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The Rat Cytomegalovirus R33-Encoded G Protein-Coupled Receptor Signals in a Constitutive Fashion

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The rat cytomegalovirus (RCMV) R33 gene is conserved among all betaherpesviruses and encodes a protein (pR33) that shows sequence similarity with chemokine-binding G protein-coupled receptors (GPCRs). Previously, the physiological significance of the R33 gene was demonstrated by the finding that an RCMV strain with R33 deleted is severely attenuated in vivo and is unable to either enter or replicate in the salivary glands of infected rats. Here, we report that RCMV pR33 is expressed as a functional GPCR that signals in an agonist-independent manner in both COS-7 and Rat2 cells. Transient expression of pR33 in COS-7 cells results in constitutive activation of phospholipase C (PLC) due to coupling to G proteins of the G\textsubscript{i} class. Interestingly, PLC activation is partially inhibited by cotransfection with G\textsubscript{i} subunits in pR33-mediated signaling. Surprisingly, PLC activation is also partially inhibited by addition of pertussis toxin (PTX), suggesting that pR33 activates not only G\textsubscript{i} but also G\textsubscript{q} proteins. The constitutive activation of G\textsubscript{q} proteins by pR33 is further demonstrated by the PTX-sensitive decrease of CRE-mediated transcription and the PTX-sensitive increase of both NF-\kappaB- and SRE-mediated transcription. In contrast to its homolog of human herpesvirus 6B (pU12), pR33 does not bind RANTES.

Cytomegaloviruses (CMVs) are species-specific betaherpesviruses that cause acute, persisting, and latent infections in both humans and animals. The ability of CMVs to induce lifelong, latent infections implies that these viruses are highly adapted to their hosts. Specifically, they will have to employ sophisticated strategies in order to remain hidden from their host’s immune system. Among the CMV genes that are likely to be involved in these strategies are those encoding homologs of crucial immune effector or regulatory proteins of the host, such as G protein-coupled receptors (GPCRs). These genes have probably been pirated by the virus during the long coevolution of pathogen and host.

GPCRs form a large and diverse family of receptors that function in signal transduction through cell membranes. They are composed of a central core domain consisting of seven transmembrane helices connected by three intracellular and three extracellular loops. The majority of these receptors activate G proteins and are capable of transducing a wide variety of messages. Within the genomes of all sequenced CMVs, genes have been identified that potentially encode homologs of host cellular GPCRs. Human CMV (HCMV) carries four of these genes: US27, US28, UL33, and UL78 (13, 14). Only two of these, UL33 and UL78, were found to have counterparts in rat CMV (RCMV) (R33 and R78, respectively [4, 5, 52]) as well as murine CMV (MCMV) (M33 and M78, respectively [17, 41]). Of the predicted amino acid sequences derived from the CMV GPCR-like genes, those encoded by HCMV US27 and US28 were found to have the highest similarity to chemokine-binding GPCRs (chemokine receptors). HCMV US28 was demonstrated to encode a functional chemokine receptor (pUS28) which binds the CC (or \beta-) chemokines RANTES, MCP-1, MCP-3, MIP-1\alpha, and MIP-1\beta (7, 8, 22, 31, 37) as well as the only known member of the CX3C (or \delta-) chemokine family, CX3CL1 (also known as fractalkine or neurotactin), which is a membrane-associated chemokine with a cytoplasmic domain (29). Upon CC-chemokine binding, pUS28 was shown to engage in intracellular signaling by the mobilization of intracellular calcium and activation of extracellular signal-related kinase 2 (7). Remarkably, pUS28 was also found to signal in the absence of exogenously added chemokines, by activating phospholipase C (PLC) as well as NF-\kappaB in transiently transfected COS-7 cells (11). This constitutive, agonist-independent activity of pUS28 can be modulated by fractalkine, which acts as a partial inverse agonist, whereas CC-chemokines appear to function as neutral antagonists (11). Another intriguing property of pUS28 was identified by Streblow and colleagues (49). They demonstrated that HCMV infection of primary arterial smooth muscle cells (SMCs) resulted in cellular migration, whereas an HCMV recombinant that lacks a functional US28 gene was unable to promote migration. The expression of pUS28 in SMCs in the presence of CC-chemokines was sufficient to induce migration. It was hypothesized by Streblow et al. that HCMV may be using SMCs as a cellular vehicle to disseminate the virus in vivo (49). As described above, the HCMV UL33 gene has homologs in...
TABLE 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Restriction site(s)</th>
<th>Source</th>
<th>Positions</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP-CN-F</td>
<td>5'-ATAAGAATTCTGACATCGGATGCGCTACG-3'</td>
<td>EcoRI</td>
<td>pEGFP-N1</td>
<td>574–603</td>
<td>+</td>
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<tr>
<td>EGFP.N1-R</td>
<td>5'-TATACGCTACGCTAGCTAGCTAACGCTGCTCC-3'</td>
<td>NcoI, BspHI</td>
<td>pEGFP-N1</td>
<td>1380–1403</td>
<td>–</td>
</tr>
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<td>R32.N-F</td>
<td>5'-TGGCAGACTTCCGAGCCAGGCCCC-3'</td>
<td>RCMV genome</td>
<td>25553–25555</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>R32.N-R</td>
<td>5'-TATCATCGGGTGTCGGCTGGTCGTC-3'</td>
<td>NcoI</td>
<td>RCMV genome</td>
<td>26253–26280</td>
<td>–</td>
</tr>
<tr>
<td>R33.N-F</td>
<td>5'-TAATCCATGAGCTGCGTGGGCGGAC-3'</td>
<td>NcoI</td>
<td>RCMV genome</td>
<td>26271–26290</td>
<td>+</td>
</tr>
<tr>
<td>R33.N-R</td>
<td>5'-GGTCTCGATCTGAGGGTTGCGGCTG-3'</td>
<td>RCMV genome</td>
<td>26474–26499</td>
<td>–</td>
<td></td>
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<tr>
<td>R33.C-F</td>
<td>5'-TCTCGGTGAGTGTCAGCCGCGCTC-3'</td>
<td>RCMV genome</td>
<td>27025–27047</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>R33.C-R</td>
<td>5'-CGTCTAGACGTCCGGCGCGGCGGAC-3'</td>
<td>XbaI</td>
<td>RCMV genome</td>
<td>27415–27438</td>
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<tr>
<td>R34.C-F</td>
<td>5'-ACTCTAGAAGCGGATCCGGCGCCCGC-3'</td>
<td>XbaI</td>
<td>RCMV genome</td>
<td>27427–27451</td>
<td>–</td>
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<tr>
<td>R34.C-R</td>
<td>5'-GGCTTCTCGGTCTTCCCCCGGCGC-3'</td>
<td>RCMV genome</td>
<td>28024–28044</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* Underlined sequences indicate restriction enzyme recognition sites; sequences in boldface type differ from that of the original sequence as described in the columns for source and positions.

