The CXCL1/GRO system has been subject to molecular piracy by certain viruses. Thus, some pox- and herpesviruses encode chemokines and/or chemokine receptors in their genomes (4). The virus-encoded chemokine receptors are structurally rather divergent and have in general evolved very different functional properties compared with their human homologs, indicating remarkable evolutionary pressure. The open reading frame 74 (ORF74) receptors constitute one cluster of synthetic genes (all encoded by ORF74) and are shared by many members of the rhadinovirus lineage (\(\gamma_2\)-herpesviruses) (Fig. 1) (5). Thus, ORF74 receptors have been cloned from murid \(\gamma_2\)-herpesvirus 68 (ORF74-MHV68), equine herpesvirus 2, human herpesvirus 8 (ORF74-HHV8), herpesvirus saimiri (ECRF3 or ORF74-HVS), and the closely related ateline herpesvirus. The ORF74 family shows the highest structural homology to mammalian CXCL2 chemokine receptor (CXCR2), the functional characterization of ORF74-HHV8 (6–8), ECRF3 (9), and ORF74-MHV68 (10) as being CXCL2 chemokine receptors supports the structural homology.

HVS is a T-cell lymphotropic virus that causes asymptomatic infection in the natural host, the squirrel monkey (Saimiri sciureus), and fatal lymphoproliferative disease when transmitted to other New World primates and some Old World tussis toxin; ORF74, open reading frame 74; MHV68, murid \(\gamma_2\)-herpesvirus 68; HHV8, human herpesvirus 8; HVS, herpesvirus saimiri; CXCR, CXCL2 chemokine receptor; IL-8, interleukin-8; NAP-2, neutrophil-activating peptide 2; IP10, interferon-inducible protein 10; GCP-2, granulocyte chemotactic protein 2; vCCL2, viral CCL2; vMIP-II, viral macrophage inflammatory protein II; SRE, serum response element; PI, phosphatidylinositol; NFAT, nuclear factor of activated T-cells; NF-\(\kappa\)B, nuclear factor \(\kappa\)B; CREB, cyclic AMP response element-binding protein; PLC, phospholipase C.
of herpesvirus-encoded chemokine receptors. The phylogenetic tree was constructed using ClustalW Version 1.5 alignment of the whole sequence, followed by analysis using the Distance program of the GCG Wisconsin package (kindly provided by Francois Talabot, Ares-Serono). Constitutively active receptors are marked in red. Solid lines indicate a more peripheral cluster association. CMV, cytomegalovirus; EHV, equine herpesvirus 2; aHSV, athele herpesvirus.

primates (11). ECRF3 from HVS was characterized as the first γ-herpesvirus-encoded CXC chemokine receptor by Ahuja and Murphy (9), with the most potent agonist being CXCL1/GROα,2 followed by CXCL8/interleukin-8 (IL-8) and CXCL7/neutrophil-activating peptide 2 (NAP-2). ORF74 from HHV8 (12) is characterized ORF74 receptor. It binds a variety of CXC chemokines; thus, pro-inflammatory and angiogenic ELR CXC chemokines act either as agonists (CXCL1–3/GROα,β,γ) or as neutral ligands (e.g. CXCL8/IL-8), whereas angiostatic non-ELR CXC chemokines act as inverse agonists (CXCL10/interferon-inducible protein 10 (IP10) and CXCL12/stromal cell-derived factor 1α). Although CXCL6/granulocyte chemotactic protein 2 (GCP-2) is an ELR CXC chemokine, it acts as a partial inverse agonist (13). The broad-spectrum chemokine antagonist viral CCL2 (vCCL2/viral macrophage inflammatory protein II (vMIP-II), encoded by HHV8, also inhibits the constitutive activity of ORF74-HHV8 (6, 8, 13–16). ORF74-HHV8 signals constitutively through multiple pathways, including Gq, Gi, and G12/13 (7, 8, 17, 18), and induces cellular transformation and production of angiogenic and inflammatory factors. Vascularized tumors develop when ORF74 is transplanted into nude mice (7), and transgenic expression of ORF74-HHV8 in mice results in Kaposi’s sarcoma-like lesions (19, 20). ORF74 from MHV68 also signals through multiple pathways upon binding of ELR motif-containing CXC chemokines (10), but it does not show constitutive activity. Thus, in general, virus-encoded receptors are more promiscuous in their interaction with (several) ligands and in their exploitation of several signaling pathways compared with their mammalian chemokine receptor homologs (1, 5).

The general knowledge of 7TM receptor signal transduction mechanisms and knowledge of the properties of virus-encoded chemokine receptors have developed considerably since the first description of ECRF3 from HVS in 1993 (9). In this study, we characterized ECRF3 on this basis and surprisingly found that it has a very interesting molecular pharmacological phenotype in being constitutively active through Gq and G12/13 (the latter shown by studying the serum response element (SRE) transcription factor in the presence of pathway-specific inhibitors), whereas it activates a broader spectrum of signaling pathways in a ligand-regulated manner, with CXCL6/GCP-2 being the most potent agonist.

EXPERIMENTAL PROCEDURES

Materials—The human chemokines were purchased from Peprotech (CXCL6/GCP-2, CXCL11/GROα, CXCL12/stromal cell-derived factor 1, and CXCL10/IP10), kindly provided by Dr. Timothy N. C. Wells (Serono Pharmaceutical Research Group, Ares-Serono, Geneva, Switzerland) (vCCL2/vMIP-II); or made in-house by Dr. Thomas P. Boesen through Escherichia coli expression, purification, and refolding (CXCL8/IL-8). The ECRF3 receptor was kindly provided by Dr. John Nicholas (Johns Hopkins Oncology Center, Baltimore, MD). ORF74 from HHV8 (GenBank™/EMBL accession number U24275) was cloned from a Kaposi’s sarcoma skin lesion biopsy taken from a human immunodeficiency virus 1-infected patient (21). LipofectAMINETM 2000 reagent and Opti-MEM I (catalog no. 51985-026) were purchased from Invitrogen. Lumistimexinated (e.g. CXCL6/GCP-2, CXCL1/GROα, myo-[3H]inositol (PT6-271), [3H]adenine (TRK311), and Bolton-Hunter reagent for iodination of CXCL6/GCP-2 and CXCL10/IP10 were from Amersham Biosciences (Uppsala, Sweden). AG 1-X8 anion-exchange resin (for phosphatidylinositol (PI) turnover) and AG-50W-X4 resin (for cAMP assay) were from Bio-Rad. Alumina, imidazole, 3-isobutyl-1-methylxanthine, and PTx (for cAMP assay) were from Sigma. U-73122, Y-27632, and C3 exoenzyme from Clostridium botulinum were from Calbiochem and Merck Biosciences (Nottingham, United Kingdom).

