Constitutive Signaling of the Human Cytomegalovirus-encoded Receptor UL33 Differs from That of Its Rat Cytomegalovirus Homolog R33 by Promiscuous Activation of G Proteins of the G_q, G_i, and G_s Classes*

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The human cytomegalovirus (HCMV) UL33 gene is conserved among all β-herpesviruses and encodes a protein that shows sequence similarity with chemokine receptors belonging to the family of G protein-coupled receptors. Here, we show that HCMV UL33 is predominantly transcribed as a spliced mRNA of which the 5' terminus is localized 55 bp upstream of the start codon. Like its homolog from rat cytomegalovirus (RCMV), R33, UL33 activates multiple signaling pathways in a ligand-independent manner. Although both receptors constitutively activate phospholipase C via Gi/o, and partially via Gi/o-mediated pathways, they exhibit profound differences in the modulation of cAMP-responsive element (CRE) activation. R33 constitutively inhibits, whereas UL33 constitutively enhances CRE-mediated transcription. For R33, the inhibition of CRE-driven transcription is entirely Gi/o-mediated. For UL33, however, CRE-mediated transcription is modulated not only through coupling to Gi/o, but also through coupling to Gq. In addition, UL33 was found to enhance CRE activation through the Rho/p38 pathway, via Gpγ. Interestingly, by studying chimeric UL33/R33 proteins, we found the C-terminal cytoplasmic tail of UL33, but not that of R33, to be responsible for the activation of Gi/o proteins. A UL33-deficient variant of HCMV was generated to analyze UL33-signaling properties in a physiologically relevant model system. Data obtained with infected cells show that HCMV induces CRE activation, and this effect is, at least in part, dependent on UL33 expression. Taken together, our data indicate that constitutive signaling of UL33 differs from that of R33 by promiscuous activation of G proteins of the G_q, Gi/o as well as G_s class. Thus, HCMV may effectively use UL33 to orchestrate multiple signaling networks within infected cells.

A large number of viruses appear to have pirated genes encoding key regulatory cellular proteins (1). It is likely that these genes play important roles in strategies that are aimed at the subversion of antiviral challenges by the immune system of the viral host. One of the most prominent examples of such strategies is employed by representatives from the β- and γ-herpesvirus subfamilies. Sequence analysis of the genomes of human cytomegalovirus (HCMV),1 Kaposi's sarcoma-associated herpes virus (KSHV), and human herpesvirus 6 and 7 (HHV-6 and HHV-7) revealed the existence of genes encoding proteins with high homology to chemokine receptors, belonging to the family of G protein-coupled receptors (GPCRs) (reviewed in Ref. 2). Because GPCRs play a crucial role in cellular communication, and chemokine receptors in particular are essential for leukocyte trafficking, the virus-encoded GPCRs (vGPCRs) may be crucial determinants of viral pathogenesis. Expression of vGPCRs may play a prominent role in immune evasion, promote virus dissemination, or modulate cellular responses of infected cells.

The β-herpesvirus HCMV can cause life-threatening systemic infections in immunocompromised individuals and has also been recognized as a risk factor for vascular diseases, like arterial restenosis and atherosclerosis (3). Within the HCMV genome, four genes encoding GPCRs have previously been identified (US27, US28, UL33, and UL78) (4). Recently, it has been suggested that the HCMV genome may contain additional putative GPCR genes (5). The US28-encoded receptor has been characterized most extensively and was shown to bind CC chemokines and the CX3C chemokine fractalkine (6, 7). In addition, we and others have recently shown that US28 signals in a constitutive manner in both transfected and HCMV-infected cells (8–10), suggesting a physiological relevance of this property.

1 The abbreviations used are: HCMV, human cytomegalovirus; KSHV, Kaposi’s sarcoma-associated herpes virus; HHV, human herpesvirus; GPCR, G protein-coupled receptor; vGPCR, virus-encoded GPCR; CMV, cytomegalovirus; RCMV, rat CMV; MCMV, mouse CMV; ORF, open reading frame; EGF, enhanced green fluorescence protein; PTX, pertussis toxin; HEL, human embryonic lung fibroblast; RACE, rapid amplification of cDNA ends; BAC, bacterial artificial chromosome, HFF, human foreskin fibroblast; m.o.i., multiplicity of infection; CRE, cAMP-responsive element; TFR, transferrin receptor; InsP, inositol phosphate; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MER, MAPK/extracellular signal-regulated kinase kinase; PLC, phospholipase C; RLU, relative light unit.

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For the other three HCMV-encoded GPCR-like genes, little information is available. The UL33 gene has homologs in all β-herpesviruses, including rat CMV (RCMV) M33, murine CMV (MCMV) M33, and the U12 genes of HHV-6A, HHV-6B, and HHV-7, which may illustrate the biological significance of this gene family (reviewed in Ref. 11). The UL33, M33, and R33 genes were found to be dispensable for viral growth in vitro (12–14). However, the biological significance of the UL33 family members has been demonstrated in studies in vitro. Recombinant RCMV and MCMV strains lacking a functional R33 or M33 gene, respectively, are unable to replicate in the salivary glands and induce a lower mortality in infected animals (13, 14). These results underline the importance of the UL33-like genes in the pathogenesis of infection.

On the basis of sequence alignments, it was suggested that the UL33 gene family members of MCMV, HCMV, HHV-6, and HHV-7 may express spliced mRNAs (13). Indeed, transcripts of M33, UL33, as well as HHV-6B U12 were demonstrated to be spliced (13, 15), although a previous report suggested that transcripts of UL33 were not spliced (12). The RCMV R33 gene, on the other hand, was found to be transcribed into an unspliced mRNA (14).

