Constitutive Signaling of the Human Cytomegalovirus-encoded Chemokine Receptor US28*

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Previously it was shown that the HHV-8-encoded chemokine receptor ORF74 shows considerable agonist-independent, constitutive activity giving rise to oncogenic transformation (Arvanitakis, L., Geras-Raaka, E., Varma, A., Gershengorn, M. C., and Cesarman, E. (1997) Nature 385, 347–350). In this study we report that a second viral-encoded chemokine receptor, the human cytomegalovirus-encoded US28, also efficiently signals in an agonist-independent manner. Transient expression of US28 in COS-7 cells leads to the constitutive activation of phospholipase C and NF-κB signaling via Gq/11 protein-dependent pathways. Whereas phospholipase C activation is mediated via Gq/11 subunits, the activation of NF-κB strongly depends on βγ subunits with a preference for the β2γ1 dimer. The CC chemokines RANTES (regulated on activation, normal T cell expressed and secreted) and MCP-1 (monocyte chemotactic protein-1) act as neutral antagonists at US28, whereas the CX4/C chemokine fractalkine acts as a partial inverse agonist with IC50 values of 1–5 nM. Our data suggest that a high level of constitutive activity might be a more general characteristic of viral G protein-coupled receptors that human cytomegalovirus might exploit this G protein-coupled receptor property to modulate the homeostasis of infected cells via the early gene product US28.

Viruses have developed a variety of strategies to evade the immune system, among which is the piracy of cellular genes that are central to the host defense system (1–3). The identification of a variety of viral genes that encode potential G protein-coupled receptors (GPCRs) or GPCR ligands is in this respect of major interest as the GPCR superfamily is essential for proper cellular communication (4). In the genome of various β- and γ-herpesviruses, like human cytomegalovirus (HCMV or HHV-5) (5–7), human herpesviruses HHV-6 (8–10), HHV-7 (11), and HHV-8 (Kaposi’s sarcoma-associated Herpesvirus) (12–15) viral genes with homology to mammalian chemokines and/or chemokine receptors have been identified. These observations suggest that these viruses exploit chemokine signaling pathways to interfere with the host immune system (1–3).

Currently, the best characterized viral GPCR is ORF74, a CXC2R homologue encoded by HHV-8 that binds a variety of CXC and CC chemokines (16–18). ORF74 signals in a chemokine-independent, constitutively active manner (18–19) and induces oncogenic transformation when transfected in NIH-3T3 cells (19). Although constitutive GPCR signaling is now a well accepted paradigm, the actual physiological relevance is still not entirely understood (20, 21). HHV-8 is considered to be the etiologic agent of Kaposi’s sarcoma, a highly vascularized tumor (22). As transgenic expression of ORF74 also results in angioproliferative lesions, resembling various symptoms of Kaposi’s sarcoma (23), the constitutive activity of ORF74 is one of the intriguing examples of a potential pathophysiological role of constitutive GPCR signaling.

In this study we report that a second viral-encoded chemokine receptor, the HCMV-encoded GPCR US28, also efficiently signals in an agonist-independent manner. The β-herpesvirus HCMV has been recognized as a risk factor for vascular diseases, like arterial restenosis and atherosclerosis, and causes life-threatening systemic infections in immunocompromised patients (24, 25). Sequence analysis of the HCMV genome has identified four genes encoding GPCRs, US27, US28, UL33, and UL78 (5), of which US28 is expressed early after viral infection (26). US28 shows the highest homology (33%) to the CC chemokine receptor CCR1 and binds CC chemokines, like RANTES and MCP-1 (6, 27), and the CX4/C chemokine fractalkine (27). US28 shows considerable HIV-I coreceptor activity (28, 29) and is known to enhance in vitro cell-cell fusion mediated by various viral proteins, including HIV-I envelope proteins (30). Moreover, US28 has been shown to induce vascular smooth muscle cell migration (31), which could provide the molecular basis for the implication of HCMV in atherosclerosis.

In this study, we show that upon transient expression in COS-7 cells US28 constitutively couples to phospholipase C and NF-κB via related, though distinct, Gq/11-protein-mediated mechanisms. Our data suggest that a high level of constitutive activity might be a more general characteristic of viral GPCRs and that HCMV might exploit this general GPCR property to modulate the homeostasis of infected cells via the early gene product US28.