Iodination of CXCL6/GCP-2 and CXCL10/IP10—The Bolton-Hunter reagent was dried under a gentle stream of nitrogen for 30–60 min. 5–10 μg of chemokine was incubated on ice with 1.5 mL of Bolton-Hunter reagent in a total volume of 50 μl of 0.1 mM borate buffer (pH 8.5) for 1 h. The reaction was terminated by addition of 0.25 ml of H2O supplemented with 0.1% (v/v) trifluoroacetic acid. The labeled chemokines were purified by reverse-phase high pressure liquid chromatography.

Transfections and Tissue Culture—Cos-7 cells were grown at 10% CO2 at 33°C in Dulbecco’s modified Eagle’s medium with Glutamax (catalog no. 21885-025, Invitrogen) adjusted with 10% fetal bovine serum, 180 units/ml penicillin, and 45 μg/ml streptomycin. HEK293 cells were grown in Dulbecco’s modified Eagle’s medium adjusted to contain 4500 mg/liter glucose with 10% heat-inactivated fetal bovine serum, 180 μg/ml penicillin, and 45 μg/ml streptomycin at 10% CO2 and 37°C. The HEK293 cell medium was modified to contain heat-inactivated fetal bovine serum and no penicillin and streptomycin during luciferase-based assays. Transfection of the COS-7 cells was performed by the calcium phosphate precipitation method (8) for the competition binding and PI turnover experiments and by the cationic lipid reagent method with LipofectAMINETM 2000 reagent in serum-free Opti-MEM I according to the manufacturer’s recommendations for the luciferase-based transcription factor experiments. HEK293 cells were transfected by the calcium phosphate precipitation method (8) for the adenylate cyclase inhibition experiments and with LipofectAMINETM 2000 reagent in serum-free Opti-MEM I according to the manufacturer’s recommendations for the luciferase-based transcription factor experiments.

Binding Experiments—COS-7 cells were transfected to culture plates 1 day after transfection. The number of cells seeded per well was determined by the apparent expression efficiency of the receptors, with the goal of obtaining 5–10% specific binding of the added radioactive ligand. 3.5 × 105 cells/well were used to test specific binding. 2 days after transfection, cells were assayed by competition binding for 3 h at 4°C using 12 pm 125I-CXCL8/IL-8, 125I-CXCL1/GROα, 125I-CXCL6/GCP-2, or 125I-CXCL10/IP10 plus unlabeled ligand in 0.5 ml of 50 mM Hepes (pH 7.4) supplemented with 1 mM CaCl2, 5 mM MgCl2, and 0.5% (v/v) bovine serum albumin. After incubation, cells were washed quickly four times with 0.4°C buffer binding supplemented with 0.5 mM NaCl. Non-specific binding was determined as the binding in the presence of 0.1 μM unlabeled chemokine. Determinations were made in duplicates.

Phosphatidylinositol Assay (PI Turnover)—1 day after transfection, COS-7 cells (2.5 × 105/well) were incubated for 24 h with 5 μCi/ml
myo-[3H]inositol in 0.3 ml/well growth medium. Cells were washed twice with 20 ml Hepes (pH 7.4), 140 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 10 mM glucose, and 0.05% (w/v) bovine serum albumin and were incubated in 0.3 ml of the same buffer supplemented with 10 mM LiCl at 37 °C for 90 min in the presence or absence of chemokines. PTx (100 ng/ml) were added 18 h prior to the experiment. Cells were extracted by addition of 1 ml/well 10 mM formic acid, followed by a 30-min incubation on ice. The generated [3H]inositol phosphates were purified on AG 1-X8 anion-exchange resin (22). Determinations were made in duplicates.

Adenylate Cyclase Inhibition Assay (cAMP Assay)—1 day after transfection, HEK293 cells (2.5 × 10$^5$/well) were incubated for 24 h with 2 µCi/ml [3H]adenine in 0.5 ml/well growth medium. Cells were washed twice with 25 ml Hepes (pH 7.2), 0.75 mM NaH$_2$PO$_4$, 140 mM NaCl, and 0.05% (w/v) bovine serum albumin, and 0.5 ml of the same buffer supplemented with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1 mM) was added together with the adenylate cyclase activator forskolin (15 µM) to the cells. Ligands were then added, and the reaction was terminated after a 15-min incubation at 37 °C. After incubation, the cells were placed on ice; the medium was removed; and the cells were lysed in 1 ml of 5% (w/v) trichloroacetic acid supplemented with 0.1 mM cAMP and 0.1 mM ATP for 30 min. The lysis mixtures were loaded onto Dowex columns, which were washed with 2 ml of water, placed on the top of alumina columns, and washed again with 10 ml of water. The alumina columns were eluted with 6 ml of 0.1 M imidazole into 15 ml of scintillation fluid (Highsafe III). Columns were reused up to 15 times. Dowex columns were regenerated by adding 10 ml of 2 N HCl followed by 10 ml of water; the alumina columns were regenerated by adding 2 ml of 1 M imidazole, 10 ml of 0.1 M imidazole, and finally 5 ml of water. The effect of 100 ng/ml PTx was tested by adding it to the cells 18 h before ligand addition. Determinations were made in triplicates.

Constitutive SRE, NFAT, and NF-kB cis-Reporting and CREB trans-Reporting Luciferase Assays—Cells were seeded at 35,000 cells/well in culture plates 24 h prior to transfection and were transfected with the cis-reporter plasmid (pSRE-Luc, pNFAT-Luc, or pNF-kB-Luc; 50 ng/well) and the receptor plasmid (0–50 ng/well). For the trans-reporting system, 50 ng/well trans-activator plasmid (pFR-Luc) was added together with 6 ng/well trans-reporter plasmid (pFA2-CREB) and receptor plasmids. 24 h after transfection, the cells were washed twice with Dulbecco’s phosphate-buffered saline, and luminescence was measured in a microplate scintillation and luminescence counter (Top-counter, PerkinElmer Life Sciences) after a 10-min incubation in 100 µl of Dulbecco’s phosphate-buffered saline together with 100 µl of LucLite substrate. All determinations were made in quadruplicates.

Ligand and Inhibitor Modification of Transcription Factor Activity—The cells were transfected with 10 ng/well receptor cDNA and the above-mentioned concentrations of reporter and activator plasmids. Chemokine ligands were added at concentrations ranging from 10$^{-11}$ to 10$^{-5}$ M. The dose-response curves. Alternatively, a constant concentration (100 nM) was added to the receptor gene dose experiments (see above). In both cases, as well as upon addition of the inhibitors PTx (100 ng/ml) and U-73122 (10 µM) to the CREB, NF-kB, and NFAT transcription factors and of PTx (100 ng/ml), C3 exoenzyme (10 µg/ml), and Y-27623 (10 µM) to the CREB, NF-kB, and NFAT transcription factors, the chemokines/inhibitors were added immediately following transfection, and luminescence was measured 24 h post-transfection as described above. All determinations were made in quadruplicates.