+ orientation of the sequence is identical to the published sequence between the indicated positions; –, sequence is complementary to the published sequence between the indicated positions.

all sequenced betaherpesviruses, which underlines the biological relevance of the UL33 gene family. At present, this family consists of six members: HCMV UL33 (13, 14), MCMV M33 (41), and RCMV R33 (5) and the U2 genes of human herpesvirus 6A (HHV-6A) (24), HHV-6B (19, 25), and HHV-7 (38). The predicted amino acid sequences of the proteins encoded by the UL33-like gene family were found to comprise several features characteristic of chemokine receptors (5, 17).

The biological significance of the UL33 family members has previously been demonstrated in studies using recombinant CMVs that carry either a disrupted UL33 (33), M33 (17), or R33 gene (5) in their genomes. In cell culture, each of these mutant viruses replicated with similar efficiency as the corresponding wild-type (WT) viruses (5, 17, 33). However, during in vivo infection, significant differences were observed between animals infected with the recombinants and those infected with the WT viruses. In contrast to their WT counterparts, viruses with M33 and R33 deleted could not be detected within the salivary glands of infected mice and rats, respectively (5, 17). This indicated that M33 and R33 play a role in virus dissemination to or replication in the salivary glands (5, 17). Furthermore, it was shown in the RCMV-rat model that R33 also has a more general function: a significantly lower mortality was seen among rats infected with RCMV from which R33 was deleted (RCMVΔR33) than among those infected with WT RCMV (5). These results stressed the importance of the UL33-like genes in the pathogenesis of CMV infection.

Although the HHV-6B member of the UL33 family, pU12, was reported to be a calcium-mobilizing receptor for several CC-chemokines (25), not much is known about the biochemical, immunological, and pharmacological processes in which the CMV members of this protein family may function. We therefore set out to study the putative signaling properties of the RCMV R33-encoded protein (pR33). We report here that pR33 is a functional GPCR that signals in an agonist-independent, constitutive fashion in COS-7 as well as Rat2 cells.

MATERIALS AND METHODS

Expression constructs. The sequence containing RCMV open reading frames (ORFs) R32 (partially), R33, and R34 (partially) (position 24384 to 29545 of the RCMV genome sequence [53]) was cloned as a PstI fragment into vector pUC119, generating plasmid RPst5. This plasmid was digested with PstI, and the resulting 2.9-kb fragment, which contains the complete R33 ORF, was treated with T4 DNA polymerase in the presence of 50 mM of dNTPs (dNTPs) in order to generate blunt ends. Subsequently, the blunt-ended fragment was cloned into the Hincl restriction site of pUC119, generating plasmid p375. Then, a PCR-based procedure was used to change the sequence around the translation initiation codon of R33, 5'-CGGTATGGACG-3', into the sequence 5'-CGCATGGACG-3', which contains an Ncol restriction site (indicated in italics). This was carried out as follows. First, the 5' end of R33 was amplified by means of PCR using primers R33.N-F and R33.N-R (Table 1) and p375 as a template. Then, the 228-bp PCR product was treated with T4 DNA polymerase and cloned into the Hincl site of R33 (5), creating plasmid p379. The 745-bp PCR product was treated with T4 DNA polymerase and subsequently digested with NcoI. The resulting Ncol-blunt fragment was cloned into p379 by using the Smal site of the polylinker and the introduced Ncol site at the translation initiation codon of R33, resulting in plasmid p3735. Finally, the 946-bp BglII-BsrGI fragment from p3735 was exchanged for the BglII-BsrGI fragment from p379, which contains the introduced Ncol site at the start codon of R33, creating plasmid p383. The 2.1-kb BamHI-Smal fragment from p383, containing ORF R33 with the introduced Ncol site, was cloned into BamHI- and HindII-digested pUC119. Subsequently, the 331-bp BamHI-Ncol fragment immediately upstream of R33 was deleted from the plasmid by digestion with BamHI and NcoI and treatment with the 5' end fragment of DNA polymerase I (Klenow), followed by closing of the linear vector with T4 DNA ligase, resulting in plasmid pUC119/R33dN. The R33 expression vector ppcDNA3/R33 was then generated by cloning the 1,680-bp KpnI fragment containing ORF R33 from pUC119/R33dN into KpnI-digested ppcDNA3 (Invitrogen, Groningen, The Netherlands). The KpnI sites of pUC119/R33dN are derived from the pUC119 polylinker, located 5' of the translation initiation codon of ORF R33, and the RCMV sequence down-stream of the ORF R33 stop codon (corresponding to position 27950 of the RCMV sequence). The orientation of ORF R33 in ppcDNA3/R33 was checked by restriction enzyme analysis.