EXPERIMENTAL PROCEDURES

Materials—ATP disodium salt, bovine serum albumin, chloroquine diphosphate, DEAE-dextran (chloride form), and pertussis toxin (PTX) were obtained from Sigma. t-Luciferin was purchased from Duchtewa Biochemie B. V. (Haarlem, The Netherlands). Cell culture media, penicillin, and streptomycin were obtained from Life Technologies, Inc.,

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* The abbreviations used are: GPCR(s), G protein-coupled receptors; HCMV, human cytomegalovirus; HIV, human herpesvirus; HIV-1, human immunodeficiency virus, type I; NF-κB, nuclear factor-κB; PTX, pertussis toxin; CMV, cytomegalovirus; InsP3, inositol phosphate.
and fetal calf serum was purchased from Integro B. V. (Dieren, The Netherlands). \( \text{nyc-2}^{-2} \text{H} \)inositol (17 Ci/mmol) was obtained from PerkinElmer Life Sciences. The human chemokines RANTES (regulated on activation, normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein-1), GRO-\( \alpha \), IP-10, and the CX\( \text{C} \) chemokine domain of human fractalkine (residues 1–76) were obtained from Peprotech (Rocky Hill, NJ).

DNA Constructs—pNF-B-Luc was obtained from Stratagene (La Jolla, CA). The cDNAs encoding for US28 (encoded by VHL/E HCMV strain) and US28-N (encoded by AD169 HCMV strain) (GenBank accession numbers L20501 and X17403, bases 219,000–220,263) inserted into pcDNA3 were a gift from Dr. R. Maggio), muscarinic m2 receptor (from Dr. S. Cotecchia) are gratefully acknowledged. The cDNA of ORF74 was inserted in pcDNA3 after polymerase chain reaction amplification. Gifts of pcDNA3-based expression vectors containing the cDNAs of CCR1 (from Dr. C. Tensen), muscarinic m2 receptor (from Dr. M. Lohse), and GRK2 and GRK2\( \text{R2}^{-2} \text{R} \) (from Dr. S. Coteccia) are gratefully acknowledged.

Cell Culture and Transfection—COS-7 cells were grown at 5% CO\( \text{2} \) at 37 °C in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 2 mM l-glutamine, 50 IU/ml penicillin, and 50 \( \mu \)g/ml streptomycin. Transfection of the COS-7 cells was performed by DEAE-dextran. The total amount of DNA in transfected cells was maintained constant by addition of the empty vector.

\( \text{3H} \)inositol Phosphate Production—Cells were seeded in 24-well plates, and 24 h after transfection they were labeled overnight in inositol-free medium (modified Eagle’s medium with Earle’s salts) supplemented with 2 mM l-glutamine, t-cysteine, l-leucine, l-methionine, l-arginine, glucose, 0.2% bovine serum albumin, and 2 \( \mu \)Ci/ml \( \text{nyc-2}^{-2} \text{H} \)inositol in the presence or absence of PTX (100 ng/ml). Subsequently, the labeling medium was aspirated, cells were washed for 10 min with Dulbecco’s modified Eagle’s medium containing 25 mM HEPES (pH 7.4), 20 mM LiCl, and incubated for 2 h in the same medium in the presence or absence of the tested chemokines. The incubation was stopped by aspiration of the medium and addition of cold 10 mM formic acid. After 90 min of incubation on ice, inositol phosphates were isolated by anion exchange chromatography (Dowex AG1-X8 columns, Bio-Rad) and counted by liquid scintillation.

Reporter-gene Assay—Cells transiently cotransfected with pNF-B-Luc and either pcDNA3 (mock) or pcDNA3-US28 were seeded in 96-well black plates (Costar) in serum-free culture medium in the presence or absence of PTX (100 ng/ml) and the tested chemokines. After 48 h, cells were assayed for luminescence by aspiration of the medium and addition of 25 \( \mu \)l of luciferase assay reagent (0.83 mM ATP, 0.83 mM Mg\( \text{Cl}_2 \), 0.78 \( \mu \)M Na\( \text{H}_2\text{PO}_4 \), 38.9 mM Tris (pH 7.8), 0.39% (v/v) glycerol, 0.03% (v/v) Triton X-100, and 2.6 \( \mu \)M diithiothreitol). Luminescence was measured for 3 s in a Wallac Victor2.

