High Constitutive Activity of a Virus-Encoded Seven Transmembrane Receptor in the Absence of the Conserved DRY Motif (Asp-Arg-Tyr) in Transmembrane Helix 3

Mette M. Rosenkilde, Thomas N. Kledal, and Thue W. Schwartz

Laboratory for Molecular Pharmacology, Department of Pharmacology, The Panum Institute, University of Copenhagen, Copenhagen N, Denmark (M.M.R., T.W.S.); and Clinical Research Unit, Hvidovre Hospital, Hvidovre, Denmark (T.N.K.)

Received January 16, 2005; accepted March 22, 2005

ABSTRACT

The highly conserved Arg in the so-called DRY motif (Asp-Arg-Tyr) at the intracellular end of transmembrane helix 3 is in general considered as an essential residue for G protein coupling in rhodopsin-like seven transmembrane (7TM) receptors. In the open reading frame 74 (ORF74) receptor encoded by equine herpesvirus 2 (EHV2), the DRY motif is substituted with a DTW motif. Nevertheless, this receptor signaled with high constitutive activity through Gi as determined by a receptor-mediated inhibition of forskolin-induced cAMP-production and by an induction of the serum response element-driven transcriptional activity through a pertussis toxin-sensitive manner. Ga and Gq were not activated constitutively as determined by the lack of inositol phosphate turnover and activities of the three transcription factors: cAMP response element-binding protein; nuclear factor-xB, and nuclear factor of activated T cells. Coexpression of the ORF74-EHV2 receptor with the promiscuous G protein Gqi4myr supported the constitutive Gi activation as determined by inositol phosphate turnover and CREB activation. The constitutive activity was inhibited by nonpeptide inverse agonists with micromolar potencies, and the chemokine CXCL6 acted as a high-affinity agonist. It is noteworthy that reconstitution of the DRY motif resulted in a 4- to 5-fold decrease of the constitutive activity. Both the wild type and the receptor with the reconstituted DRY motif were expressed at the cell surface as indicated by immunohistochemistry and enzyme-linked immunosorbent assay analysis. It is concluded that the Arg of the DRY motif in transmembrane helix 3 is not essential for G protein coupling based on the constitutive as well as the ligand-mediated activity observed for ORF74-EHV2.

G protein-coupled receptors consist of seven transmembrane α-helices (hence the name 7TM receptors) and are divided into different families based upon structural and functional properties. Chemokine receptors, that regulate leukocyte movement during homeostasis as well as during inflammation, belong to the rhodopsin-like 7TM receptors (class A) (Murphy et al., 2000). Receptors within this family contain several highly conserved regions, some of which have been shown to constitute important structural hallmarks for the activation process. An example is the conserved DRY motif in the intracellular end of TM3. The Arg is one of the most conserved residues among rhodopsin-like 7TM receptors. It is in general believed to be essential for proper G protein coupling among rhodopsin-like 7TM receptors based upon studies with charge conservative as well as charge nonconservative substitutions of this Arg and a consequent highly impaired signaling through G proteins (Rosenthal et al., 1993; Acharya and Karnik, 1996; Scheer et al., 1996; Lu et al., 1997; Wess, 1998; Gruithuijsen et al., 2004). On the other hand, other studies have shown the opposite for several 7TM receptors with gain of constitutive activity upon charge conservative as well as charge nonconservative substitutions of the Arg (Seibold et al., 1998; Fanelli et al., 1999; Scheer et al., 2000; Chen et al., 2001).

The ORF74 receptors (located in open reading frame 74) constitute one family of viral chemokine receptors encoded by...
members of the γ2-herpesviruses (Murphy et al., 2000). ORF74 receptors are found in human herpesvirus 8 (ORF74-HHV8), herpesvirus saimiri (ECRF3, also known as ORF74-HVS), murine-γ-herpesvirus 68 (ORF74-MHV68), and equine herpesvirus 2 (ORF74-EHV2) among other γ2-herpesviruses. The first three of these receptors have been characterized as CXC-chemokine binding receptors (functional homologs of CXCR2) with broad-spectrum ligand-dependent signaling and high constitutive activity through many (ORF74-HHV8), few (ORF74-HVS), or no pathways (ORF74-MHV68) (Ahuja and Murphy, 1993; Arvanitakis et al., 1997; Rosenkilde et al., 1999; Verzijl et al., 2004). The present article describes ORF74 from EHV2 for the first time. EHV2 is a widespread virus and can be isolated from almost all apparently normal horses (Telford et al., 1995). It has been associated with respiratory tract disease, conjunctivitis, and general malaise, yet the significance of EHV2 as a pathogen remains uncertain (Telford et al., 1995). ORF74-EHV2 is structurally related to the other ORF74 receptors, yet it lacks—as the only ORF74 receptor—the conserved Arg in the DRY motif and contains a DTW motif (Asp-Thr-Trp) instead (Fig. 1). Analyses of the ligand binding and signaling properties of the ORF74-EHV2 receptor uncover a high constitutive activity through Gi. The chemokine CXCL6 (chemokine nomenclature is given according the new nomenclature provided by Murphy et al., 2000) binds with nanomolar affinity and acts as an agonist, whereas chemically related nonpeptide inverse agonists inhibit the activity with micromolar potencies. It is noteworthy that a reconstitution of the DRY motif decreases the constitutive signaling.

