Molecular Pharmacology Fast Forward. Published on December 6, 2005 as DOI: 10.1124/mol.105.015222
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MOL 15222

Title page:

Chemokine-directed trafficking of receptor stimulus to different G-proteins: selective inducible and constitutive signaling by human herpesvirus 6-encoded chemokine receptor U51.

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MOL 15222

Running title page:

Running title: Chemokine-induced trafficking of HHV-6 GPCR signaling

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Document statistics:

Text pages:	35	Tables:	0
Figures:	8	References:	40
Words in *Abstract*:	189	Words in *Introduction*:	530
Words in *Discussion*:	1,458		

Non-Standard abbreviations:

$[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; cAMP, adenosine3',5'-cyclic monophosphate; CRE, cAMP response element; CMV, cytomegalovirus; G-protein, guanine nucleotide-binding regulatory protein; GPCR, G-protein-coupled receptor; HHV, human herpesvirus; InsP, inositol phosphates; ORF, open reading frame; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; vGPCR virus-encoded G-protein-coupled receptor.

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Abstract

The human herpes virus 6 (HHV-6)-encoded chemokine receptor U51 constitutively activates phospholipase C (PLC) and inhibits cAMP-responsive element (CRE)-mediated gene transcription via the activation of $G_{q/11}$-proteins. Yet, chemokines known to bind U51 differentially regulate U51 coupling to G-proteins. CCL5/RANTES induced PTX-insensitive increases in PLC activity and changes in $[Ca^{2+}]_i$, while both CCL2/MCP-1 and CCL11/eotaxin failed to stimulate PLC activity or increase $[Ca^{2+}]_i$. In contrast, all three chemokines counteracted the effects of U51 on CRE activity via the activation of PTX-sensitive $G_{i/o}$-proteins. For each of the tested chemokines, co-expression of U51 with a variety of $G\alpha$ subunits, however, revealed a distinct profile for preferred G-protein coupling, which could be shifted by modulation of the relative expression of G-proteins. These findings are consistent with a chemokine-selective trafficking of receptor stimulus to distinct G-proteins and suggest that the constitutive activity of U51 and the chemokine-induced signaling involve different active states of the receptor. By virtue of its ability to constitutively activate signaling pathways, its G-protein promiscuity and the chemokine-directed trafficking of receptor stimulus, U51 can be considered a sensitive and versatile virally encoded signaling device, potentially of importance in HHV-6 related pathologies.

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Introduction

Chemokine receptors belong to the family of G-protein-coupled receptors (GPCRs) and are involved in the regulation of the immune response, inflammation and leukocyte trafficking (Rossi and Zlotnik, 2000). Interestingly, herpesviruses, including the human cytomegalovirus (HCMV), Kaposi's sarcoma-associated virus (KSHV) and human herpesvirus 6 and 7 (HHV-6, HHV-7), contain genes that encode for proteins with homology to mammalian chemokine receptors (Murphy, 2001; Sodhi et al., 2004). A prominent feature of these virus-encoded receptors (vGPCRs) is their ability to bind a broad spectrum of chemokines and to signal in a constitutively active manner, which is not apparent for their cellular homologues (Arvanitakis et al., 1997; Casarosa et al., 2001; Casarosa et al., 2003; Waldhoer et al., 2002). The HCMV genome encodes four GPCRs, of which US28 and UL33 are capable of constitutively activating several signaling pathways linked to inflammation in HCMV-infected cells (Casarosa et al., 2003; Minisini et al., 2003). Furthermore, GPCR homologues in rat and murine CMV (R78 and M78, respectively) are required for *in vivo* virulence in the absence of defined ligands, and M78 signaling can affect immediate early mRNA accumulation (Beisser et al., 1999; Oliveira and Shenk, 2001). These findings suggest that constitutive activity of vGPCRs is of physiological relevance and allows or facilitates viruses to take control of infected cells and host immune response (Sodhi et al., 2004). In addition, some of these vGPCR show promiscuous coupling to different G-proteins (Walhoer et al, 2002; Casarosa et al., 2003; Rosenkilde et al. 2004), while cellular chemokine receptors mainly couple to Gi/o-proteins (Rossi and Zlotnik, 2000).

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HHV-6 causes widespread, persistent infection. Under immune suppressive conditions HHV-6 reactivates and is associated with inflammatory conditions including encephalitis, transplantation diseases and has been linked to multiple sclerosis (Gompels, 2004). The HHV-6 genome encodes two proteins, U12 and U51, with homology to mammalian chemokine receptors considered as major candidates to contribute to inflammatory pathologies (Gompels, 2004). Previously, we have shown that U51 binds CC-chemokines, such as CCL5/RANTES, CCL11/eotaxin and CCL2/MCP1, chemokines associated with HHV-6 linked pathologies (Milne et al., 2000; Gompels, 2004, Dockrell , 2003; Grivel et al., 2003). In addition, U51 signals in a stable cell line of hemapoetic origin, but this could be either ligand-inducible or constitutive, as the cell line also secreted chemokine ligand (Milne et al., 2000). Very recently, U51 has been shown to positively regulate HHV-6 replication and enhance cell-cell fusion in vitro (Zhen et al., 2005).