Calculations—IC$_{50}$ and EC$_{50}$ values were determined by nonlinear regression, and B$_{max}$ values were calculated using the GraphPad Prism Version 2 software.

RESULTS
The CXC Chemokine Binding Profile of ECRF3 Is Limited Exclusively to the ELR Motif-containing CXC Chemokines—Homolog competition binding analysis was performed in transiently transfected COS-7 cells with four radioligands, including two previously described agonists for ECRF3, 125I-CXCL1/GROα and 125I-CXCL8/IL-8 (9), together with 125I-CXCL10/IP10 and 125I-CXCL6/GCP-2, which are full and partial inverse agonists for ORF74-HHV8 (Table I). All three ECRF3 CXC chemokines (CXCL1/GROα, CXCL8/IL-8, and CXCL6/GCP-2) bound specifically to ECRF3 with high and remarkably similar binding affinities (IC$_{50}$) of 0.25 ± 0.07, 0.46 ± 0.17, and 0.51 ± 0.21 nM, respectively (Fig. 2, A–C). These affinities resembled the corresponding affinities for ORF74-HHV8 (Fig. 2, A–C).

![Fig. 2. Homologous competition binding to ECRF3.](http://www.jbc.org/)

The non-ELR CXC chemokine CXCL10/IP10 displayed no specific binding to ECRF3, in contrast to the high affinity for ORF74-HHV8 (Fig. 2D). Interestingly, large differences in the maximal binding capacities were assessed despite the high and similar binding affinities. Thus, B$_{max}$ was 12 ± 2.5 fmol/10$^5$ cells for 125I-CXCL1/GROα, whereas it was only 0.4 ± 0.1 fmol/10$^5$ cells for 125I-CXCL10/IP10. In contrast, 125I-CXCL6/GCP-2 displayed a B$_{max}$ of 34 ± 6.7 fmol/10$^5$ cells, nearly three times higher compared with 125I-CXCL10/IP10 and 85 times higher compared with 125I-CXCL8/IL-8. These B$_{max}$ values were in striking contrast to the almost similar values for 125I-labeled CXCL1/GROα, CXCL8/IL-8, and CXCL6/GCP-2 for binding to ORF74-HHV8 (33 ± 5.9, 39 ± 6.8, and 26 ± 9.4 fmol/10$^5$ cells, respectively), obtained in parallel with the B$_{max}$ values for ECRF3. A larger range of ELR as well as non-ELR CXC chemokines was analyzed by heterologous competition binding against 125I-CXCL1/GROα (Table I). All three GRO peptides (CXCL1-3/GROα, β, γ) bound with high affinities to ECRF3, whereas the apparent affinity of CXCL7/NAP-2 was only 155 nM. The non-ELR CXC chemokines CXCL12/stromal cell-derived factor 1 and CXCL10/IP10 displayed very low affinities for ECRF3 (1016 and 622 nM, respectively), in agreement with the absence of any specific binding for CXCL10/IP10. Previously, a selection of endogenous CC chemokines was tested (CCL3/MIP-1α, CCL5/RANTES (regulated on activation normal T-cell expressed and secreted), CCL2/monocyte chemotactic protein 1, and CCL4/MIP-1β) and found not to interact with ECRF3 (9). Therefore, no further analysis was performed in our system with respect to endogenous CC chemokines. However, the HHV8-encoded CC chemokine vCCL2/vMIP-II was found to have a surprisingly high affinity (IC$_{50}$ = 4.1 nM) (Table I) compared with its affinity of 72 nM for ORF74-HHV8 (8). In summary, ECRF3 bound a large spectrum of CC chemokines with affinities varying from 0.4 to 155 nM. The specific binding was, however, strictly limited to the ELR CXC chemokines (no binding of the non-ELR CXC chemokines), in contrast to the broad-spectrum ELR and non-ELR CXC chemokine binding properties of ORF74-HHV8 and ORF74-MHV68 (8, 10, 13).

ECRF3 Signals via G$_{q}$ in a Ligand-regulated (but Not Constitutive) Manner, with CXCL6/GCP-2 being the Most Potent and Efficacious Agonist—PI accumulation assays performed in
constitutively active G \(_\text{q}\) upon ligand binding. As expected from the initial study of ECRF3 identifying CXCL1/GRO\(\alpha\) as an agonist in Ca\(^{2+}\) release (9), this chemokine stimulated ECRF3 with a potency (IC\(_{50}\)) of 5.5 nM (Fig. 3) and an efficacy of 2.4-fold stimulation above the basal activity, a phenotype resembling the previously shown agonism (IC\(_{50}\) − 1 nM) of CXCL1/GRO\(\alpha\) toward ORF74-HHV8 (see Fig. 5B) (8). The neutral ligands for ORF74-HHV8, CXCL8/IL-8 and CXCL7/NAP-2, did not influence the G\(_{q}\) activity of ECRF3 at the maximal chosen concentration of 100 nM (Fig. 3). Surprisingly, an inverse agonist for ORF74-HHV8, CXCL6/GCP-2, turned out to be the most potent and efficacious agonist with ECRF3, with an EC\(_{50}\) of 0.42 nM and an efficacy of 5.4-fold stimulation above the basal activity (Fig. 3). Two other inverse agonists for ORF74-HHV8, CXCL10/IFN-\(\gamma\) and vCCL2/vMIP-II, had no effect on the basal activity of ECRF3; however, with respect to antagonizing the agonist-stimulated receptor, vCCL2/vMIP-II (but not CXCL10/IFN-\(\gamma\)) inhibited the CXCL6/GCP-2 (10 nM)-induced activity with a potency of 77 nM (data not shown).

Gene dose experiments for ECRF3 performed in parallel with ORF74-HHV8 demonstrated that ECRF3, in contrast to ORF74-HHV8, did not activate G\(_{q}\) in a ligand-independent (constitutive) manner (Fig. 4A). However, a comparison of the maximal ligand-stimulated activities in the gene dose setup indicated that ECRF3 could be stimulated to the same level as ORF74-HHV8 at equivalent DNA concentrations (Fig. 4B). Thus, 100 nM CXCL6/GCP-2-stimulated ECRF3 to the same level to which 100 nM CXCL1/GRO\(\alpha\) stimulated ORF74-HHV8 (at equivalent DNA concentrations), corresponding to the almost similar \(B_{\text{max}}\) values for CXCL6/GCP-2 binding to ECRF3 and for CXCL1/GRO\(\alpha\) binding to ORF74-HHV8. In contrast, 100 nM CXCL1/GRO\(\alpha\) stimulated ECRF3 with lower efficacy, corresponding to the lower \(B_{\text{max}}\) value for CXCL1/GRO\(\alpha\) binding to ECRF3.