The expression vector ppcDNA3/EGFP was generated by digestion of pEGFP-N1 (Clontech, Palo Alto, Calif.) with BamHI and Ncol and cloning the resulting 739-bp fragment into BamHI- and NoI-digested ppcDNA3. For the construction of expression vector ppcDNA3/EGFP-R33, a BspHI site was introduced immediately downstream of the enhanced green fluorescent protein (EGFP) ORF. This was carried out by means of PCR with primers GFP-CN.F and EGFP-N1-R (Table 1), using pEGFP-N1 as a template. The amplified fragment of 829 bp was treated with T4 DNA polymerase and the blunt-ended fragment was subsequently cloned into HindII-digested pUC119, generating pUC119-EGFP. Then, the 720-bp BglII-BspHI fragment from pUC119/EGFP was cloned into the introduced Ncol site at the translation initiation codon of R33 within plasmid p383-EGFP, resulting in plasmid p383-EGFP. The orientation of the EGFP ORF was checked by restriction enzyme analysis. Then, p383-EGFP was digested with Ncol and treated with Klenow. Subsequently, the DNA was digested with ApaI to generate a 2.4-kb blunt-ApaI fragment which contains the EGFP ORF fused to R33. This fragment was cloned into EcoRV- and ApaI-digested ppcDNA3, resulting in the expression vector ppcDNA3/EGFP-R33.
In order to generate the pCDNA3/R33-EGFP expression vector, a pUC119 vector was digested with XbaI and treated with T4 polynucleotide kinase, and the linear vector was closed with T4 DNA ligase. Subsequently, the vector was digested with XhoI, treated with Klenow, and closed with T4 DNA ligase, generating plasmid p380. The previously described 2.9-kb BamHI fragment containing the complete R33 ORF was cloned into the HindIII site of pre-376, resulting in plasmid p380. Then, the 768-bp XbaI-NheI fragment of p380, which contains the introduced XhoI site in the R33 ORF, was digested in 1.0 unit of REAF for 4 h at room temperature, the cells were washed three times with TBS and the OPD substrate solution (5 mM O-phenylenediamine [Sigma], 0.03% H2O2 in 0.1 M citrate-phosphate buffer [pH 5.0]) was applied for approximately 10 min. The coloring reaction was stopped by the addition of 1 M H2SO4, samples were taken from the supernatants, and the optical density was measured in a Victor2 Wallac multilabel counter at 490 nm.

EGFP-based monolayer enzyme-linked immunosorbent assay (ELISA). Transfected cells were seeded in 96-well plates (Costar). After 48 h of incubation, the monolayers were washed once with TBS-buffered saline (TBS). (50 mM Tris, 150 mM NaCl [pH 7.5]) and fixed for 30 min with 4% paraformaldehyde in PBS. Subsequently, cells were washed three times with TBS and permeabilized with 0.5% NP-40 in TBS. After 30 min, the permeabilization solution was replaced with blocking buffer (1% fat-free milk, 0.1 M NaHCO3 [pH 8.6]) and the cells were incubated for 4 h at room temperature. Then, the blocking buffer was removed and the primary solution containing 0.1% [4-14C]luciferin ([2,2]-octyl)-octanoic acid, (Merck, Darmstadt, Germany), was added. After 2 h of incubation at room temperature, the cells were washed three times with PBS and the OPD substrate solution (5 mM O-phenylenediamine [Sigma], 0.03% H2O2 in 0.1 M citrate-phosphate buffer [pH 5.0]) was applied for approximately 10 min. The coloring reaction was stopped by the addition of 1 M H2SO4, samples were taken from the supernatants, and the optical density was measured in a Victor2 Wallac multilabel counter at 490 nm.

Chemokine binding assay. Human and rat RANTES were labeled with 125I using the Iodo-gen method (Pierce, Rockford, Ill.) according to the manufacturer's protocol. 125I-labeled RANTES was separated from free iodine ([125]I) using a 25-cm, 10-ml Sephadex G-25 gel filtration column (ICN Pharmaceuticals Inc., Costa Mesa, Calif.). Iodine incorporation and specific activity were determined via precipitation of the protein by the trichloroacetic acid (TCA) method. Transiently transfected cells were seeded in 24-well plates. In saturation experiments, binding was performed for 48 h after transfection on whole cells for 3 h at 4°C using different concentrations of 125I-labeled human or rat RANTES (from 0.1 to 6 nM) in binding buffer (50 mM HEPES [pH 7.4], 1 mM CaCl2, 5 mM MgCl2, and 0.5% BSA). Non specific binding was determined in the presence of 0.1 μM unlabeled competitor (human or rat RANTES). In competition experiments, cells were incubated with 0.6 μM 125I-labeled RANTES and various amounts of unlabeled human or rat RANTES. After incubation, cells were washed four times with binding buffer supplemented with 0.5 M NaCl.