The novel virus-encoded chemokine receptor described presently is the first example of a wild-type receptor that signals constitutively as well as ligand-dependent despite a lack of the DRY motif. The signaling mode of ORF74-EHV2 is however consistent with previously published data for mutated 7TM receptors where the Arg (in the DRY motif) has been substituted by other residues (Seibold et al., 1998; Fanelli et al., 1999; Scheer et al., 2000; Chen et al., 2001). Thus, it supports the concept that G protein coupling can occur, and even be improved, in the absence of the Arg in the DRY motif.

Materials and Methods

Materials. The human chemokines were from PeproTech (Rocky Hill, NJ) (CXCL1, -5, -6, -8, and -10) or kindly provided by Timothy N. C. Wells (Ares-Serono, Geneva, Switzerland; vCCL2). ORF74 from EHV2 was cloned from EHV2 DNA kindly provided by Andrew J. Davison (University of Glasgow, Glasgow, UK) and corresponded to the GenBank accession no. AAC13861. ORF74 from MHV68 was cloned from MHV68 DNA and corresponded to the GenBank accession no. AAF19338. ORF74 from HHV8 (GenBank accession no. U24275) was cloned from a Kaposi’s sarcoma skin lesion biopsy (Kledal et al., 1997). The chimeric G protein Gaq6aq4myr (abbreviated Gq4myr) was kindly provided by Evi Kostenis (7TM-Pharma, Hørsholm, Denmark) (Kostenis, 2001) and the chimeric G protein Gqs5 was kindly provided by Bruce Conklin (University of California).
nia, San Francisco, CA) (Conklin et al., 1996). LipofectAMINE 2000 and Opti-MEM 1 were from Invitrogen (Carlsbad, CA). Lymphoid substrate solution (LuciLite) was from PerkinElmer Life and Analytical Sciences (Boston, MA). 125I-CXCL1 and -8; [myo-3H]inositol (PT6-271); Bolton-Hunter reagent for iodination of CXCL5, -6, and -10; and anti-mouse horseradish peroxidase (HRP)-conjugated antibody were from Amersham Biosciences AB (Uppsala, Sweden). AG 1-X8 anion exchange resin [for phosphatidylinositol (PI) turnover assay] and AG-50W-X4 resin (for cAMP assay) were from Bio-Rad (Hercules, CA). Alumina, pertussis toxin (PTx), imidazole, 3-isobutyl-1-methylxanthine, and anti-FLAG (M2) antibody were from Sigma-Aldrich (St. Louis, MO) and the Y-27632 compound was from Calbiochem (Merck Biosciences, Läufenfingen, Switzerland).

Iodination of CXCL5, -6, and -10. The Bolton-Hunter reagent was dried by a gentle stream of nitrogen for 30 to 60 min. Then, 5 to 10 µg of chemokine was incubated on ice with 1.5 mCi 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-performance liquid chromatography.

Transfections and Tissue Culture. COS-7 cells were grown at 10% CO2 and 37°C in Dulbecco’s modified Eagle’s medium with Glutamax (Invitrogen) adjusted with 10% fetal bovine serum (FBS), 180 U/ml penicillin, and 45 µg/ml streptomycin (PenStrep). HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium adjusted to contain 4500 mg/l glucose with the same amount FBS and PenStrep. As the COS-7 at 10% CO2 and 37°C. The stably transfected HEK-293 cells were grown in HEK-293 media supplemented with G418 (Geneticin) 0.5 mg/ml. The cell media were modified to contain heat-inactivated FBS and no PenStrep during luciferase-based assays. Transfection of COS-7 and HEK-293 cells was performed by the calcium phosphate precipitation method (Rosenkilde et al., 1999) for the competition binding, the PI turnover experiments and the cAMP inhibition experiments, and by the cationic lipid reagent method with LipofectAMINE 2000 reagent in the serum-free media Opti-MEM 1 according to the manufacturer’s description for the luciferase-based transcription factor and enzyme-linked immunosorbent assay (ELISA) experiments and the microscopy.

Binding Experiments. COS-7 cells were transfected to 12-well culture plates 1 day after transfection. Then, 3 to 5 x 10^6 cells/well were used for the test of specific binding. Two days after transfection, cells were assayed by competition binding for 3 h at 4°C using 12 to 20 µM 125I-CXCL1, -5, -6, -8, -10 plus unlabeled ligand in 0.5 ml of a 50 mM HEPES buffer, pH 7.4, supplemented with 1 mM CaCl2, 5 mM MgCl2, and 0.5% (w/v) bovine serum albumin. After incubation, cells were washed four times in 4°C binding buffer supplemented with 0.5 M NaCl. Nonspecific binding was determined in the presence of 0.1 µM unlabeled chemokine. Determinations were made in duplicates.