So far, there is no detailed information available on the signaling properties of the HHV-6-encoded GPCR U51. In the present work, we present evidence of constitutive and differential chemokine ligand-inducible signaling. U51 constitutively activates phospholipase C (PLC) and inhibits CRE-mediated gene transcription through a PTX-insensitive pathway. This constitutive activity of the receptor can be differentially modulated by human chemokines. Moreover, in systems with reduced constitutive activity induced by expression of various Gα subunits, the tested chemokines display G-protein-selective agonistic behavior. These results support the notion of chemokine-specific trafficking of receptor stimulus to different G-proteins (Kenakin, 1995*b*), suggesting the existence of chemokine-selective receptor conformations for

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these versatile vGPCRs. The widespread constitutive activity and receptor-G-protein promiscuity displayed by vGPCRs implies a common behavior within this group of GPCRs, which may contribute to herpesvirus pathogenesis.

Material and Methods

DNA constructs. The pcDNA3-derived vector containing HHV6A encoded U51 (pcDNA3-U51) was generated as previously described (Milne et al., 2000). The reporter plasmid pTLNC-21CRE was obtained from W. Born (National Jewish Medical and Research Center, Denver, CO). Gifts of pcDNA3-based vectors containing the cDNAs of $G\alpha_q$ (Dr. B. Conklin), $G\alpha_{11}$ (Dr. H. Umemori), $G\alpha_t$ (Dr. B. Defize), GRK2 and GRK2K^{220}R (Dr. S. Cotecchia) and PTX-insensitive mutant $G\alpha_{o1}$, $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$ (Dr. G. Milligan) are gratefully acknowledged.

Cell culture and transfection. COS-7 cells were cultured and transiently transfected using the DEAE-dextran method as described (Casarosa et al., 2001). In all experiments the total amount of cDNA transfected was maintained constant by addition of the empty vector (pcDNA3).

Binding experiments. Labeling of CCL5 and binding in COS-7 cells were performed as described (Gruijthuijsen et al., 2002). In saturation binding studies, cells were incubated in buffer (50 mM HEPES (pH 7.4), 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA) containing concentrations of [^{125}I]-CCL5 ranging between 0.1 and 25 nM. In competition experiments, cells were incubated with 2 nM [^{125}I]-labeled CCL5 and various amounts of unlabeled CCL2, CCL5 or CCL11. Nonspecific binding was determined in the presence of 0.1 µM

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unlabeled CCL5. Cell count was performed in triplicates in reserved wells at the end of each experiment and used to calculate number of binding sites per cell.

[^{3}H]-inositol phosphates production. Cells were labeled overnight with *myo*-[2-^{3}H]inositol (1 µCi/ml) as described (Casarosa et al., 2001) in the presence or absence of PTX (100 ng/ml), washed for 10 min with Dulbecco's modified Eagle's medium containing 25 mM HEPES (pH 7.4), 0.5% BSA and 20 mM LiCl and incubated for 2 h in the same medium with or without chemokines indicated. [^{3}H]-inositol phosphates were isolated by anion-exchange chromatography and counted by liquid scintillation.

Reporter-gene assays. CRE-driven gene transcription was measured as described (Casarosa et al., 2003). COS-7 cells were transfected with pTLNC-21CRE and indicated plasmids. Transfected cells were incubated for 18 h in the presence or absence of PTX (100 ng/ml). Then, the indicated chemokines were added together with forskolin (10^{-5} M). 24 h after transfection, CRE-driven luciferase expression was measured by luminescence in a Wallac Victor^2 microplate reader.

Calcium measurements. Agonist-stimulated increases in $[Ca^{2+}]_i$ were quantified by monitoring the fluorescence of Fluo-4 AM-loaded COS-7 cells, using an automated NOVOstar microplate reader (BMG Labtech GmbH, Offenburg, Germany). 24 h after transfection cells were treated with PTX (100 ng/ml), when indicated. 48 h after transfection, cells were loaded in Hanks' balanced salt solution containing 20 mM HEPES, 2.5 mM Probenecid, 0.5% BSA, 2 µM Fluo-4 AM and 0.02% Pluronic F-127, (pH 7.4). Cells were

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washed three times and fluorescence measured (1 data point/second, excitation 485 nm, emission 520 nm) for 10 seconds to calculate the mean basal value. After agonist addition, fluorescence was recorded for 50 seconds. Changes induced by Triton X-100 (0.25% [v/v]) injection were recorded for further 10 seconds to determine the maximal fluorescence. Basal and maximal values determined for each well were used to normalize the data. Results are expressed as percent of maximal stimulation induced by Triton X-100.

Western blot analysis. Transiently transfected COS-7 cells were lysed 48 h after transfection in PBS containing 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 2 µg each of aprotinin and leupeptin per ml, sonicated, separated by SDS-polyacrylamide gel electrophoresis, and blotted onto a polyvinylidene difluoride membrane (NEN Life Science Products, Boston, MA). In order to estimate Gα-subunit expression, increasing amounts of recombinant $G\alpha_{i1}$ (sc-4232), $G\alpha_{i2}$ (sc-4222), $G\alpha_{i3}$ (sc-4223), $G\alpha_{o1}$ (sc-4224) and $G\alpha_{q}$ (sc-4226) subunits (Santa Cruz Biotechnology Inc, Santa Cruz, CA) were run on the same gel. The antibody recognizing $G\alpha_{i/o/t/z}$ (D-15) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used in combination with a rabbit anti-goat horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA). The antibody recognizing $G\alpha_{q/11}$ (C-19) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) was used in combination with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA, USA). Protein bands were detected by an enhanced chemiluminescence assay (PerkinElmer Life Sciences, Boston,

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MA) with an Image station (NEN Life Science Products, Inc., Boston, MA) and quantified using Kodak Digital Science 1D Image Analysis Software v3.0.2. Membranes were stripped and equal loading of the lanes was verified using an antibody recognizing β-actin (AC-15) (Sigma, St. Louis, MO) in combination with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA).