Binding Experiments—Cells were seeded in 24-well plates; 48 h after transfection binding was performed on whole cells for 3 h at 4 °C using 0.1 mM \( \text{3H} \)RANTES in binding buffer (50 mM Hepes, pH 7.4, 1 mM Ca\( \text{Cl}_2 \), 5 mM Mg\( \text{Cl}_2 \), and 0.5% bovine serum albumin). After incubation, cells were washed four times at 4 °C with binding buffer supplemented with 0.5 mM NaCl. Nonspecific binding was determined in the presence of 0.1 \( \mu \)M cold competitor (RANTES or fractalkine). Western Blot Analysis—Cells were lysed 48 h after transfection in RIPA buffer (phosphate-buffered saline containing 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulphonyl fluoride, and 2 \( \mu \)g/ml of aprotinin and leupeptin), sonicated, separated by SDS polyacrylamide gel electrophoresis, and blotted to polyvi-

Statistical Analysis—All data shown are expressed as mean ± S.E. Statistical analysis was carried out by Student’s t test. p values < 0.05 were considered to indicate a significant difference.

RESULTS AND DISCUSSION

Expression of the viral chemokine receptor US28 (encoded by the HCMV VHL/E strain) in COS-7 cells resulted in an expression-dependent increase in both \( \text{3H} \)inositol phosphate production and NF-\( \text{kB} \) activation (Fig. 1A). Expression of the related US28-N, encoded by the HCMV AD169 strain, gave similar findings (data not shown). As reported in Ref. 16, the expression of the HHV-8-encoded oncogenic GPCR ORF74 also led to a pronounced constitutive activation of phospholipase C (Fig. 1B). In contrast, expression of the human CCR1 receptor, which is most homologous to US28 (6), did not result in constitutive or RANTES-mediated phospholipase C activation (Fig. 1B). These findings are in accordance with previous observations that activation of CCR1 does not result in \( \text{3H} \)inositol phosphate accumulation in COS-7 cells (32).

The constitutive activity of ORF74 was negatively modulated by IP-10 and positively by GRO-\( \alpha \) (Fig. 1B), thus acting as inverse agonist and agonist, respectively (17, 18). The US28-induced signaling was not affected by the CC chemokines RAN-

T1S or MCP-1 up to 100 nM but was inhibited by the CX\( \text{C} \) chemokine fractalkine (Fig. 1B). It has been reported that
fractalkine binds to US28 (27), but so far no functional activity of fractalkine at US28 has been described. Fractalkine inhibited the constitutive US28 signaling to phospholipase C by 37 ± 4% with an IC_{50} value of 1.6 ± 0.2 nM (n = 3; Fig. 2). As the observed inhibition of the US28 signaling is not complete, fractalkine apparently behaves as a partial inverse agonist (20, 21). The CC chemokines RANTES (100 nM) (Fig. 2, inset) and MCP-1 (data not shown) antagonized the reduction of basal US28 signaling by 10 nM fractalkine, thereby acting as neutral antagonists. RANTES and MCP-1 have previously been shown to act as agonists at US28 for G_{i} dependent signal transduction (6, 33). These data can be explained by differences in the levels of constitutive US28 signaling in the different cell systems, probably as a result of differences in expression of US28 and/or signaling moieties (20, 21). Because G_{i} proteins are known signaling partners for chemokine receptors (34), including US28 (6, 33), the activation of phospholipase C by US28 could be due to the release of G_{i}/G_{q} subunits, which can activate phospholipase C isoenzymes (35). Yet, G_{q} subunits are not implicated in the US28-mediated constitutive activation of phospholipase C. PTX treatment did not abolish the US28-mediated production of [^{3}H]inositol phosphates (103 ± 5%; n = 3), whereas for the muscarinic m2 receptor PTX, treatment inhibited the carbachol-induced increase in [^{3}H]inositol phosphate accumulation for 45 ± 1.8%. Coexpression of G_{a16} with US28 did not increase the US28 response (Fig. 3), probably because of the absence of the \( \beta \)-sensitive phospholipase C_{\beta2} in COS-7 cells (32). Instead, coexpression of US28 with either G_{a11} or G_{q} enhanced the US28-mediated production of [^{3}H]inositol phosphates (Fig. 3), whereas coexpression of G_{a12} or G_{a5} did not affect US28 responsiveness. Previously, the receptor kinases GRK2 and -3 have been reported to scavenge both G_{a11} subunits (36), as well as G_{q} subunits (37). Coexpression of US28 with GRK2 or the kinase-deficient GRK2K_{220}R (38) mutant resulted in an efficient inhibition of US28-mediated [^{3}H]inositol phosphates production (Fig. 3). In contrast, coexpression of the \( \beta \)-scavenger G_{a} did not modify constitutive US28 signaling (Fig. 3).