Phosphatidylinositol Assay (PI Turnover). COS-7 cells were transfected with receptor cDNA and without the promiscous G protein Gq4myr. One day after transfection, COS-7 cells (2.5 x 10^4 cells/well in 24-well plates) were incubated for 24 h with 5 µCi/ml of [myo-3H]inositol in 0.3 ml of growth medium per well. Cells were washed twice in 25 mM HEPES, pH 7.2, supplemented with 0.75 mM NaH2PO4, 140 mM NaCl, and 0.05% (w/v) bovine serum albumin and 0.5 ml of HBS buffer supplemented with a 1 mM concentration of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was added with increasing concentration of the adenylyl cyclase activator forskolin to the cells. After 15-min incubation at 37°C, the cells were placed on ice, 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 and placed onto 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; Sigma-Aldrich). 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. Determinations were made in triplicate.

Constitutive SRE, NFAT, and NF-kB cis-Reporting and CREB trans-Reporting Luciferase Assay. HEK-293 cells were seeded at 35,000 cells/well in 96-well culture plates 24 h before transfection and were transfected with 50 ng/well (cis)-reporter plasmid (pSRE-LUC, pNFAT-Luc, or pNF-kB-Luc) and concentrations from 0 to 50 ng/well receptor plasmid. For the trans-reporting system, 50 ng/well trans-activator plasmid (pPR-Luc) was added together with 6 ng/well trans-reporter (pPA2-CREB) and receptor plasmid. The inhibitors of Gai (PTx; 100 ng/ml) and of G12/13 pathway (the Rho-kinase inhibitor Y-27632; 10 µM) were added to the constitutive SRE-transcription factor immediately after transfection. Twenty-four hours after transfection, the cells were washed twice in Dulbecco PBS and the luminescence was measured in a microplate scintillation and luminescence counter (TopCount; PerkinElmer Life and Analytical Sciences) after 10-min incubation in 100 µl of Dulbecco’s PBS together with 100 µl of Luc-Lite substrate. All determinations were made in quadruplicate.

Microscopy. HEK-293 cells were cultured on poly-L-lysine-treated glass coverslips (50,000 cells/well) and transiently transfected with 50 ng of receptor-green fluorescent protein GFP cDNA using LipofectAMINE transfection reagent according to the manufacturer’s instructions. Two days after transfection, the cells were analyzed using a Zeiss Confocor2 LSM-FCS confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with an Argon/2 laser (488 nm) using an apochromat 63x/1.4 oil differential interference contrast immersion lens.

ELISA. Cell surface expression was measured by the use of an ELISA technique. HEK-293 cells were transfected with the N-terminal FLAG-tagged variants of the ORF74-EHV2 receptors for the ELISA assay in parallel with cells used for the CREB assay. The cells were washed twice, fixed, and incubated in blocking solution (PBS/0.3% dry milk) for 60 min at room temperature. The cells were subsequently kept at room temperature and incubated 2 h with anti-FLAG (M2) antibody in a dilution 1:300. After three washes, the cells were incubated with anti-mouse HRP-conjugated antibody in a dilution 1:4000 for 2 h. The cells were subsequently washed once, and the immune reactivity was revealed by the addition of HRP substrate according to manufacturer’s instruction.

Calculations. IC50 and EC50 values were determined by nonlinear regression and Bmax values calculated using Prism 3 software (GraphPad Software Inc., San Diego, CA).

Results

ORF74-EHV2 Displays High Constitutive Activity in the Absence of the DRY Motif. Many virus-encoded chemokine receptors signal in a constitutive manner, often through many different pathways. The ORF74-HHV8 represents the broadest spectrum of constitutive activities, within
the ORF74 receptor family, with signaling through Gq, Gi, G12/13, and downstream for the G proteins a variety of mitogen-activated protein-kinases and transcription factors (Arvanitakis et al., 1997; Rosenkilde et al., 1999, 2004; Verzijl et al., 2004). ORF74-HHV8 was therefore used as a control for the Gq, NF-κB and NFAT pathways presented below. In contrast, ORF47 from HVS signals constitutively through Gi and G12/13 (Rosenkilde et al., 2004), whereas ORF74 from MHV68 has no constitutive signaling; instead, it has a ligand-mediated signaling through Gi (Verzijl et al., 2004). This known signaling pattern of the three other ORF74-receptors was exploited in the analysis of ORF74-EHV2.

Thus, the cAMP production was measured for increasing concentrations of forskolin in cells stably transfected with ORF74-EHV2 in parallel with ORF74-HVS and ORF74-MHV68. Using this approach, ORF74-EHV2 inhibited the forskolin-induced cAMP production to the same extent as ORF74-HVS and both receptors significantly reduced the forskolin-induced cAMP-production compared with the nonconstitutively active ORF74-MHV68 (Fig. 2). This indicates constitutive activity through Gi for ORF74-EHV2.