We have previously demonstrated that the RCMV R33 gene, like US28, encodes a constitutively active receptor, modulating multiple signaling pathways (16). Also, the MCMV and HCMV homologs of M33, R33, and UL33, respectively, were shown to display constitutive activity (17). However, a detailed analysis of the signaling pathways underlying the observed constitutive activity displayed by UL33 has not been performed. As yet, no chemokines were reported to bind or modulate the activity of UL33, R33, or M33 (16, 17). Therefore, these proteins should be regarded as orphan receptors.

In this study, we compared the pharmacological behavior of the HCMV-encoded UL33 with that of its RCMV homolog R33. Because only limited information is available on the UL33 gene and protein, transcriptional analysis was performed in HCMV-infected cells, and signaling pathways activated by UL33 have been extensively delineated. Like other vGPCRs, UL33 shows a pharmacology similar to that of other members of the GPCR family. In this context, we have postulated that the UL33 gene, like US28, encodes a constitutively active receptor, modulating multiple signaling pathways (16). This is in agreement with the findings that UL33 is a constitutively active receptor and that UL33 shows a signaling profile similar to that of other vGPCRs (17, 18). Therefore, the UL33 gene family may be involved in the regulation of signaling pathways activated by UL33.

Materials and Methods

DNA Constructs—Plasmid pcDNA3/UL33(s) (gift from Dr. B. Margulies), contains the partial coding sequence of the HCMV strain AD169 UL33 gene (corresponding to position 43,253–44,425 of the HCMV AD169 genome sequence, GenBank™ data base accession number NC-001347) (12). Within plasmid pcDNA3/UL33(s), the UL33 open reading frame (ORF) starts with an ATG codon downstream of the UL33 intron. Thus, this plasmid encodes a short α version of UL33, which is truncated at its N terminus. The expression vector pcDNA3/UL33, which contains the full-length coding UL33 cDNA sequence (13), was generated as follows. First, PCR amplification was performed of the UL33 5′ coding region corresponding to positions 43,066–43,089 and 43,211–43,394 of the HCMV AD169 genome (13) using HCMV AD169 cdNA as template and primers 5'-catcactctctagggggcttc-3' (the sequence in bold type is complementary to the sequence corresponding to position 43,377–43,394 of the HCMV genome; the underlined sequence indicates the position of the BamHI site) and 5'-gagatcagctgtagggcctgc-3' (the sequence in bold type is complementary to the sequence corresponding to position 43,061–43,077 of the HCMV genome; the underlined sequence indicates the position of the SfiI site). The resulting 220-bp PCR product was then digested with BamHI and Asp718, and subsequently used to replace the BamHI-Asp718 fragment containing the 5′ coding region of UL33(s) in plasmid pcDNA3/UL33(s), thus resulting in expression vector pcDNA3/UL33.
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ng/ml). After 24 h, selected samples were stimulated with forskolin (1 μM) for a period of 6 h. Incubation was stopped by aspiration of the medium, and cells were assayed for luminescence as described previously. Luminescence was measured for 3 s in a Wallac Victor² multialarm plate reader (PerkinElmer Life Sciences, Boston, MA).

[H]Inositol Phosphate Production—Experiments in COS-7 cells were performed as previously described (8).

BAC Mutagenesis—Recombinant UL33-deficient HCMV genome was generated in E. coli using a recently established method that relies on homologous recombination of a linear PCR fragment with the HCMV BAC plasmid (21–23). The PCR product was generated with the primer pair prim5–UL33 5′-CCG ACC CCA GCA ACA ACA TTC TCT CTC CGC ACA TCA ATG ACA CTT GCA ACC GTC GTG GAA TGC CCT CCG ACC GAA TAA GTG CTC AGG AAA GCA CAA GGA CGA CGA CGA CGA GTA A-3′ (sequences homologous to the pSL-FRTKn plasmid are underlined). The amplicon contained homologies of 50 bp upstream or downstream of the positions of the deleted UL33 ORF using the plasmid pSL-FRTKn (21–23) (kindly provided by Dr. M. Wagner, Munich) as template DNA. After purification and DpnI digestion (21) this PCR fragment was inserted into pHB5 (23) by homologous recombination in E. coli, which was mediated by the recombination plasmid pKD46 leading to deletion of the native UL33 start and stop codons and the insertion of a kanamycin cassette that is flanked by Flp recombination target sites. The kanamycin gene was excised via the flanking Flp recombination target sites by Flp-mediated recombination as described previously (21, 22, 23) generating the HCMV BAC plasmid pUL33del. BAC DNA pUL33del was isolated from E. coli cultures with an alkaline lysis procedure (24) and purified with NucleoBond PC 500 columns (Machery-Nagel, Düren, Germany). Correct mutagenesis was confirmed by PCR analysis using the primer pair UL33ATG 5′-CCG ACA TTC ATG ACA CTT GCA ACC GTC GTG GAA TGC CCT CCG ACC GAA TAA GTG CTC AGG AAA GCA CAA GGA CGA CGA CGA GTA A-3′ and 30 bp upstream or downstream of the positions of the deleted UL33 ORF using the plasmid pSL-FRTKn (21–23).

Reconstitution of UL33-deficient HCMV—Mutant viruses were reconstituted by transfection of BAC DNA pUL33del into human foreskin fibroblasts (HFF) with the SuperFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Briefly, 5 × 10⁵ fibroblasts were incubated with 150 μl of medium without serum, and 30 μl of SuperFect transfection reagent. The mixture was added to 3 × 10⁶ HFF cells. Cells were washed with phosphate-buffered saline 2 h later, cultured with fresh medium. Cells were passaged 7 days after transfection. Infectious supernatants were harvested when 100% of cells showed cytopathic effects. Virus stocks were prepared on HFF. All virus titers were determined by standard assays. DNA of reconstituted virus mutants was analyzed in parallel by Southern blotting and PCR to prove the correct deletions of the UL33 gene.