Data analysis. Curve fitting and data analysis were carried out by nonlinear regression analysis using Prism 4.0 and statistical analyses with InStat 3.0 (GraphPad Software, Inc, San Diego, CA). Specific binding was calculated by subtraction of nonspecific binding from total binding, which was in all of the cases below 5 % of the total radioactivity added. Specific binding in cpm was subsequently employed to calculate number of binding sites per cell by using the specific activity of the [^{125}I]-CCL5, as previously described (Gruijthuijsen et al., 2002). Unpaired *t* test (2 groups) or one-way ANOVA test (3 or more groups) were applied. Data are expressed as mean ± S.E.M. of three independent experiments run in triplicates.

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Results

Chemokine binding properties and constitutive activity of HHV-6-encoded receptor U51. COS-7 cells were transfected with cDNA coding for the HHV-6A U51 open reading frame (Milne et al., 2000). Using [^{125}I]-CCL5 as radioligand, specific and saturable binding to one single binding site was obtained, similar to that found previously using the stable cell line U51-K562 (Milne et al., 2000) (K_d: 2.4 ± 0.8 nM, B_{max}: 53,732 ± 7,327 sites/cell) (Fig. 1A). Increasing concentrations of transfected U51 cDNA resulted in concomitant increases in the number of binding sites (Fig. 1A, inset). [^{125}I]-CCL5 was homologously displaced by unlabeled CCL5 and heterologously displaced by two other human chemokines, CCL2 and CCL11, showing similar pK_i values for each chemokine ligand (pK_i: 8.78 ± 0.19 (CCL5), 8.50 ± 0.15 (CCL11) and 8.18 ± 0.24 (CCL2), *n*=3) (Fig. 1B).

Transfection of increasing amounts of U51 cDNA in COS-7 cells resulted in a PTX-insensitive, agonist-independent enhancement of inositol phosphate (InsP) levels, indicating that the U51 protein exists in a spontaneously active conformation presumably coupling to $G_{q/11}$-proteins (Fig. 2A). In addition, U51 expression resulted in a PTX-insensitive, agonist-independent decrease in CRE-mediated transcription (Fig. 2B), whereas the constitutive activity of HHV-8 ORF74 was sensitive to PTX, as previously described (Smit et al., 2002). In the absence of forskolin, U51 expression had no detectable effect on CRE activity (data not shown), indicating the inability of U51 to activate G_s-proteins.

Involvement of $G\alpha_{q/11}$ proteins in U51-mediated signaling. The receptor kinase GRK2 is known to scavenge both $G\alpha_{q/11}$ subunits as well as $G\beta\gamma$

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subunits, thus regulating signaling through a phosphorylation-independent mechanism as well (Premont et al., 1995; Sallese et al., 2000). Accordingly, coexpression of U51 with GRK2 or the kinase-deficient mutant GRK2K^{220}R (Diviani et al., 1996) led to an effective inhibition of U51-mediated InsP production (Fig. 3A and B). In contrast, coexpression with $G\alpha_t$, known to scavenge Gβγ subunits (Claphan and Neer, 1997), did not affect U51 constitutive signaling significantly (Fig. 3C). These results suggest that, in COS-7 cells U51 constitutively activates phospholipase C interacting with endogenous $G\alpha_{q/11}$ subunits as previously described for the G_q-coupled HCMV US28 GPCR (Casarosa et al., 2001).

Treatment of COS-7 cells with 0.3 µM of the calcium ionophore A23187 but not with 0.2 µM of the protein kinase C activator phorbol ester PMA, led to a marked reduction CRE transcriptional activity (82 ± 4% reduction of the maximal response induced by 10 µM FSK, n=4), suggesting that the observed effects probably involve the modulation of $[Ca^{2+}]_i$. Moreover, treatment of COS-7 cells which endogenously express the $G_{q/11}$-coupled histamine H_1 receptor, with 10 µM histamine resulted in a PTX-insensitive partial inhibition of CRE transcriptional activity (53 ± 4% reduction of maximal response induced by 10 µM FSK, n=4) and in a PTX-insensitive increase in $[Ca^{2+}]_i$ (not shown). The H_1 receptor antagonist mepyramine (1 µM) completely inhibited the effects of histamine on CRE transcriptional activity (93 ± 3% of maximal response induced by 10 µM FSK, n=4). These data demonstrated that activation of $G_{q/11}$-mediated pathways leads to inhibition of CRE-mediated transcription in COS-7 cells, indicating that the constitutive PTX-insensitive

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effects of U51 on CRE-mediated transcription most likely involve $G_{q/11}$-proteins.

Ligand-induced modulation of signaling pathways activated by U51. To determine the effects of human chemokines on the constitutive signaling of U51, we stimulated U51-expressing cells with increasing concentrations of CCL2, CCL5 or CCL11. As shown in Figure 4A and B, CCL5 induced a small but significant dose-dependent increase in basal InsP levels in the absence of PTX (185 ± 4% *vs.* 210 ± 5%, n=4, p<0.01, basal and stimulated respectively, pEC_{50}: 8.8 ± 0.3). Moreover, in cells pretreated with PTX (100 ng/ml, 24 h) the effect of CCL5 on InsP production was further increased (180 ± 5% *vs.* 229 ± 4%, n=4, p<0.01, basal and stimulated respectively), while for CCL2 and CCL11 a small statistically non-significant effect was observed only at 100 nM (Fig. 4B). The pEC_{50} value of CCL5 was not affected by PTX-treatment (not shown). These data demonstrate that if coupling of U51 to PTX-sensitive G-proteins is prevented, CCL5-induced signaling through PTX-insensitive G-proteins is facilitated.