Gene dosage experiments in transiently transfected COS-7 cells revealed that ORF74-EHV2 has no constitutive activity via coupling to Gq because no increase in phosphatidylinositol turnover (PI turnover as a result of phospholipase C activation) was observed upon increasing concentrations of ORF74-EHV2 (Fig. 3A). Yet, upon cotransfection with the chimeric G protein Gqi4myr, a G protein α-subunit that is recognized as Gi by the receptors but transduces a Gq signal (Kostenis, 2001), a gene-dose-dependent increase in PI turnover was observed (Fig. 3A), indicating constitutive Gi activity. This increase was not observed upon cotransfection with wt Gq (Fig. 3A). The level of constitutive activity of ORF74-EHV2 corresponded to the level of activity for ORF74-HHV8 (constitutively active through Gq, Gi, and G12/13), with or without G protein cotransfection (Fig. 3B). In contrast, cotransfection of CXCR2 with the different G proteins did not result in any constitutive activity, yet in the presence of CXCL8, the CXCR2 receptor cotransfected with Gqi4myr elicited an increase in PI turnover (Fig. 3B) as expected from the Gi coupling for endogenous chemokine receptors (Murphy et al., 2000). To test for a putative Gs coupling of ORF74-

**Fig. 2.** ORF74-EHV2 inhibits cAMP-production like another constitutively active ORF74 receptor. Comparison of the receptor-mediated inhibition of forskolin-induced cAMP-production for ORF74-EHV2 (●) and two other Gi-coupled ORF74 receptors: the constitutively active ORF74 from HVS (■) (Rosenkilde et al., 2004) and the nonconstitutively active ORF74 from MHV68 (▲) (Verzijl et al., 2004) for increasing concentrations of forskolin. Data are shown as means ± S.E. (n = 4).

**Fig. 3.** ORF74-EHV2 is constitutively active through Gi. A, gene-dose experiments with measurement of PI turnover for increasing concentrations of ORF74-EHV2 in the presence of Gqi4myr (●), wt Gq (α-subunit) (■), or the vector (▲). Increasing concentrations of vector in the absence (○) and presence of Gqi4myr (○). B, PI turnover experiments of ORF74-HHV8 and human CXCR2 (test of Gqi4myr) under different receptor/G protein cotransfection settings. CXCL8 (100 nM) is added to CXCR2 under these settings. C, PI turnover experiments of ORF74-EHV2 and GIP receptor (test of Gq5) under different receptor/G protein cotransfection settings. GIP (100 nM) is added to the GIP receptor under these settings. D, gene-dose experiments with measurement of CREB activation for increasing concentrations of ORF74-EHV2 in the presence (●) or absence (○) of Gqi4myr or for increasing concentration of the vector in the presence (●) or absence (○) of Gqi4myr. E, gene-dose experiments with measurement of SRE activation for increasing concentrations of ORF74-EHV2 in the absence (●) or presence (○) of 100 ng/ml PTx or for increasing concentration of the vector in the absence (●) or presence (○) of PTx. F and G, gene-dose experiments with measurement of NF-κB (F) and NFAT (G) activation for increasing concentrations of ORF74-EHV2 (●), vector (○), or ORF74-HHV8 (▲). Data are shown as means ± S.E. (n = 3–11)
empty expression vector pcDNA3 or with Gq wt, indicating proper function of the Gq5 (Fig. 3C). The lack of Gs and Gq activity was supported by the fact that no constitutive activation of the CREB-transcription factor (that is regulated by Gs as well as by Gq; Hill et al., 2001) was observed for increasing concentrations of ORF74-EHV2 (Fig. 3D). In contrast, a pronounced gene-dose-dependent increase in CREB activity was observed upon cotransfection with GT4myr (Fig. 3D), again indicating a constitutive Gi coupling for ORF74-EHV2.

To further characterize the activation pattern of ORF74-EHV2, a panel of transcription factors were tested. Consistent with the lack of constitutive Gq activity for ORF74-EHV2, and the Gq dependence for NF-kB and NFAT (Hill et al., 2001), no constitutive activities were observed for these two factors (Fig. 3, F and G). ORF74 from HHV8 was used as a positive control for both transcription factors (Fig. 3, F and G) (Schwarz and Murphy, 2001; McLean et al., 2004). In contrast to the lack of constitutive NF-kB and NFAT activity for ORF74-EHV2, a dose-dependent increase in the SRE transcription factor was observed for increasing concentrations of ORF74-EHV2 (Fig. 3E). The SRE transcription factor activity has been reported to be regulated by Gi as well as G12/13 (Hill et al., 2001), yet no effect on the specific ORF74-EHV2-induced SRE-activity was observed upon addition of the Rho-kinase inhibitor Y-27632, indicating that there is no G12/13 component in this SRE activity (data not shown). In contrast, PTx addition resulted in a 100% decrease in SRE activity, indicating a solely Gi-mediated SRE-activity for ORF74-EHV2 (Fig. 3E). Together, these data indicate that ORF74-EHV2 is constitutively active through Gi but not through Gq, Gs, or G12/13.