Viral Nucleic Acid Isolation and Analysis—Fibroblasts were infected at an m.o.i. of 1, harvested 3 days post infection when cultures reached 100% cytopathic effect, and collected by centrifugation. Cells were lysed in a solution containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% SDS. Proteinase K digestion (500 μg/ml) was performed for 3 h at 56°C. Total DNA was extracted with phenol-chloroform and precipitated with isopropanol. Southern blot analysis was performed as described previously (25).

CRE Activation in HCMV-infected Cells—U373 cells were plated in six-well plates (~250,000 cells/well) and transiently transfected with the reporter gene pTLCN-21CRE with the calcium phosphate method. 16 h later, cells were infected with HCMV (strain AD169) or AD169-ΔUL33 at an m.o.i. of 3. At different time points after infection, cells were lysed and assayed for luciferase activity (8).

Data Analysis—Data were analyzed using the program Prism (GraphPad Software, Inc., San Diego, CA). Data are expressed as mean ± S.E.

RESULTS

Transcriptional Analysis of UL33 in HCMV-infected Cells—Although HCMV UL33 was originally thought to express an unspliced mRNA (12), Davis-Poynter and coworkers demonstrated that UL33 is transcribed as a spliced mRNA (13) (Fig. 1A). As a consequence, the UL33 gene encodes a protein that is 22 amino acids longer than the protein that was previously predicted to be encoded by UL33 (12). Nevertheless, it has not yet been established whether UL33 can also be transcribed into unspliced mRNAs, potentially coding for alternative protein products. To investigate this possibility, fibroblasts were infected with HCMV AD169. After purification of mRNA from the cells, the 5′ termini of the UL33 transcripts were assessed by rapid amplification of cDNA ends (RACE). First, the integrity of the cDNA samples was checked by using primers specific for the human transferrin receptor (TFR). As expected, the TFR-control 5′-RACE reaction produced a major DNA fragment of 2.3 kb (Fig. 1B, lane 1). Subsequently, a 5′-RACE reaction was performed using the UL33-specific antisense primer. This reaction resulted in the amplification of a major DNA species with a length of 0.9 kb (Fig. 1B, lane 2). Several minor, smaller fragments were also observed in this sample. To determine the sequence of these fragments, a portion of the 5′-RACE sample was used for subcloning into a sequencing vector. Plasmids purified from 15 independent bacterial colonies were sequenced. The resulting sequences were compared with those of the GenBank™ nucleotide sequence data base. This comparison indicated that all 15 clones contained UL33-specific sequences.

The length of the insert in 8 out of 15 clones corresponded to that of the major, 0.9-kb PCR product in Fig. 1B (lane 2). Each of these inserts was found to be the product of splicing, similarly as described previously (13). Out of these eight clones, seven were found to have the same 5′ cDNA end. This end was localized 55 bp upstream of the UL33 start codon (Fig. 1C), whereas one of these eight terminated at 31 bp upstream of the first start codon (Fig. 1C). The inserts from the other seven out of 15 clones were likely derived from the minor PCR products seen in Fig. 1B (lane 2), because these were all smaller than 0.9 kb. The 5′ cDNA ends of two of these clones were at 22 and 3 bp, respectively, upstream of the “second” UL33 ATG codon, localized downstream of the intron sequence (Fig. 1C). The 5′ cDNA termini of the other five clones were at significantly larger distances downstream from the second ATG codon (at 140, 200, 258, 259, and 283 bp, respectively).

The differences in structure of the last seven cDNA clones suggest that the corresponding cDNA fragments were the result of amplification of either truncated transcripts or truncated first-strand cDNA species. Given the relative abundance of the 0.9-kb 5′-RACE product, which constitutes ~85% of the total population of amplified products (Fig. 1B, lane 2), we conclude that HCMV UL33 predominantly encodes a spliced transcript of which the 5′ terminus is localized 55 bp upstream of the start codon within UL33 exon 1. Because cDNA corresponding to unspliced UL33 transcripts were not detected, we conclude that such mRNA species do not play a role in HCMV infection. For completeness of the study, however, a plasmid containing the cDNA corresponding to the unspliced UL33 ORF variant, designated UL33(s), was included in the signal transduction assays.

Expression of and Constitutive Signaling by UL33, UL33(s), and R33—To investigate the signaling properties of UL33, we transfected COS-7 cells with cDNAs coding for full-length UL33 and for the unspliced, shorter variant, UL33(s), which lacks the N-terminal 22 amino acids. The RCMV homolog of UL33, R33, previously found to display constitutive activity (16), was used in the different assays for comparison.

Transfection of increasing amounts of DNA encoding UL33 in COS-7 cells was accompanied by an agonist-independent increase in [3H]inositol phosphates (InsPs) production, comparable to that observed for R33 (Fig. 2A). In contrast to the observed constitutive activity displayed by UL33, UL33(s) did not induce an increase in InsP production upon transfection of either 2 μg (Fig. 2A) or 5 μg (data not shown) cDNA. To monitor the expression of UL33 and UL33(s), both proteins were tagged C-terminally with EGFP. These proteins, UL33-EGFP and UL33(s)-EGFP, displayed similar signaling activities as their native counterparts (data not shown). Their expression, as well as that of EGFP and R33-EGFP, was studied by confocal mi-
crossscopy of transfected cells. Fig. 3A shows that the 
fluorescence within cells expressing native EGFP is seen dispersed throughout the nucleus and cytoplasm. However, in cells expressing either UL33-EGFP or R33-EGFP, the fluorescent signal clearly co-localized with the cell membrane as well as with intracellular, perinuclear vesicles (Fig. 3, B and C). This indicates that UL33-EGFP, like R33-EGFP, is properly expressed on the cell surface of transfected cells. By contrast, the fluorescence in cells expressing UL33(s)-EGFP did not co-localize with the cell membrane, and seemed to be confined to intracellular compartments (Fig. 3D). This observation shows that UL33(s)-EGFP is retained intracellularly and thus explains the observed lack of constitutive signaling for UL33(s).