Interestingly, treatment of U51-expressing cells with CCL2, CCL5 and CCL11 counteracted the reduction of CRE-mediated gene transcription generated by the constitutive activity of U51 (Fig. 4C). Chemokine-induced effects were completely abolished by pretreatment with PTX, indicating coupling of U51 to $G_{i/o}$-proteins upon chemokine binding.

Cellular chemokine receptors transduce extracellular signals, in particular, chemokine-induced increases in $[Ca^{2+}]_i$ mainly through $G_{i/o}$-proteins (4), although coupling to a broader range of G-proteins has been suggested (Arai, 1996). In COS-7 cells expressing U51 only CCL5 (30 nM) induced a marked

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increase $[Ca^{2+}]_i$ (20.2 ± 3.5% of the maximal response), while CCL2 and CCL11 had no significant effect within the dose range tested (up to 30 nM) (Fig. 4D). Yet, preincubation of cells with CCL2 and CCL11 completely antagonized the effect of CCL5 on $[Ca^{2+}]_i$, thus suggesting interaction with a common binding site on U51 (Fig. 4D, inset). In contrast to cellular chemokine receptors, in U51 expressing cells the transient increases in $[Ca^{2+}]_i$ induced by CCL5 were insensitive to PTX treatment (Fig. 4E). None of the tested chemokines had detectable effects on mock-transfected COS-7 cells (CCL5, Fig. 6A and B; CCL2 and CCL11 data not shown). These results indicate that both the constitutive activity and the CCL5-induced stimulation of U51 promote receptor coupling to PTX-insensitive G-proteins, presumably of the $G_{q/11}$ family.

Promiscuous coupling of U51 to different G-proteins. To study the ability of U51 to activate different G-proteins in more detail, we examined the effects of different G-proteins, including Gα subunits of both $G_{q/11}$ family and PTX-insensitive Gα subunits of the $G_{i/o}$ family (Wise, 1997) on constitutive U51 mediated signaling. In order to estimate the levels of overexpression of the various Gα subunits achieved by transient transfection of COS-7 cells, we used defined amounts of recombinant G-proteins as standards in Western blots, along with lysates from cells transfected with U51 and each Gα subunit (Fig. 5). All Gα subunits appear to be properly expressed as determined by Western blot analysis (Fig. 5), as previously reported (Wise et al., 1997). All transfected Gα subunits were found to be expressed within a (patho)physiologycal range (Davis et al., 2000) and at comparable levels (Fig. 5). All Gα subunits of the G_i family (Fig 5 A, B and C) and $Gα_{11}$ (Fig. 5 E) were

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expressed at approximately 50 ng protein/50µg total protein while $G\alpha_{o1}$ and $G\alpha_q$ were expressed at approximately 150 ng protein/50µg total protein and 25 ng protein/50µg total protein, respectively (Fig. 5 D and E). Binding of [^{125}I]-CCL5 to U51 was not altered upon co-expression of Gαi subunits U51, only increased upon expression of $G\alpha_q$ (2.6-fold), $G\alpha_{o1}$ (5.5-fold) or $G\alpha_{11}$ (12.4-fold).

The modulating effects on basal signaling, observed upon introduction of all the Gα subunits indicated functional expression of these proteins in COS-7 cells, as previously reported (Wise et al., 1997; Casarosa et al., 2003). Co-expression of U51 with $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_{o1}$, led to a significant decrease in basal levels of InsP (Fig. 6A). Conversely, co-expression of U51 with $G\alpha_q$ or $G\alpha_{11}$ led to a small but significant increase in the basal levels of InsP, as compared to cells expressing U51 only.

Similarly, co-expression of U51 with $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_{o1}$ reversed the constitutive, U51-mediated inhibition of CRE activity to levels similar to those induced by CCL5 in the absence of exogenous G-proteins (Fig. 6B). In contrast, co-expression of U51 with $G\alpha_{11}$ or $G\alpha_q$ further enhanced the U51-mediated, constitutive inhibition of CRE activity (Fig. 6B).

In the presence of exogenous G-proteins CCL5 (Fig. 6), CCL2 and CCL11 (not shown) failed to modulate both InsP production (Fig. 6A) and CRE activity (Fig. 6B) over the basal levels induced by the constitutive activity of U51.

Chemokine-specific stimulus trafficking to different G-proteins revealed by stimulus-biased systems. To further evaluate the influence of G-protein expression on inducible U51 signaling, we utilized stimulus-biased assay