Chemokine Binding Profile of ORF74-EHV2. ORF74 receptors from three other γ2-herpesviruses (ORF74 from HHV8, MHV68, and HVS) have previously been shown to bind so-called Glu-Leu-Arg (ELR) CXC-chemokines as agonists (e.g., CXCL1–3, -6, -10). ELR CXC-chemokines contain a Glu-Leu-Arg motif before the CXC motif and act predominantly via CXCR1 and -2, whereas non-ELR CXC-chemokines act predominantly via CXCR3–6. Two of these three ORF74 receptors in addition bind non-ELR-chemokines as antagonists (e.g., CXCL10 and/or -12) (Ahuja and Murphy, 1993; Rosenkilde et al., 1999, 2004; Verzijl et al., 2004). All three receptors also bind the CC-chemokine agonist encoded by HHV8, vCCL2. The ligand binding property of ORF74-EHV2 was determined by the use of a set of different radiolabeled ELR and non-ELR CXC-chemokines. Therefore, 125I-CXCL1, -5, -6, -8, and -10 were tested and only CXCL6 displayed specific binding (IC50 of 0.7 nM; Fig. 4A), albeit with a certain degree of unspecific binding to vector-transfected cells. Thus, the unspecific binding constituted around one-third of the total binding (IC50 of 1.5 nM for the total binding, and of 6.4 nM for the unspecific binding). All radiolabeled ELR CXC-chemokines (CXCL1, -5, -6, -8, and -10) bound to human CXCR2 and the radiolabeled CXCL1, -6, -8, and -10 in addition bound to ORF74-HHV8 (data not shown). This indicates proper function of the radiolabeled chemokines. The functions of CXCL1, -5, -6, -8, -10, and vCCL2 were evaluated in the PI turnover setup (with Gq4myr co-transfection) and as expected from the competition binding profile, only CXCL6 acted on this receptor as an agonist (EC50 of 1.1 nM; Fig. 4B). None of the chemokines elicited any response in the absence of Gq4myr, indicating that the ligand-mediated stimulation indeed is through Gi and not through Gq. vCCL2 had no effect on ORF74-EHV2, which was unexpected from its broad-spectrum binding profile and antagonistic and inverse agonistic actions on endogenous as well as other virus-encoded chemokine receptors (Kledal et al., 1997; Rosenkilde et al., 1999, 2004; Verzijl et al., 2004).

Inhibition of the Activity of ORF74-EHV2 by Non-peptide Inverse Agonists. Metal ion engineering of 7TM receptors is a way to predict helical connectivity and agonistic as well as antagonistic metal ion binding sites have been created in chemokine and other 7TM receptors (Elling et al., 1999; Elling et al., 1995; Elling et al., 1999; Rosenkilde et al., 1999, 2004; Holst et al., 2001). In the PI turnover setup (with Gq4myr cotransfec-
tion) a high-potency inverse agonistic metal ion site was observed for wt ORF74-EHV2 with a potency of 5.8 μM for ZnCl₂ (Fig. 5A). Even in complex with metal ion chelators (bipyridine or phenanthroline) that by themselves had no activities on ORF74-EHV2 (Fig. 5B), the metal ion chelator complexes inhibited the constitutive activity with similar high potencies as for the metal ion alone (IC₅₀ of 2.5 for zinc-phenanthroline and 4.5 μM for zinc-bipyridine; Fig. 5B). These high potencies were similar to the potencies for metal ion-engineered 7TM receptors in which a double-His site has been introduced in the top of TM5 (position 5:01 and 5:05; Fig. 5C). For example, in ORF74-HHV8, a ZnCl₂ potency of 1.6 μM was obtained by this double-His introduction (Rosenkilde et al., 1999) compared with a potency of 212 μM for the wt receptor (Fig. 5C). Introduction of the double-His site in ORF74-EHV2 only slightly increased the potency for ZnCl₂ (IC₅₀ of 2.9 μM; Fig. 5C).

The DTW Motif in ORF74-EHV2 Is Important for Receptor Activation. All other endogenous and virus-encoded chemokine receptors contain an Arg in the DTW motif. The conserved DTW motif was therefore introduced into ORF74-EHV2 in substitution for the DTW motif ([T142R;W143Y]-ORF74, hereafter called DRY ORF74-EHV2). Functional analysis of this mutant receptor in parallel with wt revealed an interesting phenotype, because a huge decrease (4- to 5-fold) in the constitutive activity was observed for DRY ORF74-EHV2 as shown for the CREB signaling and the PI turnover (Fig. 6, A and B). Albeit low compared with wt activity, the constitutive activity of DRY ORF74-EHV2 could still be inhibited by nonpeptide inverse agonists as exemplified by ZnCl₂ (IC₅₀ of 1.6 μM) and activated by CXCL6 (EC₅₀ of 0.5 nM) (Fig. 6C) provided cotransfection of the DRY ORF74-EHV2 with Gqi4myr. Thus, like the wt receptor, the DRY ORF74-EHV2 receptor showed no constitutive or ligand-mediated activity in the absence of the promiscuous G protein, indicating Gi selectivity also for the mutant receptor (data not shown). In addition, vCCL2 showed no inhibition of the DRY ORF74-EHV2, indicating a similar ligand binding pattern for the wt and the DRY ORF74-EHV2 receptor (Fig. 4B).