UL33-mediated Inositol Phosphate Accumulation Is a G_{q/11} and Partially G_{i/o}-dependent Process—Accumulation of InsP can be achieved following activation of phospholipase C by Ga subunits of the G_{q/11} family as well as by G_{i/o} subunits (26). Previously, we have shown that the R33-mediated increase in InsP accumulation is partially mediated by the G_{i/o} subunits of activated G_{i/o} proteins (16). Also for UL33, the observed increase in InsP production can be partially attributed to involvement of G_{i/o} proteins. Incubation of cells expressing UL33 with pertussis toxin (PTX) (24 h, 80 ng/ml) led to a 40 ± 5% (n = 6) decrease of InsP production (Fig. 2, A and B). Co-expression of UL33 and G_{a_{16}}, known to sequester G_{i/o} subunits, resulted in a 43 ± 6% (n = 2) attenuation of InsP production, similar to that observed for PTX treatment (Fig. 2B). PTX treatment did not further abrogate the InsP production in cells co-expressing UL33 and G_{a_{16}}, suggesting that G_{i/o} subunits from G_{i/o} are in part responsible for the observed increase in InsP production.

The remaining increase in InsP production may be ascribed to the involvement of Ga proteins of in particular the G_{q/11} family. Co-expression of UL33 with either G_{a_{q}} or G_{a_{11}} proteins enhanced the UL33-mediated production of [3H]InsP (196 ± 14% and 144 ± 4% of UL33 basal signaling, respectively, n = 3; Fig. 2C). The contribution of both G_{a_{q/11}} as well as G_{i/o} subunits of the G_{i/o} family in the UL33-mediated accumulation of InsP production was further corroborated by use of the kinase-deficient GRK2-K220R mutant (27). This mutant has been reported to scavenge both G_{a_{q/11}} via interaction with its RGS domain and G_{i/o} subunits via binding to its pleckstrin homology domain (27). As anticipated, co-expression of the kinase-deficient GRK2K220R mutant resulted in a complete inhibition of UL33-mediated InsP production (n = 3; Fig. 2B).

G Proteins Involved in UL33-mediated Constitutive Modulation of CRE—Although R33 and UL33 both constitutively activate phospholipase C, these receptors differentially regulate CRE-mediated transcription. Expression of UL33 resulted in a concentration-dependent increase in CRE-mediated transcription (Fig. 4A). As expected, UL33(s) did not modulate CRE activity. R33, however, inhibited the forskolin-induced CRE transcription in a concentration-dependent fashion (Fig. 4B). Because basal cAMP levels are low in COS-7 cells, forskolin, known to activate adenylyl cyclase, was used to allow detection of inhibitory signaling to CRE (28). This decrease in R33-mediated CRE activation can be ascribed to involvement of G_{i/o} proteins, because PTX markedly reversed the R33-induced in-
Inhibition of forskolin-mediated CRE transcription. Interestingly, inactivation of G\textsubscript{i,o} by PTX in UL33-expressing cells led to a potentiation of the basal increase in CRE transcription (Fig. 4A), indicating that the UL33-mediated stimulation of CRE transcription is the result of the activation of both inhibitory and stimulatory pathways that converge at the level of CRE. The observed inhibition of UL33-mediated signaling to CRE can be attributed to coupling of the receptor to either G\textsubscript{i1} or G\textsubscript{i3}, as co-expression of the respective subunits resulted in a complete inhibition of UL33-induced signaling (Fig. 5A). Co-expression of G\textsubscript{o} or G\textsubscript{i1,2} had no effect on UL33-mediated signaling, suggesting selectivity of coupling for UL33 within this class of proteins. All used G\textsubscript{i,o} constructs are PTX-insensitive (via a mutation of Cys\textsuperscript{351/2} to Gly), and consequently, no increase was observed upon PTX treatment for G\textsubscript{i1} or G\textsubscript{i3}. Similarly, R33 showed preferential coupling to G\textsubscript{i1} and G\textsubscript{i3}.

**Fig. 2. UL33 and R33 induction of inositol phosphate accumulation.** A, COS-7 cells (1 \times 10\textsuperscript{6} cells) were transiently transfected with increasing amounts of cDNA encoding UL33, 2 \mu g of UL33(s), and 2 \mu g of R33. Cells were incubated in the presence (white bars) or absence (black bars) of PTX (80 ng/ml). 48 h after transfection InsP accumulation was measured. B, COS-7 cells were transiently transfected with empty vector (mock) or UL33 (1 \mu g/10\textsuperscript{6} cells) in the presence of either G\textsubscript{o} transducin or the kinase-deficient GRK2-K220R (1 \mu g/10\textsuperscript{6} cells). Cells were incubated in the presence (white bars) or absence (black bars) of PTX (80 ng/ml). 48 h after transfection InsP accumulation was measured. C, COS-7 cells were transiently transfected with UL33 (1 \mu g/10\textsuperscript{6} cells) together with cDNAs encoding G\textsubscript{o} or G\textsubscript{i1} subunits (1 \mu g/10\textsuperscript{6} cells); 48 h after transfection InsP accumulation was measured. Data are presented as percentages of UL33-mediated response, defined as absolute increase of UL33-mediated InsP accumulation above values obtained for mock transfected cells.
(Fig. 6A). All Ga\(_{i/o}\) subunits were previously shown to be properly expressed and able to signal in the presence of the adenosine A\(_1\) receptor (29) or the histamine H\(_3\) receptor.\(^2\)

To understand which other G proteins might be involved in UL33-mediated CRE activation, another panel of Ga subunits was tested. Expression of the different Ga subunits did not significantly alter the basal or forskolin-induced CRE transcription in mock cells (data not shown). Co-expression of Ga\(_{16}\), Ga\(_{12}\), and Ga\(_{13}\), did not modulate the constitutive signaling induced by UL33 (Fig. 5B). On the other hand, co-expression of Ga\(_{i/o}\), for which coupling to UL33 was already shown in the InsP

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\(^2\) P. Casarosa, G. Bongers, R. Leurs, and M. J. Smit, unpublished observations.
To investigate whether activation of Gα/H9251 subunits of the q/11 class might be responsible for a negative regulation of CRE-mediated transcription, the CRE-reporter gene was co-transfected with a constitutively active Gα/H925111 subunit (Gα/H925111-Q209L; Gα/H925111*). Expression of Gα/H925111* did not alter basal or forskolin-induced activation of CRE when compared with mock transfected cells (data not shown). Similarly, activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA, 300 nM) did not induce any change in the transcriptional activation of CRE (data not shown). Taken together, these data imply that activation of the Gq/11-PKC signaling pathway is not modulating CRE activation.