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systems allowing detection of ligand-specific effects upon differential G-protein expression (Watson et al., 2000). Cells were transfected with U51 and various PTX-insensitive mutant $G\alpha_{i/o}$ subunits and pretreated with PTX to inactivate endogenous $G_{i/o}$-proteins (Wise et al., 1997). Thereafter, the ability of chemokines to induce increases in intracellular calcium in the presence of different G-proteins was determined. Expression of the different Gα subunits appeared comparable, as shown by western blot analysis in Fig. 5. Interestingly, these studies revealed chemokine-specific G-protein activation (Fig. 7). In cells co-expressing U51 and $G\boldsymbol{\alpha}_{i1}$ or $G\boldsymbol{\alpha}_{i2}$ CCL2 and CCL11-induced increases in $[Ca^{2+}]_i$, with CCL2 being the most efficacious agonist in those conditions, while CCL5 had no effect (Fig. 7A and B). Co-expression of $G\boldsymbol{\alpha}_{i3}$, however, revealed a different rank order of efficacies of the tested chemokines. In this condition, CCL5 induced a partial response whereas CCL11 had no effect, while CCL2 remained the most efficacious agonist under these conditions (Fig. 7C). In contrast to results using $G\boldsymbol{\alpha}_i$ proteins, co-expression of U51 with $G\boldsymbol{\alpha}_{o1}$ (Fig. 7D), $G\alpha_{11}$ (Fig. 7E) or $G\boldsymbol{\alpha}_q$ (data not shown) revealed a receptor phenotype that was only sensitive to CCL5. Co-expression of $G\alpha_{o1}$ resulted in a slightly reduced pEC50 for CCL5 ($G\alpha_{o1}$=8.60 ± 0.15 *vs.* no additional Gα subunits=9.02 ± 0.09, n=4 p=0.05, unpaired t test)**.** The pEC50 values for CCL5 in the presence of $G\alpha_{11}$ or $G\alpha_q$ were comparable to the pEC50 for CCL5 without expression of additional Gα subunits (Fig. 7).

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Discussion

Most virally encoded GPCRs of a variety of β- and γ-herpesviruses signal constitutively and show promiscuous G-protein coupling, unlike their cellular homologues (see Sodhi et al., 2004 for review). In this study we show constitutive and inducible signaling combined with novel vGPCR chemokine-induced trafficking of receptor stimulus.

We report for the first time that the HHV-6-encoded GPCR U51 constitutively activates PLC, via a PTX-insensitive $G_{q/11}$-linked pathway. The involvement of $G_{q/11}$-proteins in U51 mediated signaling was corroborated by coexpression of U51 with G_q and G_{11}-proteins, GRK2 or its kinase deficient mutant GRK2K^{220}R, which scavenges $G\alpha_{q/11}$ and Gβγ subunits, or the Gβγ subunits scavenger $G\alpha_t$. Co-expression with G_q and G_{11} led to a further increase in U51-mediated InsP production, while co-expression with GRK2 or GRK2K^{220}R resulted in efficient inhibition. $G\alpha_t$ had no effect on the constitutive activity of U51, indicating involvement of $G\alpha_{q/11}$ but not Gβγ subunits, as described for other constitutively active vGPCRs (Casarosa et al. 2001, Casarosa et al. 2003).

In contrast to other vGPCRs, U51 significantly and constitutively decreases CRE-mediated gene transcription in a PTX-insensitive manner. Since cellular chemokine receptors are known to couple to $G_{i/o}$-proteins (Rossi and Zlotnik, 2000), this PTX-insensitivity was surprising. CRE activity, however, is regulated by various signals, including cAMP, $[Ca^{2+}]_i$ and/or other signaling pathways (Shaywitz and Greenberg 1999). Control experiments indicate that in COS-7 cells activation of $G_{q/11}$-proteins increases $[Ca^{2+}]_i$ and inhibits CRE-mediated gene transcription, as well. This could be explained by the

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endogenous expression of adenylyl cyclase isoform IX in COS-7 cells, which is negatively regulated by increases in $[Ca^{2+}]_i$ (Paterson et al., 2000), Thus, in non-stimulated conditions U51 can adopt an active conformation that preferentially couples to $G_{q/11}$-proteins leading to PLC activation, subsequent increases in $[Ca^{2+}]_i$ and inhibition of CRE-mediated transcription.

U51 binds a variety of human CC-chemokines among which CCL2, CCL5 and CCL11 (Milne et al., 2000). Only CCL5 significantly induced increments in $[Ca^{2+}]_i$ and modestly activated PLC over basal signaling in a G_i-independent manner. CCL2 and CCL11 rather acted as competitive ligands, antagonizing the effects of CCL5 on calcium mobilization. All three chemokines induced reversal of the U51-mediated inhibition of CRE activity. These counteracting effects of the three chemokines could be considered as negative efficacy and hence the chemokines might be classified as inverse agonists (Neubig et al., 2003). Yet, their effects were completely inhibited by PTX treatment, indicating involvement of an active state of the receptor.

Our findings indicate that CCL2, CCL5 and CCL11 act as agonists at U51, trafficking the receptor signal between $G_{q/11}$ - and $G_{i/o}$-proteins (CCL5) or only to $G_{i/o}$-proteins (CCL2 and CCL11), whereas under non-stimulated conditions U51 constitutively signals mainly to $G_{q/11}$-proteins (Fig. 8). Based on this, we speculate that chemokine binding to U51 (partly) redirects U51 signaling to $G_{i/o}$-proteins. This is consistent with our observation that co-expression of U51 with different $G\alpha_{i/o}$ subunits results in a reduced activation of PLC. When relative expression levels of $G_{i/o}$-proteins are high, a redistribution of constitutively active U51 receptor states can occur and chemokines differentially traffic U51 signaling to $G_{i/o}$-proteins. Similarly, their PTX-sensitive

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effects on CRE-mediated transcription were abolished by over-expression of $G\alpha_q$ or $G\alpha_{11}$ subunits, indicating that shifting the relative expression in favor of $G_{q/11}$-proteins, eliminates any further coupling of U51 to $G_{i/o}$-proteins.

To study the promiscuous coupling of U51, we used PTX-resistant $G\alpha_{i/o}$ subunits (Wise et al., 1997) within stimulus-biased assay systems to detect chemokine-induced trafficking of receptor stimulus to individual $G_{i/o}$-proteins (Watson et al., 2000). These PTX-insensitive $G_{i/o}$ isoforms are similarly expressed and appear functional.