The surface expression of wt and DRY ORF74-EHV2 were studied to elucidate whether the decrease in the constitutive activity was caused by a lower surface expression. Confocal microscopy of cells transiently transfected with C-terminal GFP-tagged versions of the receptors showed that both receptors were expressed at the surface, albeit with an apparent lower surface expression of DRY ORF74-EHV2 compared with wt and a certain degree of intracellular localization for both receptors (Fig. 7, A and B). A quantitative estimate of the surface expression was obtained by N-terminal FLAG-tagged versions of the two receptors and uncovered an impaired surface expression for DRY ORF74-EHV2 compared

Fig. 5. Nonpeptide inverse agonists for ORF74-EHV2. A, dose-response curves for ZnCl₂ (PI turnover) on wt ORF74-EHV4 (■) and ORF74-HHV8 (▲). B, dose-response curves for two different metal ion chelators, phenanthroline (■, □) and bipyridine (◊, ◊) in complex with zinc (■, ◊), or alone (□, ◊) on wt ORF74-EHV4. C, dose response curves for ZnCl₂ on the metal site-engineered ORF74-EHV2 (○) and ORF74-HHV8 (▲). TM5 is shown with indications of the introduced double-His site in top (at position 5:01 and 5:05). The potency of ZnCl₂ on wt receptors (shown in A) are indicated as dotted lines. Data are shown as means ± S.E. (n = 3–6).

Fig. 6. DRY-reconstitution in ORF74-EHV2 results in a highly decreased constitutive activity, yet the ligand binding is preserved. A, gene-dose experiment with measurement of CREB-activity for increasing concentrations of wt ORF74-EHV2 receptor (■ and ○), DRY ORF74-EHV2 (■ and ○), and vector control (▲ and △) in the presence (black symbols) or absence (white symbols) of Gqi4myr. B, gene-dose experiments with measurement of PI turnover (with cotransfection of the receptor with Gqi4myr) for increasing concentrations of wt ORF74-EHV2 receptor (■ and ○) and DRY ORF74-EHV2 (■ and □) in the absence (black symbols) or presence (white symbols) of 10 μM ZnCl₂. C, dose-response curves for CXCL6 (■) and ZnCl₂ (△) in the PI turnover setup (with cotransfection of the receptor with Gqi4myr) for DRY ORF74-EHV2, where 100% equals the specific basal activity for DRY ORF74-EHV2. Data are shown as means ± S.E. (n = 3).
with wt (ELISA inserted in Fig. 7, A and B). However, for equal surface expression [for example, at 50 ng of DRY ORF74-EHV2 and 25 ng of wt ORF74-EHV2 (Fig. 7)], the activity was much higher (4.5-fold) for wt than for DRY ORF74-EHV2 as exemplified in the CREB activity (Fig. 6A). The C-terminal GFP-tagged and the N-terminal FLAG-tagged receptors signaled with the same pattern as the untagged receptors (data not shown). This indicates that the activity, and not only the surface expression, was influenced by the DRY reconstitution. ORF74-HHV8 has previously been shown to be predominantly surface expressed (Schwarz and Murphy, 2001; McLean et al., 2004), and surface expression of an N-terminal FLAG-tagged version of ORF74-HHV8 measured in parallel with the two ORF74-EHV2 receptors uncovered a slightly higher expression (~2-fold) for ORF74-HHV8 compared with wt ORF74-EHV2 (ELISA values, sum ± S.E.M. of 0.002 ± 0.001, 0.019 ± 0.011, 0.033 ± 0.015, and 0.050 ± 0.015 for 0, 10, 25, and 50 ng of ORF74-HHV8/ well compared with 0.004 ± 0.001, 0.011 ± 0.003, 0.015 ± 0.004, and 0.034 ± 0.012 for the same amount of ORF74- EHV2; Fig. 7A). This difference corresponded very well to the different $B_{\text{max}}$ values obtained by $^{125}$I-CXCL6 binding to ORF74-EHV2 (Fig. 4A) compared with $^{125}$I-CXCL6 binding to ORF74-HHV8 ($B_{\text{max}}$ of 9.1 ± 2.1 and 25 ± 6.4 fmol/10^5 cells, respectively; obtained in parallel).

**Discussion**

In the present study, the molecular pharmacology of ORF74-EHV2 is described. This receptor is unique because it lacks a highly conserved Arg in the conserved DRY motif in TM3 (contains a DTW motif instead). We observed a constitutive as well as ligand-mediated signaling and a decrease in signaling upon reconstitution of the DRY motif.

