular migration, providing a molecular link between HCMV infection and the development of

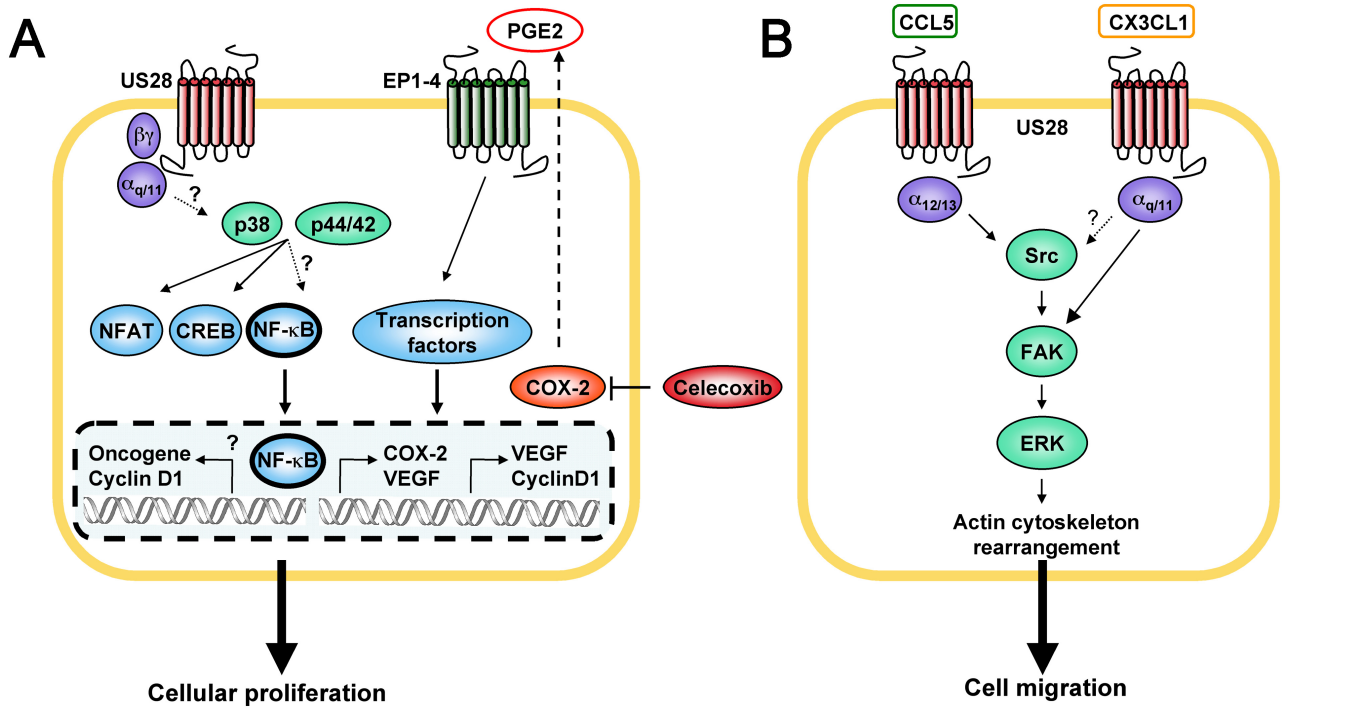

Fig. 4. US28 signaling during cell proliferation and migration. A, US28 constitutively activates $G\alpha_q$ and $G\beta\gamma$ proteins and downstream MAPKs such as p38 and p44/42. In turn, transcription factors mediate the up-regulation of oncogenes and inflammatory factors (e.g., COX-2). PGE2 is produced by the up-regulated COX-2 and further activates transcription factors after binding to its cognate receptors EP1 to -4. This positive feedback loop further up-regulates angiogenesis (VEGF) and proliferation (cyclin D1). Celecoxib inhibits COX-2 activity and impairs tumor formation and angiogenesis in vivo (Maussang et al., 2009). B, stimulation of US28-expressing cells with CCL5 and CX3CL1 activates, respectively, $G\alpha_{12/13}$ and $G\alpha_q$ proteins that both activate FAK and the downstream extracellular signal-regulated kinase (ERK). This leads to the rearrangement of actin cytoskeleton and cellular migration (Vomaske et al., 2009).