In the presence of additional $G\alpha_{i1}$ or $G\alpha_{i1}$subunits CCL2 and CCL11 selected receptor states that could couple to $G\alpha_{i1}$ and $G\alpha_{i2}$, although with different efficacies and potencies. Under these conditions CCL2 was the most efficacious and potent agonist. Interestingly, CCL5 did not induce any detectable response but was still able to specifically bind [^{125}I]-CCL5 (data not shown). In contrast, when the system was biased to $G\alpha_{i3}$, CCL2 evoked a response but CCL11 had no detectable effect, suggesting different ways for CCL2 and CCL11 to induce U51 signaling. Interestingly, the introduction of $G\alpha_{11}$ and $G\alpha_q$ induced significant increases in [^{125}I]-CCL5 binding. This was also the case for $G\alpha_{o1}$, which also recognizes receptor sates selected by CCL5 (Fig 7D). This is to be expected according to the Extended Ternary Complex Model of receptor occupancy (ETCM), since the degree to which agonist binding would be enhanced depends upon the relative stoichiometries of the G-proteins involved and their affinities for the receptor states (Watson et al., 2000). In the absence of additional Gα subunits, where U51 most probably couple to $G\alpha_{q/11}$–proteins, CCL2 and CCL11 could not induce calcium mobilization and antagonized the $G\alpha_{q/11}$–mediated effects of CCL5.

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Accordingly, CCL2 and CCL11 were unable to stimulate U51 when the system was biased to $G\alpha_q$ or $G\alpha_{11}$. Under these conditions or without additional Gα subunits, only CCL5 induced a robust calcium mobilization via PTX-insensitive $G\alpha_{q/11}$ proteins.

In cells co-expressing U51 and $G\alpha_{o1}$, only CCL5 was able to induce signaling. Co-transfected $G\alpha_{o1}$ is expressed and induced significant changes in the basal activity of U51 (Fig. 5 and 6), and slightly reduces the pEC50 to CCL5 in calcium mobilization experiments (Figure 7D). It can not be ruled out that the calcium response induced by CCL5 in $G\alpha_{o1}$ expressing cells reflects a response by endogenous $G_{q/11}$ proteins. These data reinforce the idea that each chemokine transfers U51 signaling to a distinct set of G-proteins, most likely by selecting various active receptor conformations (Fig. 8).

Recently, various examples of GPCRs exhibiting ligand-specific trafficking of receptor signals have been reported (see Kenakin, 2003 for references). Simulations with the ETCM showed that reversals of relative agonist efficacies could be affected by different ratios of two G-proteins (Kenakin, 2003). For constitutively active receptors the ETCM predicts increases in basal activity results in diminished maximal response to agonists (Chen et al., 2000), as observed here for CCL5 in cells expressing only U51 and in biased systems with added $G_{q/11}$-proteins. Moreover, modeling differential binding of agonists to multiple receptor conformations redistributes their relative abundances and the ratios of the corresponding signaling species (Chen et al., 2000). Accordingly, our data show that different chemokines (i.e. CCL2, CCL5 and CCL11) possess distinct efficacies towards U51, depending on the cellular context and the G-protein expression profile (Fig. 8).

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Agonist-directed trafficking of receptor stimulus to different G-proteins (Kenakin, 1995*b*) has been used to explain protean agonism (Kenakin, 1995*a*) invoking a redistribution of receptor species (Leff et al., 1997, Brink, 2002). For U51, the putative protean behaviour of chemokines could entirely be explained within the concept of ligand-directed trafficking of receptor stimulus. To our knowledge, results in this paper represent the first example of a chemokine/(viral)chemokine receptor system for which observations of this type can be explained directly by invoking such a mechanism. This may help to understand how the ligand-induced signaling is routed at chemokine receptors. Recently, inducible and constitutive signaling has been shown for the herpesvirus saimiri ECRF3 and equine herpesvirus 2 chemokine receptor (Rosenkilde et al., 2004, Rosenkilde et al., 2005). Ligand-induced and constitutive signaling occurred through different G-proteins, suggesting that the G-protein selectivity is unique for each vGPCR. Our current study on U51 takes these observations further in demonstrating not only selectivity in constitutive signaling but also selectivity in chemokine-induced signaling depending on the expression of specific G-proteins.

In conclusion, we present evidence for the existence of differential chemokine-induced activation of a constitutively active vGPCR, HHV-6 U51. The constitutive activity of U51 and the chemokine-induced signaling involve activation of different molecular pathways. U51 exists in a constitutively active state preferentially coupled to $G_{q/11}$-proteins, which can be differentially redistributed to different $G_{i/o}$-proteins upon binding of different chemokines. Considering the widespread observation of constitutive activity and receptor-G-protein promiscuity for (v)GPCRs, these observations on ligand-dependent

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re-directed signaling could be more common than previously recognized and may help to interpret the currently widespread claim of protean behavior of receptor ligands, particularly in different cellular contexts (Kenakin, 2003). Ligand-selected U51 conformations may be favored in membrane microdomains, such as lipid rafts, linked with $G_{i/o}$-proteins or caveolins linked with $G_{q/11}$-proteins (Oh and Schnitzer, 2001). This may be important for infections *in vivo* where resting lymphocytes lack caveolin, thereby affecting chemokine receptor activity (Venkatesan et al., 2003). Moreover, marked increases in G-proteins of the $G_{i/o}$ family have been reported upon differentiation of hematopoietic cells, which are well known targets of HHV-6 (Davis et al. 2000). These increases are much larger that the levels of overexpression induced by our transient transfections, suggesting that our observations are of physiological relevance. Thus, in a conceivable scenario in which U51 is expressed after HHV-6 infection in different cell types with distinct G-protein composition and chemokine expression profile, U51 signaling properties will lead to altered signaling, which could affect HHV-6 infection and its associated pathologies.