Statistical analysis. All data shown are expressed as mean ± standard error. Statistical analysis was carried out by Student’s t test. P values of <0.05 were considered to indicate a significant difference.
RESULTS

RCMV pR33 and pR33-EGFP signal constitutively in COS-7 cells. To test whether the RCMV R33-encoded protein (pR33) functions as a GPCR in vitro, we set out to examine the effect of pR33 expression on the accumulation of inositol phosphates (InsP) in COS-7 cells. To this end, the cells were transiently transfected with either of four different expression constructs, which encode native RCMV pR33 (pcDNA3/R33), pR33-EGFP (pcDNA3/R33-EGFP), pEGFP-R33 (pcDNA3/EGFP-R33) or no protein (pcDNA3). At 24 h after transfection, the cells were incubated with 3H-labeled inositol, and at 48 h after transfection, the accumulation of radiolabeled InsP was measured. As shown in Fig. 1A, transfection of increasing amounts of both pcDNA3/R33 and pcDNA3/R33-EGFP resulted in increased [3H]InsP production within the cells. The InsP production in cells transfected with pcDNA3/R33 and pcDNA3/R33-EGFP only (4.0 μg/10^6 cells) was 3.2 times higher than that in cells transfected with an equal amount of pcDNA3 only. Interestingly, a significant difference in InsP levels was not observed between pcDNA3- and pcDNA3/R33-EGFP-transfected cells (data not shown). Taken together, these data indicate that (i) these proteins activate signaling pathways leading to increased InsP production rates and (ii) these proteins signal in an agonist-independent fashion.

To examine the expression levels as well as intracellular localization of the R33-encoded proteins, both ELISA and confocal microscopy were performed. Since appropriate anti-pR33 antibodies were unavailable, we could investigate only the expression of the EGFP fusion proteins by virtue of the presence of the EGFP tag in these proteins. The semiquantitative, ELISA-based monolayer assay showed that transfection of increasing amounts of pcDNA3/R33-EGFP resulted in increasing intracellular levels of pR33-EGFP (Fig. 1B). Contrast, expression of pEGFP-R33 was not detected (data not shown). These expression data are in agreement with the activity of these EGFP-tagged proteins in the InsP accumulation assay.

The cellular expression of pR33-EGFP was studied further by confocal microscopy. Figure 1D shows that the EGFP fluorescence colocalizes with intracellular, perinuclear vesicles as well as with the cell membrane of COS-7 cells transfected with pcDNA3/R33-EGFP. This expression pattern differs considerably from that seen in nonfused EGFP-expressing COS-7 cells, in which EGFP is seen dispersed throughout the nucleus and cytoplasm (Fig. 1C). These results strongly suggest that pR33-EGFP is properly expressed on the cell surface of transfected cells. In order to identify the intracellular vesicles to which pR33-EGFP localized, we stained the cells either with a c marker (Bodipy TR ceramide) or with antibodies directed against ER-specific SERCA proteins. However, as shown in Fig. 1E and F, the EGFP signal does not colocalize with the signals that were generated with these compartment-specific markers. This indicated that pR33-EGFP localizes to intracellular compartments other than ER or trans-Golgi. Nevertheless, other attempts to identify these compartments have not yet been undertaken. Whether the presence of pR33-EGFP in these compartments is the consequence of high level of expression of this protein is not known.

To evaluate the specificity of the constitutive activity of pR33 in the InsP accumulation assay, we compared the activity of this protein with that of two mammalian β-chemokine receptors, CCR3 and CCR10, with which pR33 shares some homology, and a constitutively active viral GPCR, HCMV pUS28. As shown in Fig. 1G, cells transfected with either pcDNA3/R33 or pcDNA3/US28 produced significantly higher levels of InsP than cells transfected with either pcDNA3 (mock) or the mammalian GPCR expression constructs. These data show that constitutive activity of GPCRs in the InsP assay is not a general phenomenon but rather a characteristic of a subset of receptors, as was recognized previously (11).

In summary, we have found pR33-EGFP to colocalize with the cell membrane of transfected COS-7 cells and to induce signaling pathways in an expression level-dependent fashion, leading to the production of InsP. Since both pcDNA3/R33-EGFP- and pcDNA3/R33-transfected cells demonstrate similar activities in the InsP assay, we infer that pR33 is expressed on the surface of transfected cells in a similar fashion as was demonstrated for pR33-EGFP.

pR33 couples to G proteins of the Gq and G16 classes. An intracellular rise in InsP levels is predominantly achieved following activation of PLC by G protein α subunits of the q class (Goq). In addition, PLC activity can be stimulated by free G protein βγ subunits (16). To investigate whether the constitutive rise in InsP accumulation that is seen in cells expressing pR33 and pR33/EGFP is indeed mediated through the interaction of these proteins with heterotrimeric G proteins, COS-7 cells were transfected with both pcDNA3/R33 and constructs expressing either the Gα transducin subunit (Goα), GRK2, or GRK2K220R. Goq is known to sequester βγ subunits, whereas both GRK2 and GRK2K220R not only scavenge βγ subunits but also specifically inhibit Goq activation (10, 20, 40). In addition, in contrast to GRK2K220R, GRK2 can phosphorylate GPCRs, leading to internalization and degradation of the receptors (18). As shown in Fig. 2A, cotransfection of pcDNA3/R33 and pcDNA3/Gαq resulted in a lower level of InsP accumulation (80%) than transfection of pcDNA3/R33 (basal) alone (100%). However, even lower levels of InsP were seen after cotransfection of pcDNA3/R33 and either pcDNA3/GRK2 or pcDNA3/GRK2K220R (25 and 40%, respectively). These InsP levels are similar to those measured in pcDNA3-transfected cells (30%). Together, these results suggest that the InsP production is mediated via coupling of pR33 to βγ as well as Goq subunits. Since COS-7 cells endogenously express only two members of the Gα class, Gαq and G11 (56), it is likely that the pR33-induced PLC activation is mediated by the α subunits of Gαq and/or G11. In order to confirm this interaction of pR33 with the α subunits of Gαq11 and to investigate the putative interaction with another member of the Gαq family, Gα16, which is not expressed endogenously by COS-7 cells, cells were cotransfected with both pcDNA3/R33 and constructs expressing either Goαq, Goα11, or Goα16. As shown in Fig. 2B, cotransfection of pcDNA3/R33 with pcMV5/Goα11 resulted in a higher level of InsP production (145%) than transfection of pcDNA3/R33 alone (100%), whereas cotransfection of pcDNA3/R33 with either pcDNAs/Goαq or pC1H1/Goα16 did not change InsP production levels significantly compared to pcDNA3/R33 alone. These findings suggest that pR33 spe-
cifically activates \( G_{11} \) in a constitutive fashion but does not couple constitutively to \( G_q \) or \( G_{16} \).