Phospholipase C (PLC) is activated mainly through an interaction with the \(\alpha\)-subunit from G\(_{q}\) (23); however, the \(\beta\)-y-dimer released from activated G\(_{q}\) is yet another way to activate PLC (24). The \(\beta\)-y-dimer has the highest affinity for the PLC\(\beta2\) isoform compared with PLC\(\beta1\) and PLC\(\beta3\); and because COS-7 cells have a very low content of PLC\(\beta2\), PLC activation in these cells is believed to be through interaction with G\(_{q}\) (25). We performed PI turnover experiments in the presence of the G\(_{q}\) inhibitor PTx (100 ng/ml) and observed no inhibition of the efficacy of CXCL6/GCP-2-induced ECRF3 activation or of CXCL1/GRO\(\alpha\)-induced ORF74-HHV8 activation in the presence of PTx (Fig. 5, A and B, respectively). This supports G\(_{q}\) activation of PLC. In fact, we observed an increase in the efficacies as well as in the basal constitutive activity of ORF74-HHV8, but not in the basal non-constitutive activity of ECRF3 (Fig. 5, A and B). PTx had no effect on cells transfected with the empty expression vector (data not shown).

**ECRF3 Activates a Broad Range of Downstream Transcription Factors in a Ligand-regulated (but Not Constitutive) Manner**—Many virus-encoded chemokine receptors have been shown to signal constitutively through a broad range of transcription factors (26–31). In a gene dose setup using luciferase-based reporter systems for CREB, NF-\(\kappa\)B, and NFAT (three transcription factors with a known dependence on G\(_{q}\) activation) and receptors transiently expressed in HEK293 cells (Fig. 6), none of the transcription factors were constitutively activated by ECRF3. In contrast, all three were activated by increasing doses of ORF74-HHV8 in parallel with ECRF3 (Fig. 6, A–C, for CREB, NF-\(\kappa\)B, and NFAT, respectively) until a plateau was reached. This plateau was probably due to limitations in the content of intracellular signaling mediators from the cell surface to nucleus. The PI turnover experiments are not de-

### Table 1

**Constitutive and Ligand-regulated Activities of ECRF3**

| Chemokine | IC\(_{50}\) (nM) | Hill | EC\(_{50}\) (nM) | Hill |
|-----------|-----------------|------|-----------------|------|
| CXCL1/GRO\(\alpha\) | 0.25 ± 0.07 | −0.88 ± 0.07 | (6) |
| CXCL2/GRO\(\beta\) | 0.34 ± 0.13 | −1.12 ± 0.27 | (3) |
| CXCL3/GRO\(\gamma\) | 0.17 ± 0.06 | −0.84 ± 0.05 | (3) |
| CXCL5/NAP-2 | 0.10 ± 0.07 | 0.27 (3) |
| Non-ELR CXCLs | 1016 ± 593 | −1.27 ± 0.27 | (4) |

**Fig. 3.** ECRF3 activates G\(_{q}\) in a ligand-regulated manner. Transiently transfected COS-7 cells were used for the PI turnover assays. Chemokine dose-response curves are shown for ECRF3, CXCL6/GCP-2 (○), CXCL1/GRO\(\alpha\) (■), CXCL6/GCP-2 activation of ECRF3 (●) and CXCL1/GRO\(\alpha\) activation of ECRF3 (▲) and ORF74-HHV8 (□). Arrows indicate agonist stimulation of ECRF3 (black) and ORF74-HHV8 (gray). All experiments were done in parallel (\(n = 5\)).

**Fig. 4.** ECRF3 is not constitutively active through the G\(_{q}\) pathway. Transiently transfected COS-7 cells were used for the PI turnover assays. Increasing amounts of receptor/vector cDNA varying from 2.5 to 20 \(\mu\)g \(\times 10^6\) cells were used for the transfection. A, basal activities of ECRF3 (○), ORF74-HHV8 (●), or pcDNA3-transfected cells (vector; ▼); B, agonist (10\(^{-7}\) M)-stimulated basal activities of ECRF3 (○) and ORF74-HHV8 (●). CXCL6/GCP-2 activation of ECRF3 (●) and CXCL1/GRO\(\alpha\) activation of ECRF3 (▲) and ORF74-HHV8 (□). Arrows indicate agonist stimulation of ECRF3 (black) and ORF74-HHV8 (gray). All experiments were done in parallel (\(n = 5\)).
Constitutive and Ligand-regulated Activities of ECRF3

Inhibitors of two different enzymes in the early branches of the signal transduction pathways were used to describe the $G_q$ versus $G_i$ dependence of the transcriptional activity for the two receptors. We tested the effect of 100 ng/ml PTx (Gi inhibitor) and 10 μM U-73122 (PLC inhibitor) on the CXCL6/GCP-2 dose-response curves for ECRF3 (Fig. 7, A–C) and on the constitutive activity of ORF74-HHV8 (Fig. 7, D–F). The effect of the inhibitors was remarkably similar for the two receptors. For CREB activation, PTx had only very little effect, in agreement with the $G_q/G_i$-dependent nature of this pathway (32), whereas PTx resulted in a surprisingly high inhibition of 45–60%, and concomitant application of these two inhibitors totally abolished the NF-κB activity of both receptors (Fig. 7, B and E). NFAT is traditionally described as being $G_q$-dependent (32); and consistent with this, we found that U-73122 efficiently inhibited the activities by 55–70% inhibition of activity. Concomitant application of the inhibitors increased the inhibition to 85–95% (Fig. 7, A and D). The NF-κB activation of ECRF3 by CXCL6/GCP-2 was totally inhibited by U-73122, whereas the activation of ORF74-HHV8 was inhibited by 70%, consistent with the $G_i$-dependent nature of NF-κB (32). PTx resulted in a remarkably high inhibition of 95–100%, and the combination of these two inhibitors resulted in almost total inhibition of the NF-κB activity in both receptors (Fig. 7, B and E). NFAT reporter (pNFAT-Luc) (C and F). Shown is the effect of the Gi inhibitor PTx (100 ng/ml) and the PLC inhibitor U-73122 (10 μM), applied either alone (●, PTx; ▲, U-73122) or together (●), on the CXCL6/GCP-2 dose-response curve for ECRF3 (A–C) or the constitutive activity of ORF74-HHV8 (D–F). The activities in the absence of inhibitors are indicated (○). The effect of the inhibitors on vector-transfected cells is indicated (○ and □). The experiments for each transcription factor were done in parallel ($n = 3–10$).