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Acknowledgments

C.P.F. acknowledges Dr. S. A. Fratantoni for critical revisions.

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Footnotes

This work was funded in part by The Netherlands Organization for Scientific Research (Jonge Chemische Wetenschappen) (C.P.F. and D.V.), Dutch Technology foundation (STW) (D.V. and H.F.V.) the Royal Netherlands Academy of Arts and Sciences (M.J.S.). U.A.G. thanks the support in part by the Royal Society and the Biotechnology and Biological Sciences Research Council (UK) at LSHTM.

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Legends for figures

Figure 1. Chemokine binding profile at U51. (A) Saturation binding using [^{125}I]-CCL5. COS-7 cells were transfected with 5 µg of U51 cDNA. 48 hours after transfection, [^{125}I]-CCL5 binding was measured. Nonspecific binding was determined in the presence of 0.1 µM unlabeled CCL5. Results are presented as specific binding (number of binding sites/cell). A representative experiment of three independent experiments, performed in triplicate is shown. Inset: specific binding of [^{125}I]-CCL5 (10nM) to COS-7 cells transfected with increasing amounts of U51 cDNA. (B) Displacement of [^{125}I]-CCL5 binding at U51 by CCL2, CCL5 and CCL11. COS-7 cells were transfected with 5 µg of U51 cDNA. 48 hours after transfection, cells were incubated with 2 nM [^{125}I]-CCL5 in the presence of various concentrations of the displacing chemokines. Nonspecific binding was determined in the presence of 0.1 µM unlabeled CCL5. Data are presented as percentage of U51 total specific binding. The average of three experiments, each performed in triplicates, is shown.

Figure 2. Constitutive activation of signaling pathways by U51. (A) PLC activity. COS-7 cells (1x10^6 cells) were transiently transfected with increasing amounts of U51 cDNA. 48 h after transfection InsP accumulation was measured, in cells treated with or without PTX (100 ng/ml). (B) CRE-mediated gene transcription. COS-7 cells (1x10^6 cells) were transiently transfected with increasing amounts of U51 cDNA, HHV-8 ORF74 (2 µg), or empty vector (mock, 5 µg) and pTLNC-21CRE (5 µg) and incubated in the presence or absence of PTX (100ng/ml). 18 h after transfection 10 µM forskolin was added. 24 h after transfection, CRE-driven luciferase expression was determined. The asterisk denotes a statistically significant difference *vs.* basal

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levels in cells expressing only U51 without PTX treatment. The average of four experiments, performed in triplicate, is shown.

Figure 3. Regulation of U51-mediated constitutive activity by GRK2. COS-7 cells ($1x10^6$ cells) were transiently cotransfected with U51 (2 µg) plus 5 µg vector (control, 0) or increasing amounts of GRK2 (A), GRK2-$K^{220}R$ (B) or $G\alpha_t$ (C) cDNAs as indicated. 48 h after transfection U51-mediated InsP accumulation was measured. Data are expressed as percentage of maximal stimulation in control cells. The average of three experiments, performed in triplicate, is shown.

Figure 4. Ligand-induced modulation of signaling pathways by U51. (A) Chemokine effect on U51-mediated PLC activity. COS-7 cells were transiently transfected with cDNA encoding U51 (2 µg/10^6 cells). Cells were incubated with the indicated chemokines (100 nM), and InsP production was measured. Results are expressed as percentage of vector-transfected (mock) control cells. The average of four experiments, each performed in triplicates, is shown. The asterisk denotes a statistically significant difference *vs.* non-treated U51 expressing cells. (B) Concentration-response curves for CCL2, CCL5 and CCL11. Cells were incubated with increasing concentrations of the indicated chemokine, and InsP production was measured. Results are expressed as percentage of maximal response induced by CCL5. The average of three experiments, each performed in triplicates, is shown. The effects shown for CCL2 and CCL11 were measured in PTX-treated cells. (C) Effects of various chemokines on U51-modulatory effects on CRE-mediated gene transcription. COS-7 cells (1 x 10^6 cells) were transiently transfected with U51 cDNA (2 µg) or empty vector (mock, 2 µg) and pTLNC-21CRE (5 µg)

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and incubated in the presence or absence of PTX. The asterisks denote a statistically significant difference *vs.* basal levels in non-treated U51-expressing cells. Results are expressed as percentage of the maximal response induced by forskolin in vector-transfected (mock) cells. The average of four experiments, performed in triplicates, is shown. (D) Effects of chemokines on $[Ca^{2+}]_i$. COS-7 cells were transiently transfected with U51 cDNA, (2 µg/10^6 cells). 48 h after transfection, cells were loaded with Fluo-4 AM and exposed to chemokines indicated (30 nM). Inset: preincubation of U51 expressing cells (10 min) with 30 nM CCL2, or CCL11 on CCL5-induced (30 nM) $[Ca^{2+}]_i$ mobilization. (E) Insensitivity to pertussis toxin of CCL5-mediated $[Ca^{2+}]_i$ mobilization. COS-7 cells were transiently transfected with U51 cDNA, (2 µg/10^6 cells) and treated with or without PTX (100 ng/ml) for 24 h. 48 h after transfection, cells were loaded with Fluo-4 AM and exposed to CCL5 (30 nM). Results are shown as percent maximal response induced by Triton X-100. A representative experiment of three independent experiments, performed in triplicate is shown.