cardiovascular diseases. Because chemokines are major players in the promotion of inflammation and cellular migration, the chemokine receptor US28 was identified as a molecular effector involved in cell migration. Deletion of US28 from the viral genome abolished HCMV-induced cellular migration (Streblow et al., 1999). Binding of chemokines to US28 was necessary to induce chemotaxis. It is noteworthy that different ligands activate specific signaling pathways in a cell type-dependent manner (Waldhoer et al., 2003; Vomaske et al., 2009). CCL5 and CX3CL1 both bind to US28 with nanomolar affinities, but they activate different G proteins, such as $G\alpha_{12/13}$ in the case of CCL5 and $G\alpha_q$ by CX3CL1. One of the main downstream effectors of G protein activation is the focal adhesion kinase (FAK) (Streblow et al., 2003; Vomaske et al., 2009). Upon CCL5 binding, US28 interacts with and activates Src kinase, which phosphorylates FAK and promotes actin cytoskeleton rearrangement (Streblow et al., 2003; Vomaske et al., 2009). Although the direct interaction of US28 with Src has not been established with CX3CL1, such a mechanism may be occurring (Fig. 4B). Of interest, the actions of these ligands seemed to be cell-specific. CCL5 was able to induce a robust US28-mediated chemotactic activity in smooth muscle cells, whereas CX3CL1 presented a neutral antagonist effect. In contrast, CX3CL1 but not CCL5 stimulation mediated the migration of US28-expressing macrophages (Vomaske et al., 2009). As such, ligand-induced US28 signaling is cell type-specific, which may be of importance during atherogenesis, promoting the recruitment of macrophages and smooth muscle cells in the atherosclerotic plaques and inflamed lesions.

HCMV-Encoded US28 as Drug Target

The oncomodulatory properties of HCMV described by Cinatl et al. (1996) are important for the future studies of the US28 receptor. In contrast to our observation that US28 presents oncogenic properties in NIH-3T3 cells, previous studies have shown that US28 induces apoptosis in human embryonic kidney 293T (Pleskoff et al., 2005). Thus, cells possessing oncogenic properties (i.e., with genetic mutations and altered cellular gene regulations) may be a prerequisite for US28 to exert its tumorigenic potential. Because of the link between HCMV infection and neoplasia such as glioblastoma and colon cancer (Harkins et al., 2002; Miller, 2009), the role of US28 in such pathologies needs to be examined. Besides direct cellular transformation, US28 may also be able to induce oncogenesis in a paracrine manner, as exemplified for the KSHV-encoded chemokine receptor ORF74. US28-expressing NIH 3T3 cells were shown to secrete high levels of VEGF, implying that US28 induces angiogenesis. Besides in vitro studies, the generation of transgenic animals expressing US28 will be a very valuable tool for understanding the importance of this vGPCR in HCMV-related diseases.

To block US28 constitutive activity, we generated compounds that can act as inverse agonists. The first nonpeptidergic compound, VUF2274, was derived from an antagonist against the chemokine receptor CCR1 (Casarosa et al., 2003). This compound inhibited US28-mediated signaling activity in transfected cells and the constitutive activity of US28 observed in HCMV-infected cells (Casarosa et al., 2003). Such inverse agonist compounds seem to be promising antiviral drugs, but their low potency still requires further optimization (Hulshof et al., 2005; Hulshof et al., 2006).