Previously, it has been reported that PLC-activating \( \beta\gamma \) subunits can be released by \( G \) proteins of the \( G_{i0} \) class (16). To examine if these \( G_{i0} \) proteins are also involved in pR33-mediated constitutive PLC activation, we incubated pcDNA3- and pcDNA3/R33-transfected cells in the presence of a \( G_{i0} \) inhibitor (PTX), and measured the InsP accumulation. Figure 2A shows that the InsP accumulation level in pcDNA3/R33-transfected cells is significantly lower in the presence of PTX (75%) than in the absence of PTX (100%), indicating that at least part of the pR33-induced InsP production is mediated via \( G_{i0} \) proteins.

pR33-induced inhibition of CRE-mediated transcription.

The interaction of pR33 with \( G \) proteins of the \( G_{i0} \) class was studied in more detail using the CRE reporter assay. This assay allows detection of the inhibitory effect of activated \( G_{i0} \) subunits on adenylate cyclase (AC). The CRE assay is based on
the artificial stimulation of AC with forskolin, which results in an increase of intracellular cAMP levels. The cAMP activates the CRE-binding protein (CREB), which can then bind and activate a CRE that controls transcription of a reporter gene (the luciferase gene). Hence, stimulation of cells carrying the CRE-luciferase gene with forskolin leads to increased production of luciferase transcripts and, subsequently, to higher levels of the luciferase protein, which can be quantified by measuring its activity. As described above, activated G_o/i/o subunits inhibit AC. Consequently, activation of these subunits by a GPCR in the CRE assay will result in lower luciferase levels and, thus, lower luciferase activity. To investigate whether pR33 can activate G_o/i/o subunits, we transfected COS-7 cells with the CRE-luciferase reporter construct and with either pcDNA3, pcDNA3/R33, or pcDNA3/R33-EGFP. Figure 3A shows a plasmid concentration-dependent inhibition of luciferase activity in both pcDNA3/R33- and pcDNA3/R33-EGFP-transfected cells, which indicates that pR33 can indeed constitutively activate G_o/i/o subunits. As expected, this activation can be inhibited by PTX, as shown in Fig. 3B. In order to study the specificity of this activity, we also tested constructs expressing either CCR3, CCR10, or US28 in the CRE assay. However, in contrast to pcDNA3/R33, these constructs did not induce an activity significantly different from that induced by pcDNA3 alone (Fig. 3C).

**pR33-mediated activation of NF-κB- and SRE-driven transcription.** To assess the effect of constitutive pR33 signaling on the NF-κB promoter element and SRE, two additional reporter assays were used. In these assays, plasmid constructs are used in which the luciferase gene is placed under the control of either multiple SREs or the NF-κB promoter element. If receptor signaling induces upregulation of the intracellular concentration of activated NF-κB or serum response factor (SRF), an increase in activity will be measured in the NF-κB and SRE assay, respectively. Figure 4A shows that pcDNA3/R33-transfected cells have a significantly higher activity in the NF-κB assay than pcDNA3-transfected cells. As in the CRE assay, the pR33-induced activity was completely blocked in the presence of PTX (Fig. 4A). A similar observation was made for the activity of pR33-EGFP in the NF-κB assay (data not shown). We conclude that pR33 induces an upregulation of activated NF-κB that is mediated via a G_o/i/o protein-dependent pathway(s). Of the other GPCRs that we tested in the NF-κB assay, only pUS28 was found to be active (Fig. 4A). As described previously, the activation of NF-κB by pUS28 is dependent not on G_o/i/o protein-dependent pathways (since it is unaffected by the addition of PTX) but rather on G_{11} protein-dependent pathways (11).

Figure 4B shows that pcDNA3/R33-transfected cells also have a significantly higher activity in the SRE assay than pcDNA3-transfected cells. Again, this activity as well as the activity of pcDNA3/R33-EGFP-transfected cells (data not shown) was blocked in the presence of PTX, indicating that pR33 induces an upregulation of activated SRF that is mediated via a G_o/i/o protein-dependent pathway(s). A moderate but significant activity in the SRE assay was also measured in cells transfected with constructs expressing pUS28 (Fig. 4B). However, in contrast to the activity of pR33, the activity of pUS28 was not significantly affected by addition of PTX.