These data indicate that, in contrast to the homologous CC chemokine receptor CCR1 (32), US28 interacts with endogenous G_{a11} subunits in COS-7 cells and thereby constitutively activates phospholipase C. A large number of GPCRs can also couple to phospholipase C upon coexpression of G_{a10}, an hematopoietic specific member of the G_{q} class of proteins (39). Expression of G_{a16} enhanced, for example, the agonist-induced inositol phosphate production mediated by the muscarinic m2 receptor 2.2-fold, as previously reported (40). However, US28 shows a remarkable level of selectivity for G_{a11} and G_{q} over G_{a16} for the coupling to phospholipase C in COS-7 cells. Besides the US28-mediated modulation of phospholipase C activity, we also observed a constitutive activation of NF-\( \kappa \)B activity upon expression of US28 in COS-7 cells (Fig. 1A). This effect was not observed for the homologous CCR1 receptor (data not shown). The constitutive stimulation of NF-\( \kappa \)B activity was not modulated by RANTES or MCP-1 (up to 100 nM; data not shown), but fractalkine again behaved as an apparent partial inverse agonist. The US28-mediated increase in NF-\( \kappa \)B expression of the fractalkine, and InsP release was measured. Inset, US28-transfected cells were incubated with fractalkine (10 nM) in the presence or absence of RANTES (100 nM, added 10 min prior to fractalkine), and InsP accumulation was measured. Data are presented as percentage of US28-mediated response and defined as absolute increase of US28-mediated InsP accumulation above values obtained for mock-transfected cells. A representative experiment of three experiments, each performed in triplicate, is shown. The asterisk indicates a statistically significant difference (p < 0.05) versus receptor only.

**Fig. 2.** Inhibition of US28-mediated inositol phosphates accumulation by fractalkine. COS-7 cells were transiently transfected with cDNA encoding US28 (2 \( \mu \)g/10^6 cells). Cells were incubated with various concentrations of fractalkine, and InsP release was measured. Inset, US28-transfected cells were incubated with fractalkine (10 nM) in the presence or absence of RANTES (100 nM, added 10 min prior to fractalkine), and InsP accumulation was measured. Data are presented as percentage of US28-mediated response and defined as absolute increase of US28-mediated InsP accumulation above values obtained for mock-transfected cells. A representative experiment of three experiments, each performed in triplicate, is shown. The asterisk indicates a statistically significant difference (p < 0.05) versus receptor only.

**Fig. 3.** Effect of various G_{a} subunits on US28-mediated inositol phosphates accumulation. COS-7 cells were transiently transfected with cDNA encoding US28 (2 \( \mu \)g/10^6 cells) in the presence of cDNAs encoding the indicated G_{a} subunits (2 \( \mu \)g/10^6 cells) and were assayed for InsP accumulation after 48 h. Expression of G_{a} subunits by themselves did not give a rise in [^{3}H]inositol phosphate production (data not shown). Inset, the effect of coexpression of GRK2 and the mutant GRK2K_{220}R or G_{a} transducin (4 \( \mu \)g/10^6 cells) on the US28-mediated InsP production. Data are expressed as percentage of US28-mediated response. Representative experiments performed in triplicate are shown, and each experiment was repeated at least three times. The asterisks indicate a statistically significant difference (p < 0.05) versus receptor only.
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activity was reduced by fractalkine for 42 ± 4% (n = 3) with an IC50 value of 5 ± 0.2 nm.