HCMV-Encoded UL33, UL78, and US27 as Additional Modulators of Cellular Signaling

Viral GPCRs other than US28 also deserve attention in the search for molecular effectors of HCMV-derived pathogenesis. In particular, the UL33 gene is constitutively active and is part of a gene family that is conserved among herpesviruses (Gruijthuijsen et al., 2002; Waldhoer et al., 2002). In HCMV-infected cells, UL33 is responsible for the constitutive CREB-mediated gene transcription, whereas US28 constitutively induces inositol phosphate formation. Thus, the constitutively active vGPCR UL33 and US28 may play different roles in infected cells, but they may also present overlapping functions. In fact, US28 is not present in mouse and rat CMV, and it is not conserved in all CMV species, whereas UL33 is. Mouse and rat CMV encode UL33 genes that are able to induce cellular migration, mimicking the described chemotactic function of the HCMV-encoded US28 gene (Streblow et al., 1999, 2005; Melnychuk et al., 2005). The M33 protein induces migration of mouse cells toward mCCL5, and deletion mutants of mouse CMV lacking M33 or rat CMV missing the ORF R33 induce impaired cellular migration compared with their respective WT viruses (Melnychuk et al., 2005; Streblow et al., 2005). It has been shown that UL33 could compensate for the lack of M33 in a mouse CMV deletion mutant, suggesting conserved biological functions of the UL33 gene family (Case et al., 2008). Signaling studies in transfected cells and infected cells will determine what signaling pathways other than CREB activation are attributed to UL33 and how the receptor activity may be implicated in viral life cycle and/or viral pathogenesis.

The other genes conserved among CMV species belong to the UL78 family. Studies of rat and mouse CMV showed that the UL78 genes are important for viral replication (Oliveira and Shenk, 2001; Kaptein et al., 2003). However, deletion of the UL78 gene from the HCMV genome did not impair viral replication (Michel et al., 2005). Because the UL78 protein is not constitutively active and its ligands are still unknown, signaling studies seem premature at this point. In a similar manner, the HCMV-encoded US27 gene (which is not present in mouse and rat CMV) has not shown constitutive signaling, and its role for HCMV remains puzzling. Deorphanization of both US27 and UL78 genes is a first step that will allow characterization of the signaling properties of these receptors.

HHV-6- and -7-Encoded GPCRs

The two other members of the β-HHV subfamily are the HHV-6 and HHV-7 viruses. Infection with HHV-6 and HHV-7 are responsible for skin diseases in children, namely exanthem subitum (Caselli and Di Luca, 2007). In addition, HHV-6 is increasingly recognized as a pathogen responsible for the development of encephalitis and multiple sclerosis in immunocompromised patients (Christensen, 2007). Both HHV-6 and HHV-7 encode for two vGPCRs, U12 and U51. U12 and U51 genes from HHV-6 and HHV-7 bind different broad spectra of human chemokines. HHV-6 U51 binds chemokines from the CC (CCL2, 5, 7, 11, 13, 19, and 22), CX3C (CX3CL1), and XC (XCL1) families, as well as the KSHV-encoded chemokine vCXCL2 (Milne et al., 2000; Catusse et al., 2008), whereas HHV-7 U51 ligands are strictly from the CC family (CCL17, 19, 21, and 22) (Tadagaki et al., 2005).

Likewise, U12 from HHV-6 and HHV-7 bind the CC chemokines CCL2, CCL3, CCL4, and CCL5 (Isegawa et al., 1998) and CCL17, CCL19, CCL21, and CCL22 (Tadagaki et al., 2005), respectively (Fig. 1). Constitutive signaling has so far been measured only for the HHV-6-encoded U51 gene (Fitzsimons et al., 2006).

HHV-6-Encoded U51 Contributes to Immune Evasion

Similar to the HCMV-encoded US28 receptor, the HHV-6-encoded U51 gene was shown to scavenge CCL5 from the supernatant of U51-expressing and HHV-6-infected cells (Milne et al., 2000; Catusse et al., 2008). In contrast to US28, U51 chemokine sink function was attributed to both transcriptional repression of the CCL5 gene and the scavenging of CCL5 protein. In fact, the constitutive activity of U51 leads to the down-modulation of several immunological factors. The U51-expressing myeloid cell line K562 exhibited constitutive $G\alpha_q$ protein activation as measured by inositol phosphate formation, and CCL5 stimulation further increased U51 signaling. To identify targets of constitutive signaling, a microarray analysis restricted to targets of infection and immunity indicated that U51 down-regulated the transcription of the friend of GATA2, zinc finger protein (FOG-2) gene (Catusse et al., 2008). FOG-2 belongs to the family of FOG proteins that are transcriptional repressors involved in the development of Th1 and Th2 immune responses (Kurata et al., 2002; Mariani et al., 2004). As such, U51 ligand-dependent and -independent signaling may influence gene expression during immune responses.