Interestingly, co-expression of Gαs increased UL33-mediated signaling to CRE (204 ± 9% of UL33 basal signaling, n = 6, Fig. 5B), suggesting that UL33 coupling to Gαs is, at least in part, responsible for CRE activation. As a control, the effect of Gαs was monitored in R33-transfected cells. Only a minor increase in CRE activation was detected for both mock and R33-transfected cells (data not shown). Similarly, activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA, 300 nM) did not induce any change in the transcriptional activation of CRE (data not shown). Taken together, these data imply that activation of the Gq/11-PKC signaling pathway is not modulating CRE activation.

Additional Downstream Signaling Components Involved in UL33 Signaling to CRE—CRE transcription is known to be regulated by elevation of cAMP following Gαs activation, leading to activation of protein kinase A (PKA), which subsequently phosphorylates CREB, initiating CRE transcription (30). However, besides PKA also other signaling entities, including, e.g. PKC, MAPKs (p44/p42, p38, and JNK), and small G proteins, are known to regulate CRE transcription in a tissue-specific manner (30). UL33-mediated increase in CRE transcription is not mediated via p44/p42 MAPK, phosphoinositide 3-kinase, or protein kinase C signaling pathways as their respective specific inhibitors U0126 (MEK inhibitor), wortmannin (phosphoinositide 3-kinase inhibitor), and bisindolylmaleimide (PKC inhibi
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Fig. 6. Effect of various Go subunits on the R33-mediated CRE activation. A, COS-7 cells were transfected with pTLNC-21CRE and either pcDNA3 (mock) or R33 (1 µg/10⁶ cells), together with cDNAs encoding the indicated Go subunits (1 µg/10⁶ cells). Cells were incubated in the presence (white bars) or absence (black bars) of PTX (80 ng/ml). After 24 h, cells were stimulated with forskolin (1 µM) for additional 6 h, then CRE-driven luciferase expression was measured. Data are presented as relative light units (RLUs). B, COS-7 cells were transfected with pTLNC-21CRE and either pcDNA3 (mock) or R33 (1 µg/10⁶ cells), together with cDNA encoding the Go subunit (1 µg/10⁶ cells). Cells were incubated in the presence (white bars) or absence (black bars) of PTX (80 ng/ml). After 24 h, cells were stimulated with forskolin (1 µM) for additional 6 h, then CRE-driven luciferase expression was measured. Data are presented as relative light units (RLUs).

tor) did not abrogate the UL33-induced signaling to CRE (Fig. 7A). These inhibitors, however, do inhibit signaling of the KSHV-encoded receptor ORF74 to p44/p42 MAPK in COS-7 cells, indicating proper effectiveness (31). The specific inhibitor of p38, SB203580, on the other hand, markedly inhibited the UL33 effect (43 ± 5% inhibition of UL33 basal signaling, n = 3; Fig. 7A).

Upstream activators of p38 are, among others, small G proteins of the Rho family (32, 33). Co-expression of C3 exoenzyme, which is known to inactivate these G proteins (by ADP-ribosylation of Asn⁴¹) (34), resulted in a marked inhibition of UL33-induced activation of CRE transcription (40 ± 3% inhibition of UL33 basal signaling, n = 3), similar to that obtained with the p38 inhibitor SB203580 (Fig. 7B). Co-expression of C3 exoenzyme or treatment with the p38 inhibitor SB203580 did not alter R33 signaling (data not shown). These data suggest that UL33, differently from R33, engages the Rho/p38 pathway in its signaling to CRE (Fig. 7B).

To examine whether the Goα and Rho pathway are connected, we determined the effect of the C3 toxin on Goα-mediated signaling. Co-expression of C3 exoenzyme did not abrogate the increase in CRE transcription induced by expression of constitutively active Goα (Goα/R201E; Goα*), indicating that the Goα and Rho pathway do not coincide (Fig. 7C).

Next, we investigated which upstream signaling components were involved in UL33-mediated activation of Rho. Goα₁₂ and Goα₁₁, known to activate Rho (35), did not play a role in the UL33-mediated increase in CRE transcription, as can be seen in Fig. 5B. It has previously been reported that also Gβγ subunits can activate small GTPases of the Rho family, resulting in activation of p38 (36). Consequently we determined the role of Gβγ subunits in UL33-mediated activation of CRE by co-expression of Goα. As can be seen in Fig. 7 (B and C), Goα markedly attenuated the UL33-induced transcription of CRE (49 ± 5% inhibition of UL33 basal signaling, n = 3), while not affecting Goα* signaling, used as a control. Co-expression of C3 toxin and Goα did not lead to a further decrease in UL33-mediated signaling, suggesting that Gβγ subunits, through activation of the Rho/p38 pathway, may lead to activation of CRE-mediated transcription (Fig. 7B).