Fig. 6. Transcription factor activity of ECRF3 compared with ORF74-HHV8. HEK293 cells were transiently transfected with ECRF3 (A–F) or ORF74-HHV8 (A–C) together with the transcription activation reporter CREB (pFA2-CREB) and the trans-activator (pFR-Luc) (A and D), the NF-κB reporter (pNF-κB-Luc) (B and E), and the NFAT reporter (pNFAT-Luc) (C and F). A–C, test for constitutive activation of the transcription factors CREB, NF-κB, and NFAT, respectively, by increasing receptor CDNA concentrations from 0 to 50 ng/well for ECRF3 (●), ORF74-HHV8 (▲), and the empty expression vector pcDNA3 (□). The experiments for each transcription factor were done in parallel for the two receptors and the expression vector. D–F, ligand-regulated activation of the different transcription factors with dose-response curves for the full agonist CXCL6/GCP-2 (●) upon ECRF3-mediated activation of CREB (D), NF-κB (E), and NFAT (F) ($n = 3–10$). RLU, relative light units.

Fig. 7. Similar regulation of transcription activation by ECRF3 and ORF74-HHV8. HEK293 cells were transiently transfected with ECRF3 (A–F) or ORF74-HHV8 (A–C) together with the transcription activation reporter CREB (pFA2-CREB) and the trans-activator (pFR-Luc) (A and D), the NF-κB reporter (pNF-κB-Luc) (B and E), and the NFAT reporter (pNFAT-Luc) (C and F). Shown is the effect of the Gi inhibitor PTx (100 ng/ml) and the PLC inhibitor U-73122 (10 μM) of the G, inhibitor PTx (100 ng/ml) and the PLC inhibitor U-73122 (10 μM), applied either alone (●, PTx; ▲, U-73122) or together (●), on the CXCL6/GCP-2 dose-response curve for ECRF3 (A–C) or the constitutive activity of ORF74-HHV8 (D–F). The activities in the absence of inhibitors are indicated (○). The effect of the inhibitors on vector-transfected cells is indicated (○ and □). The experiments for each transcription factor were done in parallel ($n = 3–10$).
inhibition (Fig. 7, C and F). Thus, NF-κB and NFAT were substantially influenced by the PLC inhibitor U-73122, in agreement with the known Gα dependence of these transcription factors (32). Surprisingly, both transcription factors were also inhibited by PTx, indicating that Gα plays a role in the regulation of these pathways. The content of the PLCβ isoform in HEK293 cells makes it most likely that the PTx effect on NF-κB and NFAT activation is through inhibition of βγ-mediated PLC activation. In fact, PTx inhibition of constitutive and/or ligand-induced NF-κB activation by ORF74-HHV8 has been reported previously by several groups (26, 27, 31).

**ECRF3 Constitutively Activates the Gi Pathway, and Agonists and Inverse Agonists Further Regulate This Signaling**—Most (if not all) of the endogenous chemokine receptors characterized so far signal in a PTx-sensitive and ligand-dependent manner through inhibition of adenylate cyclase (3). In addition, ORF74-HHV8 also constitutively activates Gi (26, 27, 31). To study constitutive Gi signaling of ECRF3, transiently transfected HEK293 cells were treated with forskolin. Stimulation of ECRF3 with CXCL6/GCP-2 resulted in inhibition of the forskolin-induced adenylate cyclase activity, with an EC50 of 0.3 mm (Fig. 8A). In contrast, CXCL1/GROα acted as a partial agonist (Fig. 8A), whereas CXCL8/IL-8 had no effect within the chosen concentrations (data not shown). The antagonist vCCL2/vMIP-II acted as an inverse agonist, as it inhibited the constitutive activity with a surprisingly high potency (EC50) of 0.14 mm, i.e. the highest potency ever observed for vCCL2/vMIP-II with any receptor yet tested (13, 21, 33). The chemokine modulations could be eliminated by the presence of 100 ng/ml PTx, indicating Gi as an involved G-protein (data not shown). In theory, cells expressing constitutively active Gi-coupled receptors display lower levels of cAMP production upon forskolin-induced adenylate cyclase stimulation compared with control cells (34). Consistent with this, we observed slightly lower levels of forskolin-induced adenylate cyclase stimulation in ECRF3-expressing cells compared with control cells. Importantly, PTx addition eliminated this difference, supporting the constitutive nature of Gi activation by ECRF3 (Fig. 8B).

**Constitutive and Ligand-regulated Activity of the Transcription Factor SRE by ECRF3 and ORF74-HHV8**—The SRE transcription factor has been shown to be dependent on Gi as well as G12/13, the latter via activation of Rh and Rho kinase (32, 35, 36). A gene dose experiment revealed constitutive signaling for ECRF3 (Fig. 9, A and B) as well as for ORF74-HHV8 (Fig. 9B) expressed in transiently transfected COS-7 cells through the SRE pathway. The constitutive activity of ECRF3 was ~2.5-fold higher than the basal cell activity (Fig. 9A), whereas the constitutive activity of ORF74-HHV8 was ~10-fold higher than the basal cell activity (Fig. 9B). Furthermore, both receptors where activated further by their respective agonists. Thus, CXCL6/GCP-2 stimulated the ECRF3-mediated SRE activity with a potency of 64 pM (Fig. 9C), whereas CXCL1/GROα stimulated the ORF74-HHV8-mediated SRE activity with a potency of 181 pM (Fig. 9D).
A similar scenario of constitutive and ligand-regulated activities was obtained in transiently transfected HEK293 cells (data not shown). PTx (100 ng/ml) was applied to determine the G\(_i\) contribution to the constitutive and ligand-regulated SRE activities. For both receptors, PTx was found to inhibit (albeit not eliminate) the constitutive activity. Thus, PTx resulted in a 47–55% reduction of the basal ECRF3 activity (Fig. 9A) and a 60–65% reduction of the basal ORF74-HHV8 activity (Fig. 9B). With respect to the ligand-mediated activities, PTx (100 ng/ml) resulted in a 50% reduction in the efficacy of CXCL6/GCP-2-induced ECRF3 activity, and the potency was concomitantly shifted 0.6-fold to the right. PTx did not reduce the efficacy of CXCL1/GRO\(_\alpha\) for ORF74-HHV8, but the potency was shifted 4-fold to the right. Thus, G\(_i\) contributed to the SRE activities of both receptors; however, since PTx only reduced (but did not eliminate) the activities, we investigated the G\(_{12/13}\) contribution by application of inhibitors of the G\(_{12/13}\)-dependent signal mediators Rho and Rho kinase. Thus, we applied the C3 exoenzyme (C3 transferase) from \textit{C. botalimum}, which irreversibly ADP-ribosylates and inactivates Rho (37, 38), and the Rho kinase inhibitor Y-27632 (39) to the constitutive as well as ligand-mediated activities of ECRF3 and ORF74-HHV8. Both inhibitors resulted in a reduction (but not elimination) of the SRE activities of both receptors (Fig. 9, E and F). Thus, the C3 exoenzyme (10 \(\mu\)g/ml) inhibited the constitutive activities by 34% and 46% for ECRF3 and ORF74-HHV8, respectively, whereas the ligand-regulated signaling (10 nM CXCL6/GCP-2 for ECRF3 and 10 nM CXCL1/GRO\(_\alpha\) for ORF74-HHV8) was inhibited by 65–67%. The SRE activities of the two receptors were similarly reduced by Y-27632 (10 \(\mu\)M) since the constitutive activities were inhibited by 31–34%, whereas the ligand-regulated activities were inhibited by 34% for ECRF3 and by 71% for ORF74-HHV8. The two inhibitors did not influence basal cellular SRE activity (data not shown). In summary, the constitutive and ligand-regulated SRE activities of both receptors were dependent on G\(_i\) as well as G\(_{12/13}\) activation.