**pR33 is unable to bind RANTES.** We previously reported that pR33 shows similarity with CC-chemokine-binding GPCRs (5, 54). In addition, the pR33 homolog from HHV-6B, pU12, was shown to bind CC-chemokine RANTES (24). To test whether RANTES could also bind to and/or modulate the constitutive activity of pR33, we transfected COS-7 cells with pcDNA3/R33 and, as a positive control, pcDNA3/pUS28 and monitored the binding of either 125I-labeled human or rat RANTES by the transfected cells. Although pUS28 was found to bind human and rat RANTES with a moderate affinity (K_D = 0.9 and 15.8 nM, respectively) (Fig. 5B), binding of these chemokines by pR33 was not detected (Fig. 5A). Furthermore, the addition of human or rat RANTES in the culture medium of pcDNA3/R33-transfected COS-7 cells did not modulate the activity of pR33 in the InsP production, NF-κB, and SRE

**FIG. 2.** (A) Effects of G_o transducin subunits, GRKs, and PTX on pR33-mediated InsP accumulation. COS-7 cells were transiently transfected with either pcDNA3 or pcDNA3/R33 in the presence of either pcDNA3/G_o1 (Go1), pcDNA3/GRK2 (GRK2), or pcDNA/GRK2K220R (GRK2K220R) (each construct at 2.0 μg of DNA/10^6 cells). The InsP accumulation was subsequently assessed either in the presence (+) or absence (−) of PTX in the culture medium. (B) Effects of G_o1, G_o16, and G_o11 on pR33-mediated InsP accumulation. COS-7 cells were transiently transfected with either pcDNA3 or pcDNA3/R33 in the presence of either pcDNA3/G_o1, pcC1H1/G_o16, or pcCMV5/G_o11, and the InsP accumulation was assessed after 48 h. Representative experiments performed in triplicate are shown. Each experiment was repeated at least three times. The asterisks indicate statistically significant differences (P < 0.05) between specific results and the result from pcDNA3/R33-transfected cells. Statistically significant differences (P < 0.05) were not found between results from the pcDNA3-transfected cells. Error bars, standard error of the mean.
assays (Fig. 5C). The addition of rat RANTES in the CRE assay resulted in a further inhibition of CRE-mediated transcription in pcDNA3/R33-transfected cells (Fig. 5C). However, a similar inhibitory effect of rat RANTES was seen in pcDNA3-transfected cells (Fig. 5D), indicating that this was not a specific pR33-mediated effect. Taken together, these findings suggest that, unlike HHV-6B pU12, RCMV pR33 does not bind and is not responsive to RANTES. In addition, several human chemokines of the CC (eotaxine, Teck, MCP-1), CX3C (fractalkine), and CXC class (IP-10, IP-9, GRO-α, and interleukin-8 [IL-8]) were not able to change the activity of pR33 significantly in any of the signaling assays used in this study (data not shown).

pR33- and pR33-EGFP-induced stimulation of SRE-mediated transcription in Rat2 cells. In previous experiments, GPCR activity was measured in COS-7 cells, which are well characterized and highly suitable for signal transduction studies. However, since these cells are not likely to represent appropriate hosts for efficient replication of the species-specific RCMV, we set out to test the activity of pR33, pR33-EGFP, and pEGFP-R33 in rat cells (Rat2). These cells have previously been demonstrated to support full replication of RCMV (e.g., see reference 51). We first investigated the expression of pR33-EGFP in Rat2 cells by confocal microscopy and found the expression pattern of this protein to be similar to that in COS-7 cells (data not shown). This suggests that pR33-EGFP may be properly expressed as a functional GPCR in Rat2 cells.

To test whether pR33-EGFP and pR33 are capable of signaling in Rat2 cells, we transfected these cells with either pcDNA3, pcDNA3/R33 (R33), pcDNA3/CCR3 (CCR3), pcDNA3/CCR10 (CCR10), or pcDNA3/US28 (US28) at 0.5 μg of DNA/10⁶ cells. Data are presented as percentages of the result for the control (pcDNA3-transfected cells in the absence of PTX). Representative experiments performed in triplicate are shown, and each experiment was repeated at least three times. An asterisk indicates data that are statistically significantly different (P < 0.05) from that for the control. Error bars, standard error of the mean.

**FIG. 3.** pR33-mediated inhibition of forskolin-induced, CRE-driven luciferase expression. (A) COS-7 cells (10⁶) were transiently transfected with reporter plasmid pTLNC-21CRE (5.0 μg of DNA/10⁶ cells) and with various amounts of either pcDNA3, pcDNA3/R33, or pcDNA3/R33-EGFP (in total, 2.0 μg of DNA/10⁶ cells). When amounts of pcDNA3/R33 or pcDNA3/R33-EGFP lower than 2.0 μg were used, pcDNA3 was added to include a total of 2 μg of pcDNA3-derived plasmid per transfection. At 18 h after transfection, the cells were stimulated with forskolin. Six hours later, the CRE-driven luciferase expression and activity was quantified, and expressed as relative light units (RLU). (B) Influence of PTX on the pR33-mediated inhibition of CRE-driven expression. PTX (100 ng/ml) was added to the culture medium at 2 h after transfection. (C) Influence of various receptors on forskolin-stimulated, CRE-driven expression. The luciferase activity was determined similarly as described above, after transient transfection of COS-7 cells with either pcDNA3, pcDNA3/R33 (R33), pcDNA3/CCR3 (CCR3), pcDNA3/CCR10 (CCR10), or pcDNA3/US28 (US28) at 0.5 μg of DNA/10⁶ cells. Data are presented as percentages of the result for the control (pcDNA3-transfected cells in the absence of PTX). Representative experiments performed in triplicate are shown, and each experiment was repeated at least three times. An asterisk indicates data that are statistically significantly different (P < 0.05) from that for the control. Error bars, standard error of the mean.
FIG. 4. pR33-mediated NF-κB and SRE activation. COS-7 cells (10⁶) were transiently transfected with either reporter plasmid pNF-κB-Luc (A) or pTLNC-3SRE (B) (5.0 μg DNA/10⁶ cells) and with either pcDNA3, pcDNA3/R33 (R33), pcDNA3/CCR3 (CCR3), pcDNA3/CCR10 (CCR10), or pcDNA3/US28 (US28) at 0.5 μg of DNA/10⁶ cells. At 48 h after transfection, luciferase activity was measured. The experiments were carried out either in the presence (+) or absence (−) of PTX in the culture medium. Representative experiments performed in triplicate are shown, and each experiment was repeated at least three times. Single asterisks indicate differences statistically significant (P < 0.05) from data from pcDNA3-transfected cells (in the absence of PTX). Double asterisks (**) indicate statistically significant differences (P < 0.05) between data from PTX-treated and -untreated cells that were transfected with the same expression construct. Error bars, standard error of the mean.