Signaling by UL33 and R33 Chimeric Proteins—Because the C-terminal tail is an important determinant of receptor signaling in general, and UL33 and R33 exhibit marked differences in their C-tails, we constructed receptor chimeras in which their C-tails were either partially or completely exchanged (Fig. 3E). Exchange of the entire C-terminal tails was made at a conserved tyrosine residue at positions 306 and 305 in UL33 and R33, respectively (R33-Y-UL33 and UL33-Y-R33), and the partial exchange of the C terminus was made at a conserved arginine residue at positions 328 and 327 in UL33 and R33, respectively (R33-R-UL33 and UL33-R-R33). To confirm proper expression of the chimeric receptors, we also generated C-terminal EGFP-tagged variants of these proteins, which were studied by confocal microscopy. As shown in Fig. 3 (F, H, and I), the fluorescence patterns of the EGFP-tagged chimeras, R33-R-UL33-EGFP, UL33-R-R33-EGFP, and UL33-Y-R33-EGFP were similar to that seen for UL33-EGFP and R33-EGFP, showing co-localization with the cell membrane of transfected cells as well as with intracellular vesicles. However, in cells expressing R33-Y-UL33-EGFP, fluorescence was seen exclusively within intracellular compartments (Fig. 3G), indicating that R33-Y-UL33-EGFP is not properly delivered to the cell membrane.

Subsequently, the chimeric proteins were tested in the various signaling assays. In each of these assays, the activity of the chimeric receptors was similar to that of their EGFP-fused counterparts (data not shown). Fig. 8A shows that, in comparison with UL33, UL33-R-R33 is impaired in activation of Goα proteins, leading to an increased basal level of CRE activation (Fig. 8A) as well as an impaired accumulation of InsP (Fig. 8C). This impairment is even more pronounced for the chimera UL33-Y-R33, which appears to be completely incapable of Goα coupling. In agreement with these observations, UL33-R-R33 showed a lower sensitivity to PTX than UL33, whereas the activity of UL33-Y-R33 was not significantly influenced by PTX.

The activity of R33-R-UL33, both in the CRE reporter gene assay (Fig. 8B) and in the InsP assay (Fig. 8D), was not signif-
significantly different from that of R33. By contrast, replacement of the entire C-terminal tail of R33 by that of UL33 resulted in a mutant (R33-Y-UL33) that did not display activity in any of the assays used (Fig. 8, B and D). As shown above (Fig. 3G), this lack of activity can be attributed to the inability of R33-Y-UL33 to be expressed on the cell membrane.

Fig. 7. Additional pathways involved in the UL33-mediated CRE activation. A, COS-7 cells were transfected with pTLNC-21CRE and UL33 (1 μg/10⁶ cells). Cells were incubated in the presence of different specific inhibitors (purchased from Calbiochem, San Diego, CA): wortmannin (phosphatidylinositol 3-kinase inhibitor; 100 nM), SB203580 (p38 inhibitor; 2 μM), UO126 (MEK inhibitor; 1 μM), and bisindolylmaleimide (PKC inhibitor; 100 nM). One day after transfection, CRE-driven luciferase expression was measured. Data are presented as percentages of UL33-mediated response. B, COS-7 cells were transfected with pTLNC-21CRE and UL33 (1 μg/10⁶ cells), together with combinations of cDNAs encoding Gαs transducin and C3 toxin (1 μg/10⁶ cells for each construct). One day after transfection, CRE-driven luciferase expression was measured. Data are presented as percentages of UL33-mediated response. C, COS-7 cells were transfected with pTLNC-21CRE and either pcDNA3 (mock) or Gαs* (corresponding to Gαs-R201E, constitutively active mutant; 1 μg/10⁶ cells), together with cDNAs encoding Gα transducin or C3 toxin (1 μg/10⁶ cells). One day after transfection CRE-driven luciferase expression was measured. Data are presented as relative light units (RLUs).
Construction of the Deletion Mutant HCMV AD169-\textit{ΔUL33}—To analyze the function of the UL33 protein in HCMV-infected cells, a \textit{UL33}-deficient variant of HCMV AD169 (AD169-\textit{ΔUL33}) was generated by mutagenesis of the AD169 genome as infectious BAC in \textit{E. coli}.

To this end, nearly the entire coding region of UL33 was deleted (Fig. 9A). The recombinant virus AD169-\textit{ΔUL33} was reconstituted in fibroblasts, as described under “Material and Methods.”

To confirm the deletion of UL33 ORF in the mutant genome, Southern blot analysis was performed. Viral DNA was extracted from fibroblasts infected either with HCMV AD169 or AD169-\textit{ΔUL33}, digested, separated, and hybridized with the entire UL33 coding region, used as probe. As can be seen in Fig. 9B, no signal was detected for the mutant strain, proving that the UL33 gene has been deleted. As a positive control, the integrity of the \textit{US28} and the \textit{UL78} ORFs was checked with the respective probes (Fig. 9B). No differences could be detected in the fragment length of both regions.

Propagation of the recombinant AD169-\textit{ΔUL33} virus also revealed that the UL33 gene product is not essential for viral growth in tissue culture, because the titers of wild-type and mutant virus stocks produced by infection of fibroblasts were identical (Fig. 9C).

Analysis of UL33-mediated Signaling in HCMV-infected Cells—We next investigated whether UL33 is capable of acti-
Fibroblasts were infected at an m.o.i. of 0.01 with either wild-type HCMV AD169 or AD169-H9004 BamHI for UL78 in the culture medium obtained with AD169 (was extracted from cells infected with either wild-type HCMV AD169 or AD169-

U373 cells were transiently transfected with the reporter plasmid pTLNC-21CRE and subsequently infected with wild-type HCMV AD169, FSK samples of mock infected cells were stimulated with forskolin (indicated as *numbering corresponding to EMBL/GenBankTM accession no. X17403 is shown.

RLUs relative light units ( was obtained in transfected COS-7 cells. Most interestingly, we found a marked increase of CRE activation in HCMV-infected cells, as suggested by results obtained in transfected COS-7 cells.

To this end, U373 cells were transiently transfected with the reporter-gene pTLNC-21CRE and subsequently infected with HCMV AD169 or AD169-ΔUL33 or mock infected. CRE activation was measured at 6, 24, and 48 h post-infection (Fig. 9, D–F). As internal control for transfection, some samples of mock infected cells were stimulated with forskolin (10 μM) for the last 6 h before each read-out. Forskolin-induced CRE activation was comparable at the different time points (∼60,000 relative light units), suggesting that no major changes in transfection efficiency among the different time points are present.