**DISCUSSION**

In this study, we have characterized the molecular pharmacological profile of ECRF3 in terms of ligand binding and signaling activities and compared it with that of ORF74-HHV8. Different ELR CXC chemokines bound to ECRF3 with high and similar affinities, yet the maximal binding capacities varied dramatically for the three radioligands \(125\text{I}-\text{CXCL10}/\text{IP10}\), \(125\text{I}-\text{CXCL8}/\text{IL-8}\), and \(125\text{I}-\text{CXCL8}/\text{IL-8}\). ECRF3 did not bind the non-ELR CXC chemokine \(125\text{I}-\text{CXCL10}/\text{IP10}\), in contrast to ORF74-HHV8. Signal transduction analysis revealed activation of a broad range of pathways for ECRF3, similar to ORF74-HHV8. However, in contrast to ORF74-HHV8, which promiscuously activated all pathways constitutively, the ECRF3 receptor activated G\(_i\) and G\(_{12/13}\) (measured via SRE activation) constitutively, whereas the other pathways (G\(_{11}\) and the transcription factors NFAT, CREB, and NF-\(\kappa\)B) were activated solely in a ligand-regulated fashion.

**Ligand Repertoire for ECRF3 Compared with Other ORF74 Receptors**—In the initial study of ECRF3, CXCL1/GRO\(_\alpha\) was found to be the most potent agonist, followed by CXCL8/IL-8 and CXCL7/NAP-2 (9). In agreement with this, ORF74-HHV8 was later shown to recognize the GRO peptides (CXCL1–3) as agonists, with CXCL1/GRO\(_\alpha\) being the most potent agonist (6, 8, 13). Also ORF74 from HHV8 responds to GRO-related ELR motif-containing chemokines since the murine chemokines MIP-2 and KC (homologs of CXCL1/GRO\(_\alpha\)) in addition to LIX (homolog of CXCL6/GCP-2 and CXCL5/ENA78) act as agonists for this receptor (10). Our analysis of ECRF3 confirms that CXCL1/GRO\(_\alpha\) indeed is a highly potent agonist; however, another ELR CXC chemokine, CXCL6/GCP-2 was the most potent and efficacious agonist for ECRF3, with a 13 times higher potency and a 2.2 times higher efficacy with respect to G\(_i\) activation compared with CXCL1/GRO\(_\alpha\). The reason for the recognition of CXCL6/GCP-2 as the most potent agonist for ECRF3 and as an inverse agonist for ORF74-HHV8 (13) remains to be explained. The ELR CXC chemokines are chemottractants of neutrophils and are angiogenic, whereas the non-ELR CXC chemokines attract lymphocytes and are in general angiotropic (40–42). Two ELR CXC chemokines (CXCL8/IL-8 and CXCL6/GCP-2) bind to the structurally related CXCRI and CXCRII (43, 44), whereas the rest, e.g. the GRO peptides (CXCL1–3), are selective for CXCRII (45, 46). A selective non- peptide agonist (SB 225002) has been developed for CXCRII (47), and chemokines have been shown to bind differently to the two receptors (48, 49) and to be expressed differently (50). Thus, CXCL1/GRO\(_\alpha\) and CXCL6/GCP-2 (and CXCL8/IL-8) differ with respect to receptor recognition and physiological roles, which could explain the choice of CXCL6/GCP-2 as a full agonist for ECRF3. The lack of agonistic properties of CXCL8/IL-8 for ECRF3 expressed in COS-7 or HEK293 cells is in contrast to the initial agonistic properties for ECRF3 expressed in \textit{Xenopus laevis} oocytes (9). However, we observed a very low \(B_{\text{max}}\) for CXCL8/IL-8 compared with the \(B_{\text{max}}\) values for CXCL6/GCP-2 and CXCL1/GRO\(_\alpha\), and it could be anticipated that the lack of agonistic properties in our system was due to this diminutive \(B_{\text{max}}\) value for CXCL8/IL-8.

In this study, we observed no specific binding of the non-ELR CXC chemokine \(125\text{I}-\text{CXCL10}/\text{IP10}\) to ECRF3, and binding analysis with \(125\text{I}-\text{CXCL10}/\text{IP10}\) revealed a very low affinity for CXCL10/IP10. Thus, the binding and inverse agonistic properties of CXCL10/IP10 observed for ORF74-HHV8 (8, 13, 14, 51) and the antagonistic properties of murine and human IP10 (CXCL10) for ORF74-HHV8 (10) are not shared by ECRF3. This lack of CXCL10/IP10 binding (and lack of modulation and down-regulation of ECRF3 by CXCL10/IP10) is, however, explainable in light of the cellular tropism for HVS since HVS has tropism for T-cells, in contrast to the B-cell tropism of HHV8 and MVH8 (8, 51). CXCR3 is highly up-regulated and expressed abundantly in activated T-cells, and the CXCR3 ligands (e.g. CXCL10/IP10) are present in high concentrations in T-cell-rich areas (1, 52). Thus, an interaction of ECRF3 with CXCL10/IP10 and consequent inhibition would be potentially harmful for HVS.

vCCL2/vMIP-II encoded by HHV8 is an unnatural ligand for ECRF3; and during \textit{in vivo} settings, this ligand-receptor pair will never meet due to the virus-host discrepancy. Nevertheless, vCCL2/vMIP-II serves important purposes as determined in studies of virus-encoded receptors due to the promiscuous binding to the majority of endogenous and viral CC, CXC, and CX\(_C\) chemokine receptors, in most cases as an antagonist (8, 14, 21, 33) and more seldom as an agonist (53). We employed vCCL2/vMIP-II as a prototype antagonist and observed inverse agonistic and antagonistic properties of this chemokine for ECRF3.