DISCUSSION

The biological significance of the RCMV R33-like genes in the pathogenesis of CMV infections has previously been demonstrated in studies with recombinant viruses (5, 17). In contrast to their WT counterparts, RCMV and MCMV mutant viruses lacking a functional R33 or M33 gene, respectively, were unable to replicate in the salivary glands and displayed lower mortality rates in infected animals (5, 17). Although these findings illustrated the importance of the R33-like genes, not much was known about the biochemical or pharmacological properties of the proteins encoded by these genes. Here, we report that R33 encodes a membrane-associated protein that functions as a GPCR. Furthermore, we demonstrate for the first time that a member of the R33-like gene family encodes a constitutively active receptor that activates multiple signaling pathways in both COS-7 and Rat2 cells.

Currently, the concept of constitutive signaling by GPCRs is well accepted. It has been demonstrated for multiple endogenously expressed GPCRs, such as the H3 receptor in the rat brain (36). Although the biological relevance is still poorly understood, it is clear that constitutive activity can be of major pathophysiological significance. Various diseases, including proliferative disorders, have been described that are caused by GPCRs that are constitutively active due to naturally occurring mutations in these receptors (42, 43, 46, 48).

Previously, two other virus-encoded GPCRs were demonstrated to be constitutively active. The Kaposi’s sarcoma-associated herpesvirus (KSHV) ORF74-encoded GPCR (KSHV-GPCR) and HCMV pUS28 both signal in an agonist-independent fashion, resulting in constitutive activation of various signaling pathways (2, 3, 11, 35, 45, 47). KSHV is thought to be the causative agent of AIDS- and non-AIDS-related Kaposi’s sarcoma as well as Castleman’s disease (12), and the KSHV-GPCR has been proposed as a key mediator of both the proliferative and angiogenic properties of KSHV (2, 3, 12, 28, 35, 45, 47, 57). As described in the introduction, several interesting pUS28 activities have been suggested to be of importance in the pathogenesis of HCMV infections. However, it is still unclear which role constitutive signaling by pUS28 plays in these activities. Similarly, the physiological importance of constitutive pR33 signaling remains speculative.

Both RCMV pR33 and HCMV pUS28 were found to constitutively induce activation of PLC. Upon ligand binding, chemokine receptors also activate PLC, and this is predominantly achieved by release of βγ subunits from activated, PTX-sensitive G proteins, which stimulate the PLC isoform PLC-β2 (30, 50). In addition, some chemokine receptors can activate PLC by direct interaction with the α subunits of Gq class proteins like Gqα, Grq11, Grq14, Grq15, or Grq16 (1, 30, 50). Similarly, the constitutive pR33-induced PLC activation involves Gq, as well as Gαo classes of G proteins, whereas constitutive pUS28-mediated PLC activation is solely dependent on Gq class proteins (11). Additionally, pR33 shows a remarkable level of selectivity for Gq11 over Gαq while pUS28 interacts both with Gαq and Gq11 to enhance PLC activity in COS-7 cells. A wide variety of GPCRs, including chemokine receptors, can couple to PLC via activation of Gq16, a hematopoiesis-specific member of the Gq class (34). Even receptors that selectively couple to Gαq proteins, like the muscarinic acetylcholine M2 receptor, induce InsP₃ production in the presence of PTX when Gq16 is present (34). Surprisingly, pR33-mediated constitutive PLC activation is not enhanced by expression of Gq16. Since GPCR-induced PLC activation via Gq16 has been demonstrated as an agonist-mediated response, it is possible that pR33 might interact with Gq16 only upon ligand binding and not in a ligand-independent fashion. On the other hand, pR33 might not be capable of Gq16 activation at all, as has been shown previously for certain CC-chemokine receptors (30). Nevertheless, we were not able to investigate these notions because at present no ligands for pR33 have been identified.

As indicated above, pR33 constitutively couples to Gq proteins, and this interaction stimulates the pR33-induced PLC activity. Like PLC stimulation by Gq-coupled chemokine receptors, this effect might be mediated by the release of Gq βγ...
subunits, because the pR33-induced PLC activity in the presence of PTX was similar to that seen in the presence of βγ scavengers. Since COS-7 cells lack the PLC isoform that is most sensitive to stimulation by Gi-derived subunits (PLC-2), it is likely that the pR33-induced βγ-mediated signal activates other PLC isoforms expressed in COS-7 cells, i.e., PLC-1 and PLC-3 (16, 27, 56).