Most interestingly, we found a marked increase of CRE activation in AD169-infected cells compared with mock infected cells (Fig. 9, D–F). Virus-induced CRE activation is already present at the early time point (12 ± 1-fold over mock infected cells; Fig. 9D) and is further increased at later time points (430 ± 50- and 318 ± 20-fold over mock infected cells at 24 and 48 h, respectively; Fig. 9, E and F). Importantly, cells infected with AD169-ΔUL33 showed a lower activation of CRE at each time point examined (Fig. 9, D–F): the residual CRE activation is 47 ± 3%, 62 ± 1%, and 45 ± 5% of WT-HCMV-induced signaling at 6, 24, and 48 h after infection, respectively. These data strongly imply that UL33 is at least in part responsible for virus-induced CRE activation.

**DISCUSSION**

Viruses have evolved various ways to alter intracellular signaling pathways (1). By means of expression of pirated GPCRs, viruses are suggested to coordinate and regulate cellular signal transduction both spatially and temporally to enhance the degree of signal specificity according to its own needs. As such, vGPCRs would significantly contribute to viral pathogenesis. Direct evidence that vGPCRs indeed contribute to pathology is available for several of these receptors. For instance, KSHV ORF74 was found to act as an oncogene (37). Moreover, transgenic expression of ORF74 induces an angioproliferative disease resembling Kaposi’s sarcoma (38). Furthermore, the GPCR genes of RCMV (R33 and R78) and MCMV (M33 and M78) were each found to play a crucial role in the pathogenesis of viral infection (13, 14, 39, 40).

Our current data and those reported earlier by us and others (8, 9, 10, 16, 17) indicate that CMV effectively uses vGPCRs to orchestrate signaling networks within the cell during its viral life span. Previously, four genes encoding vGPCRs have been identified in the HCMV genome (US27, US28, UL33, and UL78) (4). Although a recent study has suggested that the HCMV genome contains an additional 11 genes that putatively encode proteins possessing 7 transmembrane domains (5), these genes seem to lack other sequences characteristic of the family of GPCR genes. Of the four putative HCMV genes, only
US28 and UL33 have hitherto been demonstrated to encode functional GPCRs (Refs. 8 and 17, and this study). Previously, US28 has been shown to constitutively activate a variety of signal transduction cascades, including PLC (8), MAPK pathways (41), and various transcription factors (8, 17). Moreover, we and others have recently shown that also in HCMV-infected fibroblasts US28 constitutively activates PLC (9, 10), further emphasizing the physiological relevance of the constitutive signaling of US28 after viral infection. Here, we show that another HCMV-encoded receptor, UL33, displays constitutive signaling in transfected as well as in HCMV-infected cells.

Until now, the structure of the UL33 cDNA was predicted on the basis of sequence analyses as well as the finding of an intron near the 5' end of the UL33 open reading frame. In this study, we have shown that UL33 predominantly encodes a spliced transcript of which the 5' terminus is located 55 bp upstream of the start codon. No evidence was found for the existence of an unspliced UL33 transcript, which could potentially encode a shorter version of UL33 such as the one used in this study, i.e., UL33(s). The observed lack of membrane expression and signaling of UL33(s) indicates that it is indeed unlikely that this hypothetical UL33 gene product is relevant in HCMV infection.

As reported previously (10, 16, 17, 42), our present results with UL33 indicate that a high level of constitutive activity appears to be an important property of vGPCRs. Viruses might exploit this inherent GPCR property to modulate homeostasis of infected cells. UL33 is, like R33, M33, and US28, positively coupled to PLC, generating an increase in inositol phosphate production. For both UL33 and R33, activation of PLC is mediated not only by Gq11 proteins, but also partially by G16 proteins (Fig. 10A). Gaq11 and Gb2 subunits from G16 appear to be the most likely components activating PLC. UL33 and its RCMV counterpart R33 differ, however, in their ability to modulate activation of CRE transcription. R33 constitutively inhibits signaling to CRE, measured as a reduction of forskolin-induced signaling. Inhibition of CRE-driven transcription by R33 is entirely G16-mediated (Fig. 10B), preferably through Ga1 and Gai (Fig. 6A). By contrast, UL33 appears to possess a broader range of G protein coupling. Although UL33 interacts with G proteins of the a16 class (most likely Ga1 and Gai), additional signal transduction routes play a more prominent role with respect to CRE activation; as a result, the overall effect of UL33 signaling is a transcriptional activation of CRE (Fig. 10B). CREB is regulated by a concerted action of various signaling kinases such as PKA, PKC, MAPKs, including p44/p42 and p38 (30), many of which are up-regulated early after CMV infection (41). UL33-mediated transcriptional activation of CRE appears to be mediated by coupling to Ga1, on one hand, and signaling through Rho and p38, via Gb2, on the other. Because co-expression of Ga1 and C3 exoenzyme does not result in a further decrease in UL33-mediated signaling to CRE, it is likely that signaling pathways converge. Rho is known to activate p38 via MKK3/6 (36), leading to phosphorylation of CREB, possibly via MSK-1 (43).

Interestingly, we noticed that co-expression of Ga1 reduced UL33-mediated CRE transcription. Apparently, this effect was not due to a direct negative regulation of CRE, as shown by data obtained either with the constitutively active Ga1* subunit or in the presence of PKC activator PMA. As an alternative explanation, we suggest that overexpression of Ga1 is actually scavenging the receptor from coupling to G16 subunits, resulting in a reduction of CRE activation. In line with this hypothesis, we found that overexpression of Ga1 strongly reduced UL33-mediated InsP accumulation (data not shown). Similar results were previously shown for the a16-adrenoceptor, which can couple to both Ga1 and Gai subunits. Co-expression of either Ga1 or Gai subunits resulted in an agonist-mediated inhibition or increase of cAMP levels, respectively (44). The interaction with an overexpressed G protein excludes interaction with others; therefore, the availability of G proteins of different classes can significantly alter the signaling response for broadly coupled receptors.