**Constitutive and Non-constitutive Activities of ECRF3**—Besides the broad-spectrum ligand binding and the exploitation of several signal transduction pathways, the majority of virus-encoded receptors are unique due to the high degree of constitutive activity they usually display through several pathways (Table II) (6, 8, 26, 30, 54–58), in contrast to the quiescent endogenous chemokine receptors. In general, high constitutive activity is not common for endogenous 7TM receptors (59, 60); however, some receptors display high constitutive activity, e.g. the ghrelin and histamine receptors (61, 62), whereas the majority display low to undetectable levels of constitutive activity (60). In this study, we have characterized signaling pathways...
in close proximity to the receptor (G, Gi and G12/13) and signaling mediators farther downstream (transcription factors CREB, NFAT, NF-κB, and SRE). In contrast to the rest of the constitutively active virus-encoded receptors, we found, for ECRF3, selectivity in the constitutive activation of two of the three G-proteins investigated (G, and G12/13) but not (Gi). The constitutive Gi activity was measured directly by looking at the inhibition of cAMP production in transiently transfected HEK293 cells, in which vCCL2/vMIP-II functioned as an inverse agonist and CXCL6/GCP-2 functioned as an agonist (Fig. 8A) and in which PtX reversed the constitutive adenylate cyclase inhibition by ECRF3 seen after forskolin treatment (Fig. 8B). We also measured the constitutive Gi activity indirectly from the SRE activity in the presence of pathway-specific inhibitors since SRE has been described as being dependent on Gi, as well as on (Gi, Gi, and G12/13) (32, 35, 36). The constitutive SRE activity was 4–5-fold lower for ECRF3 compared with ORF74-HHV8 (tested in parallel) (Figs. 9B and 10 and Table III). We applied the Gi inhibitor PtX and the Rho and Rho kinase inhibitors C. botulinum C3 exoenzyme and Y-27632, respectively (Rho and Rho kinase are downstream mediators of Gi12/13 activation) (32, 35, 36), and observed partial inhibition of the constitutive as well as ligand-regulated activities of all three inhibitors, indicating a contribution of Gi as well as Gi12/13 to the SRE activation of these two receptors. The Gi contribution to the basal and ligand-regulated activities of ECRF3 was ~50% as judged from the influence of the inhibitors (47–55% inhibition by PtX concomitant with 34–67% inhibition by Y-27632 and C3 exoenzyme) of the constitutive and ligand-regulated activities of ECRF3 (Fig. 9, A, C, and E). For ORF74-HHV8, the Gi pathway was most important for the basal SRE activity (60–65% inhibition by PtX concomitant with 31–46% inhibition by Y-27632 and C3 exoenzyme, respectively) (Fig. 9, B and F), whereas Gi12/13 was most important for the ligand-regulated signaling (small effect of PtX and 65–71% inhibition by C3 exoenzyme and Y-27632) (Fig. 9, D and F). Thus, using these inhibitors, it became clear that the Gi activity (from SRE activation) was 6–7-fold lower for ECRF3 than for ORF74-HHV8, whereas the Gi12/13 activity was 3–4-fold lower for ECRF3 than for ORF74-HHV8 (Fig. 10 and Table III). Gi12/13 activation by ORF74-HHV8 is consistent with previously published data (31).

We observed no constitutive activity with respect to PI turnover (Gi), CREB, NFAT, and NF-κB for ECRF3 as opposed to a high constitutive activity for ORF74-HHV8 through all these pathways (Figs. 4 and 7), again tested in the same cell type and at the same time as ECRF3. The lack of constitutive activation of CREB, NF-κB, and NFAT (Fig. 7) was expected from the lack of constitutive PLC activation (Fig. 4) and the general Giq dependence of these transcription factors (32). However, the pathway inhibitors revealed that only the CREB activation was solely Giq-dependent, whereas the NF-κB and NFAT activation was G, and Gq-dependent for both receptors (Fig. 7). Thus, despite the direct and indirect evidence of constitutive Gi activity for ECRF3 (Figs. 8 and 9), there was no detectable constitutive activation of NF-κB and NFAT. Activation of the Gi dependence of NF-κB activation by ORF74-HHV8 has previously been shown in HeLa, COS-7, and lung endothelial cells (26, 27, 31). The lack of constitutive Gi activity was observed in terms of both PI turnover (Fig. 3) and CREB activation (Fig. 6); and for both assays, we could not detect any increase in basal activities, not even low activation corresponding to one-seventh of the ORF74-HHV8 activation. From our studies of the ORF74 receptor encoded by MHV68, we have been able to detect even very low increases in activation, as low as one-seventh to one-tenth of the corresponding activities of ORF74-HHV8.

**Ligand-regulated Activities of ECRF3**—Despite the lack of constitutive activity for Gi, CREB, NF-κB, and NFAT, we observed a brilliant ligand-regulated signaling through all pathways for ECRF3. Thus, CXCL6/GCP-2 induced a 5.4-fold increase above the basal non-constitutive Gi activity (Fig. 5A) and Gi activity for ECRF3 (Figs. 8 and 9), resulting in a maximal stimulation similar to the maximal CXCL1/GROα-mediated stimulation of ORF74-HHV8 (Fig. 4), tested in parallel for the two receptors. The PI turnover was not inhibited by the presence of PtX, indicating that the PLC activity is indeed a result of Gi activity in COS-7 cells and not mediated by the βγ-subunits released from activated Gi. In fact, an increase in efficacy for both receptors (as well as in the constitutive activity of ORF74-HHV8) occurred in the presence of PtX (Fig. 5B), indicating that inhibition of Gi potentiates PLC activation. This phenomenon has been observed before for ORF74-HHV8 expressed in endothelial cells (27) and is explainable by the law of mass action since we found that ECRF3 couples to several G-proteins, (Gi, Giq, and G12/13) the same has been shown for ORF74-HHV8 (6, 27, 29, 31, 63). A receptor in the active state couples only to one G-protein at a given time (64), and elimination of active Gi by PtX could raise the probability for an interaction with another G-protein and consequently result in an increase in PI turnover, as we observed for both receptors.

The CREB, NF-κB, NFAT, and SRE transcription factors were activated 2–5-fold above the basal activity of ECRF3 by CXCL6/GCP-2 (Figs. 6 and 9). SRE activation has never been characterized before for ORF74-HHV8. We observed a high constitutive SRE activity for ORF74-HHV8 that was increased further by CXCL1/GROα, whereas the constitutive ECRF3 activity was increased further by CXCL6/GCP (Fig. 9, C–F).