**Ligand Binding and Signaling of ORF74-EHV2.** CXCL6 was identified as the only chemokine for ORF74-EHV2 among the different CXCR-chemokines known to bind the other ORF74 receptors (Fig. 4A). The agonistic action of CXCL6 is not surprising, because CXCL6 also acts as a full agonist for ORF74-HVS (Rosenkilde et al., 2004) and for ORF74-MHV68 (M. M. Rosenkilde, unpublished data). However, the narrow binding spectrum is rather surprising in light of the broad-spectrum binding for the other ORF74 receptors (Ahuja and Murphy, 1993; Rosenkilde et al., 1999; Rosenkilde et al., 2004; Verzijl et al., 2004). In particular, the lack of vCCL2 action surprises because this chemokine inhibits many virus-encoded and endogenous chemokine receptors (Kledal et al., 1997; Rosenkilde et al., 1999, 2004; Verzijl et al., 2004). However, because equine CXC-chemokines are inaccessible at the moment, the true ligand binding spectrum of ORF74-EHV2 remains to be elucidated.

The activation pattern of ORF74-EHV2 was investigated by different assays measuring downstream events in the signaling cascade. Only one G protein (Gi) of four tested (Gi, Gq, Gs, and G12/13) was activated by ORF74-EHV2. This sole Gi activation differed from the promiscuous signaling through at least two and often three G proteins (Gi, Gq, and G12/13) for the other ORF74 receptors.

**Identification of Nonpeptide Inverse Agonists for Virus-Encoded Chemokine Receptors.** Naturally occurring metal ion binding sites are found occasionally (e.g., in the neurokinin 3 receptor) (Rosenkilde et al., 1999). In ORF74-EHV2, a natural-occurring high-potency inverse agonistic metal ion site was identified (Fig. 5). The obtained micromolar potencies were similar to the potencies obtained for nonpeptide inverse agonists for the human cytomegalovirus-encoded chemokine receptor US28 (Casarosa et al., 2003). Studies of virus-encoded chemokine receptors have uncovered an importance, although not mandatory, for virus survival in vivo (Davis-Poynter et al., 1997; Lee et al., 2003). Thus, ORF74-EHV2 could potentially be important for EHV2 life cycle. The development of nonpeptide inverse agonists will be important for the elucidation of the roles of these receptors in virus life cycle and as novel putative antiviral therapies and the nonpeptide inverse agonists presented here constitute, in principle, putative lead compounds in the development of antiviral drugs targeting ORF74-EHV2.

**The DRY Motif Is Highly Conserved and Important for Receptor Activity.** Activation of G proteins by 7TM receptors involves major movements of the transmembrane helices, in particular TM6 and TM7. Observed from the intracellular side of the membrane both helices move away from TM3, as shown for rhodopsin (Farrens et al., 1996). Observed from the extracellular side, these helices approach the intracellular side of the membrane both helices move away from TM3, as elucidated by metal ion engineering in the β2-adrenergic receptor (Elling et al., 1999). The helical movements allow high-affinity binding of the G protein, and the Arg in the DRY motif is considered to be essential here by forming intramolecular interactions to constrain the receptor in inactive and/or active conformations (discussed below). The absence of an Arg in the DRY motif is very seldom among

---

**Fig. 7.** Confocal microscopy of wild-type and DRY ORF74-EHV2. Cells were transiently transfected with C-terminal GFP-tagged versions of wt ORF74-EHV2 (A) and DRY ORF74-EHV2 (B). Insets present a quantitative estimate of the surface expression obtained by ELISA analyses of N-terminal FLAG-tagged receptors for increasing concentrations of DNA in transiently transfected cells. Data are shown as means ± S.E. (n = 5).
rhodopsin-like 7TM receptors. We analyzed 362 human rhodopsin-like 7TM receptors and only 15 lacked this Arg (~4%). In three cases, a Lys was found instead (~1%), and other residues were found in the rest. It is noteworthy that most of the Arg- (or Lys)-lacking receptors were found among olfactory and orphan receptors that all remain to be characterized. The C5A binding protein C5L2 (with a DLC motif) is one example of an Arg-lacking 7TM receptor. C5L2 has no G protein coupling; however, the G protein coupling is gained upon introduction of the Arg instead of the Leu (Okinaga et al., 2003). Many 7TM receptors (e.g., vasopressin, adrenergic, muscarinic, rhodopsin, and chemokine receptors) are dependent upon the Arg for proper signaling, because nonconservative substitutions often eliminate G protein signaling, whereas conservative substitutions retain activity, albeit with a lower efficacy (Rosenthal et al., 1993; Acharya and Karnik, 1996; Scheer et al., 1996; Lu et al., 1997; Wess, 1998; Grujthuijsen et al., 2004). On the other hand, many other receptors (e.g., adenosin A3, α1b- and β2-adrenergic and oxytocin receptors) are more active in the absence of the Arg, because conservative and nonconservative substitutions reveal gain of constitutive activity (Seibold et al., 1998; Fanelli et al., 1999; Scheer et al., 2000; Chen et al., 2001).