Hijacking of Human Receptors by Viral GPCRs

Viral GPCRs directly modulate cellular signaling in a ligand-induced and/or constitutive manner, as outlined in the preceding sections. In addition, viral GPCRs can also affect the properties of human receptors by means of receptor hetero-(di/oligo)merization and/or downstream signaling cross-talk.

Receptor Heteromerization

GPCRs have long been considered to exist and function as monomeric entities. However, during the last decade, accumulating evidence revealed that GPCR proteins physically interact with each other. GPCR heteromers are considered to be unique functional entities next to GPCR monomers and homomers (Ferré et al., 2009). Heteromerization of different GPCR proteins can alter the functional characteristics of the individual partners, which includes trafficking, ligand binding, and signaling (Levoye et al., 2006b; Milligan, 2006; Springael et al., 2007).

Human chemokine receptor heteromers (e.g., CCR2/CCR5, CCR2/CXCR4, and CCR5/CXCR4) display negative binding cooperativity for their cognate ligands when both chemokines are simultaneously administrated (Springael et al., 2007). Hence, chemokine receptor heteromers can only bind a single ligand with high affinity. Heteromerization of the human orphan GPCR GPR50 with the MT1 melatonin receptor fully impairs high-affinity ligand binding as well as G-protein and β-arrestin coupling to the MT1 protomer (Levoye et al., 2006a).

The appearance of foreign, virally encoded chemokine or orphan receptor proteins (in or) on infected human cells and their subsequent heteromerization with human GPCR proteins may significantly alter the functional properties of the latter. This receptor hijacking might be an additional, yet underappreciated, mechanism by which the cellular homeostasis can be changed in favor of the virus. We recently identified heteromers between the EBV-encoded GPCR BILF1 and various human chemokine receptor that are involved in B lymphocyte migration (Vischer et al., 2008). The functional consequences of heteromerization of this constitutively active, orphan GPCR BILF1 with these human chemokine receptors are currently under investigation. In addition, similarly to the EBV-encoded vGPCR BILF1, the HCMV-encoded GPCRs UL78 and US27 proteins, which seem to be silent, may be able to heteromerize with other cellular receptors to modulate cellular signaling pathways (Vischer et al., 2008).

Signaling Cross-Talk

US28 constitutively activates NF-κB transcription factors in a pertussis toxin-insensitive manner via $G\alpha_{q/11}$ proteins (Casarosa et al., 2001; Bakker et al., 2004). This constitutive activation cannot be further stimulated by chemokine binding to US28 (Bakker et al., 2004). However, coexpression of US28 with the human chemokine receptor CCR1 unmasks the activation of NF-κB transcription factors in response to CCL5 (Bakker et al., 2004). It is noteworthy that this $G\alpha_{i/o}$-coupled CCR1 does not activate normally NF-κB signal transduction pathways. Binding of CCL5 to US28 is not involved in this NF-κB stimulation, as indicated by CCL5-induced NF-κB signaling in cells coexpressing CCR1 and the N-terminal deletion mutant Δ22-US28, which is not able to bind CCL5 (Casarosa et al., 2003). Hence, CCL5 binding to CCR1 induces NF-κB activation in a pertussis toxin-sensitive manner via $G\alpha_{i/o}$ proteins. However, this CCR1-mediated activation of NF-κB signaling is strictly dependent on the constitutive activity of US28, because the signaling-impaired US28 mutant $R^{129}A$ failed to reveal this CCL5-induced signaling (Waldhoer et al., 2003; Bakker et al., 2004). Whether this effect of the constitutively active US28 on CCR1 signaling involves direct physical interactions between these GPCRs or is at the level of intracellular signal networks is currently under investigation.

Conclusion

Herpesviruses have acquired several genes encoding chemokine receptors into their genome. Both chemokine binding and the constitutive modulation of signaling pathways by vGPCRs participate in autocrine or paracrine neoplastic transformation, migration of cells involved in atherosclerosis development, and helping the virus to escape immune surveillance. In addition, the modulation of other chemokine receptors signaling by heteromerization or signaling cross-talk is another way for vGPCRs to reorchestrate cellular communication. The crucial roles of vGPCRs during pathogenesis and viral life cycle may represent opportunities for the development of innovative antiviral therapies targeting these viral receptors.

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