The apparent divergent and opposite signaling properties of the UL33 and R33 receptors prompted us to investigate the differences between these proteins in more detail. Sequence comparison indicated that the observed differences in activity might be due to their divergent C-terminal tails. The predicted amino acid sequence of UL33 has a relatively long C-terminal tail in comparison with R33. Remarkably, R33 contains a unique stretch of 11 consecutive Pro residues (polyproline stretch) near the C terminus. Although polyproline motifs have previously been shown to mediate binding to a variety of conserved protein domains, such as Src homology 3 (SH3) and WW domains (45), the role of this motif in the function of R33 is yet
unknown. To investigate the role of the C-terminal tails in the differential signaling profiles of UL33 and R33, we generated UL33/R33 chimeras. Interestingly, the study of these chimeras showed that the C-terminal tail of UL33 appears to be the determinant for signaling of UL33 via Go/α. Exchanging (part of) the C terminus of UL33 for the C terminus of R33, resulted in a loss of Go/α coupling, as observed in both the InsP accumulation assay and CRE reporter gene assay.

Conversely, exchange of the C terminus of R33 for that of UL33, did not significantly alter R33-mediated signaling, suggesting that sequences other than the C terminus are important for coupling of R33 to Go/α. It is interesting to note that R33 contains a basic motif (314KR316) that has recently been shown to play a crucial role in proper receptor expression of CCR5 (46). It was found that substitution of the C-terminal tail of CCR5, which comprises this basic motif, for that of CXCR4, which lacks it, resulted in a mutant that was defective in trafficking to the cell surface, whereas the CXCR4 chimera that exchanged the C terminus for that of CCR5 displayed a normal, cell surface expression pattern. In analogy, the lack of the basic motif from chimera R33-Y-UL33 may explain the inability of this protein to be expressed properly on the cell surface and, thus, its inactivity in any of the signaling assays. Additionally, the UL33 tail contains an RXR motif (316RMR318), known to be implicated in protein retention in the endoplasmic reticulum (52). It is possible that this motif is masked in the UL33 receptor, but is exposed within chimera R33-Y-UL33, thereby conferring endoplasmic reticulum localization.

To date, UL33 homologs from rodent CMVs, R33 and M33, remain orphan receptors, as chemokines, which either modulate the activity of, or bind to, these receptors, have not yet been found (16, 17). Similarly, the CC chemokines RANTES (regulated on activation normal T cell expressed and secreted), and MIP-1α did not modulate UL33 constitutive activity nor showed any binding to this receptor (data not shown). The recent identification of a small non-peptidergic inverse agonist for US28 by its modulating effect on US28-mediated constitutive signaling (9), demonstrates that the UL33-mediated signaling assays described in this study may be suitable screening systems for the identification of ligands acting at UL33. The availability of such a ligand would be of help in elucidating the role of UL33 in HCMV infection.

Little is known about the mechanisms underlying the pathogenesis of human cytomegalovirus. It is known that, following CMV infection, effector responses such as inositol lipid hydrolysis, kinase activation, and arachidonic acid metabolism are increased within the host cell and are required to direct and control early viral gene expression and DNA replication (47). Here we show that HCMV also induces a strong activation of CRE, at early and late times post infection. In this regard, it is important to note that the HCMV major immediate-early promoter contains four consensus CRE sites (48, 49). Because the immediate-early promoter of HCMV constitutes a primary genetic switch, essential for the progression of viral infection and reactivation, regulation of CRE activation during the infectious course might be of (patho)physiological importance. Importantly, data obtained with the deletion mutant virus AD169-ΔUL33 strongly indicate that UL33 is involved in virus-induced CRE activation. At all time points, AUL33 induces approximately only half the response of WT virus. These results cannot be ascribed to an impaired infectivity of the deletion mutant virus, because we have shown that viral growth kinetics are essentially the same for WT and ΔUL33-HCMV (Fig. 8C). These findings confirm the UL33-mediated CRE activation in a physiologically relevant model system. Infection of cells with HCMV offers a significant model for the pharmacological study of UL33, because in this condition UL33 expression is regulated by the virus and its constitutive signaling is not a potential artifact due to overexpression. Interestingly, UL33 contribution to CRE activation was already present at early time post infection. Because UL33 is mainly transcribed at late times of infection (13), it is likely that UL33 expression on the virion envelope, as described previously (12, 50), activates signal transduction pathways immediately upon infection, after fusion of the viral envelope with the cell membrane. Competition of CRE activation in cells infected with ΔUL33 CMV or mock infected also indicates that UL33 is not the only player involved in HCMV-induced CRE activation. It is suggestive to propose US28 as a candidate for the residual CRE activation, because it was shown that this receptor can also activate CRE in transiently transfected COS-7 cells, similarly to UL33 (17). We are currently investigating in more detail the signaling pathways engaged by HCMV in CRE activation. Interestingly, it was recently shown that HCMV infection induces p38 phosphorylation (51). The role of UL33 and US28 in HCMV-induced p38 activation and its possible link to CRE activation, as suggested by results obtained in COS-7 cells, will be further examined.

In conclusion, it is apparent from these and earlier studies that CMV-encoded receptors regulate cellular signaling via the constitutive activation of a range of G proteins. In this study, we show that UL33 constitutively modulates several pathways via promiscuous activation of G proteins of different classes. Initial experiments performed with HCMV-infected cells suggest that the signaling properties of UL33 are of physiological relevance and that HCMV may effectively use UL33 and US28 to orchestrate multiple signaling networks within infected cells.
Differential Signaling by CMV-encoded Receptors UL33 and R33

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