The Asp/Glu (67%/20%, respectively; Mirzadegan et al., 2003) in the DRY motif also influence receptor signaling because nonconservative substitutions reveal constitutive activity in some (Scheer et al., 1996; Burger et al., 1999; Ballesteros et al., 2001), but not all receptors (Lu et al., 1997; Capra et al., 2004). In contrast, the third position (Tyr in 67%, Phe in 11%, and Trp in 4%; Mirzadegan et al., 2003) is in general not important for receptor signaling and expression, as shown by mutational analysis (Lu et al., 1997; Wess, 1998).

The functional significance of the Arg (and Asp) in the DRY motif is supported by structural analyses. Thus, the crystal structure of (the inactive) rhodopsin has uncovered that the guanidinium group of the Arg forms hydrogen bonds with residues in TM3 and -6 and that it furthermore interacts directly with the neighboring Asp by ion pair formation (Wess, 1998; Okada and Palczewski, 2001). Supportive to this, structural and functional analyses have suggested ionic interactions to a conserved Glu in TM6 in addition to the neighboring Asp for the Arg in the nonconstitutively active β2-adrenergic receptor (mutagenesis of both acidic residues resulted in increased basal activity; Ballesteros et al., 2001). In summary, structural and functional analyses of many 7TM receptors have revealed that the Arg (in the DRY motif) is important for a stabilization of the receptor in inactive conformations. Yet, because many other 7TM receptors are dependent upon the Arg for proper activation, it must be concluded that the Arg is important for a stabilization of active as well as inactive receptor conformations, conceivably through intramolecular interactions with different residues. The activation pattern of wt ORF74-EHV2 substantiates that signaling can occur independently of an Arg, which is consistent with observations in many other receptors (Seibold et al., 1998; Fanelli et al., 1999; Scheer et al., 2000; Chen et al., 2001).

Is the Structure of ORF74-EHV2 Highly Different from Other 7TM Receptors? A detailed sequence analysis reveals that ORF74-EHV2 contains many structural hallmarks for rhodopsin-like 7TM receptors (Fig. 1). Thus, the extracellular regions contain the two conserved Cys involved in the disulfide bridge between TM3 and the extracellular loop 2 (EC2) (Schwartz and Holst, 2003). It also contains the two Cys responsible for making the chemokine-receptor related disulfide bridge between the N terminus and EC3 (Murphy et al., 2000). The transmembrane helices contain certain differences from the majority of rhodopsin-like 7TM receptors, yet these are often preserved among the other ORF74-receptors. TM1 is rather “normal” with the conserved Gly-Asn motif in position 16 and 17 (1:16 and 1:17), whereas TM2 lacks the conserved Asp and Leu (2:10 and 2:17). However, these are also missing in other ORF74 receptors. TM3 contains the conserved Cys in position 1 (3:01) and lacks the DRY motif (DTW instead). TM4 lacks the Trp (4:10), yet again this is the case also for the other ORF74 receptors. TM5 is basically normal, because ORF74-EHV2 contains the conserved Phe (5:13), Pro (5:16), and Tyr (5:24). TM6 contains some of the conserved residues in the CWxP motif (Cys, Trp, x, Pro motif in position 12–16) (Shi et al., 2002), because Trp and Pro are conserved, whereas Cys is substituted by a Thr. Yet, in all other ORF74 receptors this position contains amino acids different from Cys. Finally, TM7 contains the conserved Tyr (7:20) but not the Asn (7:16) and the Pro (7:17). These residues together with the conserved Phe in helix 8 constitute the NPxEx(x)5,6F motif with importance for receptor activation (Fritze et al., 2003). The Asn (7:16) is missing in all other ORF74 receptors, whereas the Pro (7:17) is missing in some of the ORF74 receptors. The importance of the NPxxY(x)5,6F motif has been characterized thoroughly, and the Tyr and Phe (both conserved in ORF74-EHV2) are the most important residues for signaling (Fritze et al., 2003). In conclusion, the structural analysis does not reveal any obvious explanation for the evolved Arg-independent G protein coupling mode.

Why Are the Virus-Encoded Receptors Constitutively Active? Many herpes and poxviruses encode 7TM receptors, and functional properties of these in immune eva-

sion have been suggested (Lee et al., 2003). The constitutive activity observed for the majority provides an obvious and easy way to interfere with cell reprogramming and gene expression, because no ligand is needed initially, and the activity is changeable in both directions upon ligand binding. However, the functional significance of the constitutive activity remains to be elucidated either by the development of high-potency inverse agonists or by the development of transgenic viruses that express receptors with no constitutive activity. Independently of the functional significance of the constitutive activity among viral 7TM receptors, the ORF74-EHV2 presented here is of particular interest from a general 7TM receptor structure-function point of view, because it has adapted an Arg (in DRY)-independent mode of ligand-mediated as well as constitutive G protein-dependent signaling.

Acknowledgments

We thank Evi Kostenis and Hans R. Luttichau for critical comments to the manuscript. We thank Inger Smith Simonsen and Rikke Larsen for excellent technical assistance. We thank Peter Baade (7TM-Pharma) for assistance with the receptor alignment. We thank Kristine Nerregaard for assistance with the confocal microscope and Ulrik Gether for the use of the microscope.
References