In addition to PLC activation, both pR33 and pUS28 also induce NF-κB-mediated transcription in a constitutive fashion. In the case of pUS28, this constitutive effect is due to the activation of Gt proteins via the release of Gtβγ subunits (PLC-β2). In contrast, pR33-mediated NF-κB activity is completely inhibited by PTX, indicating that this activity of pR33 is mainly due to the activation of Gt10 proteins. Similarly, pR33 induces SRE-mediated transcription and inhibits CRE-mediated transcription in a PTX-sensitive, constitutive fashion. These two signaling parameters, however, are not affected by pUS28. Together with the PLC data, these findings illustrate that although pR33 and pUS28 are both virus-encoded receptors which signal constitutively, they are completely different with regard to their respective signaling profiles.

It is likely that the PTX-sensitive, pR33-induced signaling to CRE, SRE, and NF-κB, may be mediated by the Gi2 protein, since COS-7 cells do not endogenously express any other PTX-sensitive G proteins apart from Gi2 (27). Previously, signaling by Gi2 has been demonstrated to be linked to downregulation of cytokine expression (32). Additionally, PTX-sensitive NF-κB-mediated transcription has been shown to influence the expression of cytokines, chemokines, growth factors, and cell adhesion molecules as well as some acute-phase proteins (15). The induction of NF-κB-mediated transcription has also been linked to inflammatory events associated with various disease states, such as autoimmune diseases and atherosclerosis (15). Whether or not (the constitutive activity of) pR33 plays a role in any of these processes during RCMV infection in vivo will have to be addressed in future studies.

A factor that may influence the effect of (constitutive) pR33 signaling in vivo is the presence of endogenous ligands. Interaction of pR33 with endogenous ligands may modulate constitutive activity as well as (de)activate additional signaling pathways. Previously, modulation of constitutive activity by agonists and inverse agonists has been demonstrated for KSHV-GPCR.

FIG. 5. pR33 does not bind rat RANTES. (A) Binding of human and rat RANTES by pUS28 and pR33. COS-7 cells were transiently transfected with either pcDNA3, pcDNA3/US28 (US28), or pcDNA3/R33 (R33). Binding was performed 48 h after transfection on whole cells using 6 nM 125I-labeled human or rat RANTES. Nonspecific binding was determined in the presence of 0.1 μM unlabeled competitor (human or rat RANTES). (B) Human and rat RANTES competition curves on pUS28. COS-7 cells were transiently transfected with pcDNA3/US28 and incubated with 0.6 nM 125I-labeled human RANTES and various amounts of unlabeled human or rat RANTES. (C) Influence of human and rat RANTES on pR33-mediated constitutive signaling. COS-7 cells were transiently transfected with either pcDNA3 or pcDNA3/R33. Human or rat RANTES was added to the supernatants, both in the [3H]inositol phosphate production assay (InsP) and in the reporter gene assays (CRE, NF-κB, and SRE). Data are presented as percentages of pR33 activity (100%). (D) Influence of rat RANTES on pcDNA3- and pcDNA3/R33-transfected cells in the CRE assay. Error bars, standard error of the mean.
and pUS28 in vitro. The constitutive activity of the KSHV-GPCR is modulated positively by GRO-α and negatively by IP-10 and SDF-1α, which are thus acting as agonist and inverse agonists, respectively (23, 35, 44, 45). Nevertheless, not all ligands that bind to a certain GPCR also affect the constitutive activity of that GPCR. This was previously shown for the KSHV-GPCR ligand IL-8 (23, 44). Similarly, pUS28 binds and responds to various CC-chemokines, whereas none of these CC-chemokines have been reported to modulate the constitutive activity of pUS28. By contrast, fractalkine was identified as a partial inverse agonist for pUS28, inhibiting both constitutive PLC activation and NF-κB-mediated transcription (11).

The identification of agonists and, in particular, inverse agonists of constitutive viral GPCR signaling might provide valuable tools for elucidation of the role of this constitutive activity in the pathogenesis of viral infection. In addition, inverse agonists may be regarded as putative leads for antiviral drug design. In order to identify potential (inverse) agonists of pR33, we tested several chemokines for modulation of pR33-mediated constitutive activity. However, neither the human chemokines of the CC (RANTES, etoxeine, Teck, MCP-1), CX3C (fractalkine) or CXC class (IP-10, IP-9, GRO-α, IL-8) nor the CC-chemokine rat RANTES was able to change the activity of pR33 significantly in any of the signaling assays used in this study. In addition, pR33 was unable to bind RANTES, in contrast to its homolog from HHV-6B (pU12). The latter protein was shown to bind RANTES, MCP-1, and MIP-1α as well as MIP-1β, resulting in an intracellular calcium flux (25). These findings illustrate that individual members of the R33 family can have significantly different ligand-binding characteristics.

Taken together, we found pR33 to activate and inhibit various signaling pathways in the absence of exogenously added ligands. However, it cannot be ruled out that the activity of pR33 is the result of binding of yet-unidentified, endogenously produced ligands. In this respect it is interesting that a novel CC-chemokine was identified, CCL28, which is a ligand for both CCR10 and CCR3 (39, 55). This chemokine, also known as mucosa-associated epithelial chemokine, is expressed in human and murine epithelial cells of diverse mucosal tissues and directs chemotaxis of CCR10-expressing lymphoid cells. Interestingly, CCL28 transcripts are most abundant in the salivary glands (39). With respect to the sequence similarity between CCR10, CCR3, and pR33, we hypothesized that the ability of RCMV to establish a successful infection within the salivary glands could be dependent on the interaction of a CCL28-like chemokine with pR33 (54). This putative interaction of pR33 with the rat homolog of CCL28 is currently under investigation.

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