**Quantitative Comparison of ECRF3 and ORF74-HHV8** Signal Properties—Not only did the two receptors express very similarly as determined from the Bmax values for their full agonists (125I-CXCL6/GCP-2 binding to ECRF3 with a Bmax of 34 ± 6.7 fmol/10^6 cells and 125I-CXCL1/GROα binding to ORF74-HHV8 with a Bmax of 33 ± 5.9 fmol/10^6 cells), the total signaling capacities or efficiencies of ECRF3 and ORF74-HHV8 were also very similar. Thus, both in terms of G (Fig. 4) and G12/13.
TABLE III
Quantitative comparison of ECRF3 and ORF74-HHV8 signaling properties

| Type of comparison                        | Comparison ratio | G-proteins          |
|-------------------------------------------|------------------|---------------------|
| Constitutive signaling of ORF74-HHV8 vs. ECRF3 (C/C) | C (ORF74-HHV8/C (ECRF3)) | NDCA\(^a\) 6–7 3–4 4–5 |
| Ligand-regulated signaling of ORF74-HHV8 vs. ECRF3 (L/L) | L (ORF74-HHV8/L (ECRF3)) | 0.3 0.25 0.6 0.4 |
| Total activity (C + L) of ORF74-HHV8 vs. ECRF3 | C + L (ORF74-HHV8/C + L (ECRF3)) | 1 1.1 0.98 1 |
| Ligand-regulated vs. constitutive signaling (L/C) | ECRF3 | NDCA\(^a\) 4–5 4–5 4–5 |
|                                           | ORF74-HHV8 | 0.5 0.2 1 0.5 |

\(^a\) This type of comparison was done under the assumption that the relative levels of receptor expression did not vary among experiments.

The signaling pathways were determined in transiently transfected COS-7 cells (in parallel for the two receptors). The ratios were calculated from the data in Fig. 4 (G\(_1\)) and in Fig. 9 (SRE). The ratios for G\(_1\) and G\(_{12/13}\) were calculated from the SRE experiments (SRE included in last column) in the presence or absence of pathway-specific inhibitors (Fig. 9). NDCA, no detectable constitutive activity.

**Fig. 10.** Quantitative comparison of ECRF3 and ORF74-HHV8 signaling properties. A, the ligand-regulated (gray bars) and constitutive (black bars) activities of ECRF3 and ORF74-HHV8 (expressed in parallel in transiently transfected COS-7 cells) are shown here for the three different G-proteins (G\(_1\), G\(_2\), and G\(_{12/13}\)) activated by both receptors. 100% equals the total activity (ligand-regulated + constitutive) of ORF74-HHV8 for each pathway. B, the signaling pattern with respect to SRE activation is also provided since the analysis of this transcription factor provided the data for the G\(_1\) and G\(_{12/13}\) activities (by addition of pathway-specific inhibitors to SRE activity). The constitutive ORF74/ECRF3 activity ratio for each pathway is given by the height of the black bars; the ligand-regulated ORF74-HHV8/ECRF3 activity ratio is given by the height of the gray bars; and the ligand-regulated/constitutive activity ratio for each receptor in each pathway is given by the height of the gray versus black bars. (Table III summarizes these ratios.)

and G\(_{12/13}\) coupling, based on SRE activation in the absence/presence of inhibitors (Fig. 9), we observed a total stimulation in the same range for these two receptors (Fig. 10, full bars; and Table III). However, whereas ORF74-HHV8 displayed very high constitutive signaling (between 50 and 80% of the maximal signaling capacity) (Fig. 10, black bars; and Table III), in all signaling pathways that it had the ability to signal through, ECRF3 displayed clear constitutive activity (14–20% of the maximal signaling capacity) only through the G\(_1\), G\(_{12/13}\), and SRE pathways (Fig. 10, black bars; and Table III). In contrast to ORF74-HHV8, no sign of constitutive signaling was observed for ECRF3 through G\(_1\) or through the other tested transcription factors, despite the fact that the full agonist for ECRF3 stimulated this receptor to the same maximal signaling capacity as that observed for ORF74-HHV8 (Fig. 10 and Table III). Since the maximal signaling capacities in the various pathways were rather similar for the two receptors, this means that the ligand-mediated signaling was responsible for the major part of the signaling capacity for ECRF3 (ligand-regulated/constitutive signaling ratio (L/C) > 1) and only a minor part for ORF74-HHV8 (L/C < 1), as most clearly seen in Fig. 10 (gray versus black bars) and in Table III. The lowest ligand contribution compared with constitutive signaling was found for the G\(_1\) coupling of ORF74-HHV8 (L/C = 0.2). The G\(_2\) activity of ECRF3 (assessed by the cAMP inhibition experiments in HEK293 cells) (Fig. 8) resulted in a surprisingly low ligand regulation compared with the constitutive activity (L/C = 0.8). Thus, for this measurement (but not for the G\(_1\) component of SRE), the constitutive signaling slightly exceeded the ligand-regulated signaling (assuming that CXCL6/GCP-2 and vCCL2/vMIP-II are full agonist and full inverse agonist, respectively). This discrepancy in the ligand-regulated versus constitutive component of G\(_1\) signaling could be a reflection of the very different steps at which we measured in the G\(_1\) signaling cascade. Adenylyl cyclase inhibition is close to the G-protein, whereas SRE is far away and influenced by several regulators from the G-protein to the nucleus. In addition, adenylyl cyclase inhibition is dependent on pre-activation with forskolin. Since forskolin activates adenylyl cyclase in all cells, the actual window of G\(_1\) activity is therefore dependent on transfection efficiency.

In summary, the ligand-regulated signaling compared with the constitutive signaling was greater for ECRF3 than for ORF74-HHV8 in all cases. The ligand-regulated activities were also greater for ECRF3, whereas the constitutive activities were greater for ORF74-HHV8 (Fig. 10 and Table III).

**What Are the Roles of Virus-encoded 7TM Chemokine Receptors?**—Experimental evidence from receptor knockout studies indicates a certain importance of the chemokine receptors in the virus-host interplay. For instance, it has been shown that knockout of the murine cytomegalovirus-encoded receptor M33 results in a virus strain with decreased virulence and with less severe host infection (65). Similarly, deletion mutants of ORF74 from MHV68 point to a receptor function in the early reprogramming of the virus-infected cells and upon virus reactivation from latency (66, 67). Transgenic expression of ORF74-HHV8 in mice has shown that this receptor is important for Kaposi’s sarcoma development (19, 20) as a side effect of high constitutive activity since abrogation of the constitutive activity decreases the appearance of Kaposi’s sarcoma-like lesions (68).

In conclusion, virus-encoded chemokine receptors serve important functions, although they are not mandatory for virus survival. From a molecular pharmacology point of view, these receptors constitute a unique opportunity to study basic principles of ligand recognition, the activation mechanism of 7TM receptors, internalization, and recycling pathways as examples of targeted evolution, i.e. the receptors obtained from the host and through “combinatorial chemistry” structurally and functionally varied by random mutagenesis. The receptor phenotypes are highly interesting, with most of them being constitutively active. This study of ECRF3 addresses differences among virus-encoded chemokine receptors characterized so far with respect to ligand repertoire and signaling properties.
The CXC Chemokine Receptor Encoded by Herpesvirus saimiri, ECRF3, Shows Ligand-regulated Signaling through Gq, Gi, and G12/13 Proteins but Constitutive Signaling Only through G12 and G13 Proteins

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