The observed constitutive activation of NF-κB by US28 is of potential pathophysiological relevance, as NF-κB is an ubiquitously expressed transcription factor that plays a critical role in the regulation of inducible genes in immune response and inflammatory events associated with, for example, atherosclerosis (41–43). Activation of NF-κB-mediated transcription has been reported in human aortic smooth muscle cells after CMV infection via PTX-sensitive G proteins. Yet, as observed for the production of [3H]inositol phosphates, Gαi is not involved in the US28-induced increase in NF-κB activity, as PTX treatment did not affect the US28 response (104 ± 5%; n = 3). Previous studies have indicated that depending on the cell type and GPCR, NF-κB-mediated transcription can also be stimulated following activation of G12/13 or Gγ11 proteins (44). Yet, in COS-7 cells, only the expression of the activated form of Gα11 (Gα11 Q209L, referred to as Gα11*) subunits resulted in significant activation of NF-κB (Fig. 4A). In line with these findings, coexpression of the wild-type Gα11 or Gαi (Fig. 4A) increased the US28-mediated constitutive NF-κB signaling, whereas the basal NF-κB activity in mock-transfected cells was not affected. These observations clearly imply the involvement of the Gαγ11 proteins in the US28-mediated signaling to NF-κB. As found for the activation of phospholipase C, GRK2 and its kinase-deficient mutant inhibited the increase in NF-κB activity (Fig. 4B, inset). Yet, in contrast to the US28-induced phospholipase C activation, US28-mediated NF-κB activity was fully inhibited by coexpression of Gαi (Fig. 4B). The involvement of βγ subunits in constitutive US28 signaling to NF-κB was further strengthened by coexpression experiments with various βγ subunits (Fig. 5, inset). Of the different combinations tested, only the Gβ2 and Gγ1 further significantly increased the US28-mediated activation of NF-κB (Fig. 5). These data corroborate previous findings that GPCRs can show a clear specificity for specific βγ subunit combinations (45).

Whereas US28 activates phospholipase C via Gαγ11 subunits, our data suggest that besides αγ11 subunits, βγ subunits are also involved in the NF-κB activation by US28. The apparent coinvolvement of αγ11 and βγ subunits suggests that NF-κB activation is due to βγ subunits that are released upon US28 interaction with Gαγ11 proteins, although release of βγ subunits from other G proteins cannot be ruled out. As previously observed after stimulation of the bradykinin B2 receptor in Hela cells (44), Gβγ subunits appear to be essential but not exclusive signaling moieties for the NF-κB signaling by US28.

Expression of the various Gβγ subunits by themselves did not increase NF-κB signaling (data not shown). Moreover, our observation that expression of activated Gα11 initiates NF-κB signaling indicates that Gαγ11 subunits also trigger a signaling pathway that converges to NF-κB. Protein kinase C activation is a likely candidate for this αγ11-mediated pathway (46). Activation of NF-κB via βγ subunits probably involves the activation of phosphatidylinositol 3-kinase and Akt (44), two recently identified signaling partners for GPCRs (47, 48). Additional experiments need to be performed to further delineate the mechanisms of US28-mediated NF-κB activation. The specific roles of the αγ11 and βγ subunits, especially, will require further clarification.

In conclusion, in comparison to its closest human homologue...
CCKR1, it is interesting to note that the viral GPCR US28 signals without the need for an agonist and is using a larger diversity of G proteins and chemokines to affect cellular signaling pathways. For the first time, we show that US28 signals to phospholipase C via Go_{q/11} subunits and NF-κB via both Go_{q/11} and G_{q} in a constitutively active manner. It is tempting to speculate that these characteristics of a promiscuous GPCR allows US28 to affect a broad range of cells upon CMV infection. The constitutive activation of the ubiquitous GPCR US28 by the early viral gene product US28 could be of major importance for viral action. US28 has been shown to cause smooth muscle cell migration upon HCMV infection without the addition of exogenous chemokines (31). The basal US28-mediated migration was antagonized for 80% by neutralizing antibodies against MCP-1, which was released in an autocrine fashion (31). Constitutive signaling by US28 could be responsible for the remaining migratory response of the HCMV-infected smooth muscle cells. Moreover, if US28 is expressed on viral particles, it would also be immediately present on the membrane of CMV-infected cells and, by means of its constitutive activity, could modulate the cellular response. We also show for the first time a functional response to the CX_{C} chemokine fractalkine, i.e., acting as an inverse agonist at US28. Fractalkine is a quite unique GPCR ligand as its chemokine-like domain is linked to a transmembrane segment (49). Accordingly, fractalkine is membrane-bound, and its interaction with US28 has been suggested to be involved in the viral transfer between cells (27). The action of fractalkine as an inverse agonist suggests that inhibition of constitutive US28 activity by fractalkine expressed on the membrane of a target cell might give the appropriate signal to an CMV-infected, US28-expressing cell to allow CMV entry into the target cell. Because no data are currently available on the expression of US28 on the viral particle or on the role of fractalkine in CMV infections, future investigations should substantiate these suggestions and indicate if US28 can be regarded as an interesting drug target in HCMV-related disorders.

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