Figure 5. Expression of Gα subunits. (A to D) Expression of $G\alpha_{i/o}$. COS-7 cells (1 x 10^6 cells) were transiently transfected with cDNA encoding U51 (2µg) and different Gα subunits (2µg each) or empty vector (4µg). Expression of $G\alpha_{i/o}$ subunits was determined by Western blot analysis using an antibody against $G\alpha_{i/o/t/z}$ (top panels). To ensure equal loading of the lanes, the blots were stripped and checked for expression of β-actin (bottom panels). Increasing amounts of recombinant $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ or $G\alpha_{o1}$ subunits were run on the same gel to estimate Gα-subunit expression. (E) Expression of $G\alpha_{q/11}$. COS-7 cells (1 x 10^6 cells) were transiently transfected with cDNA encoding

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U51 (2µg) and $G\alpha_q$ or $G\alpha_{11}$ subunits (2µg each) or empty vector (4µg). Expression of $G\alpha_{q/11}$ subunits was determined by Western blot analysis using an antibody against $G\alpha_{q/11}$ (top panel). To ensure equal lane loading, the blots were stripped and checked for expression of β-actin (bottom panel). Increasing amounts of recombinant $G\alpha_q$ subunits were run on the same gel to estimate Gα-subunit expression. Quantification of the bands (bar diagrams) was done with Kodak Digital Science 1D Image Analysis Software v3.0.2. A representative experiment of three independent experiments, is shown.

Figure 6. Effect of various Gα subunits on the chemokine-induced and constitutive signaling of U51 (A) Effect of co-expression of U51 with various Gα subunits on U51-induced PLC activity. COS-7 cells (1 x 10^6 cells) were transfected with either U51-encoding cDNA (basal, 2 µg) or empty vector (mock, 2 µg) and cDNAs encoding different Gα subunits (2 µg/10^6 cells) or not. Cells were incubated with CCL5 (100 nM) when indicated, and InsP accumulation was measured. Results are shown as percentage of vector-transfected (mock) control cells. (B) Effect of co-expression of U51 with various Gα subunits on U51-modulated CRE-mediated gene transcription. COS-7 cells (1 x 10^6 cells) were transfected with either U51-encoding cDNA (basal, 2 µg) or empty vector (mock, 2 µg), and cDNAs encoding different Gα subunits (2 µg) or not and pTLNC-21CRE (5 µg). Cells were incubated with CCL5 (100 nM) when indicated, and CRE-driven luciferase expression was determined. Results are shown as percent maximal response induced by forskolin in vector-transfected cells. The asterisks denote a statistically significant difference *vs.* basal levels present in U51-expressing cells (p<0.05,

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n=3). In both panels, the average of three experiments, performed in triplicate, is shown.

Figure 7. Responses to chemokines in G-protein biased assay systems. COS-7 cells were transfected with 2 µg pcDNA3-U51/10^6 cells and 2 µg/10^6 of different Gα subunits or pcDNA3 vector alone. 24 h after transfection cells were exposed to 100 ng/ml PTX. 48 h after transfection, cells were loaded with Fluo 4-AM and exposed to increasing concentrations of chemokines. Calculated pEC_{50} values in the presence of the different Gα subunits were: for CCL2, 9.92 ± 0.17 (A, $Gα_{i1}$), 9.53 ± 0.16 (B, $Gα_{i2}$) and 9.91 ± 0.19 (C, $Gα_{i3}$) (*p*=0.25, not significant); for CCL5, 8.88 ± 0.18 (C, $Gα_{i3}$), 8.60 ± 0.15 (D, $Gα_{o1}$), 9.05 ± 0.11 (E, $Gα_{11}$), and 9.02 ± 0.09 (F, no additional Gα subunits) (*p*=0.15, not significant, ANOVA test). Data are shown as percent maximal response induced by Triton X-100. The average of four experiments, performed in triplicate, is shown.

Figure 8. Schematic representation of constitutive and chemokine induced signaling of U51. The scheme is based on the Three State Model of agonist action (35). U51 exists in a resting conformation (middle) and at least two active conformations, which interact with different G-proteins. The active conformation 1 (right) interacts with $G_{q/11}$-proteins and the active conformation 2 (left) interacts with $G_{i/o}$-proteins. In the absence of an agonist the distribution of the receptor states is governed by the equilibrium constants L and M. In view of the observed constitutive activity of U51 the equilibriums are spontaneously displaced to the active conformation 1, resulting in constitutive activation of $G_{q/11}$-proteins. The presence of an agonist redistributes the receptor states, trafficking the signaling of U51 to different G-proteins. CCL5,

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a more pleiotropic agonist, selects $G_{q/11}$- and $G_{i/o}$-coupled active states, resulting in CCL5-induced increases in PLC activity and $[Ca^{2+}]_i$ mobilization. Conversely, CCL2 and CCL11 select only $G_{i/o}$-coupled active states. The redistribution of receptor states induced by CCL2 and CCL11 results in an apparent decrease in the inhibition of CRE activity induced by the spontaneously favoured active state 1. In the presence of exogenous $G\alpha_{i/o}$ subunits CCL2- and CCL11-differentially induced $[Ca^{2+}]_i$ mobilization.

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Figures **Mol# 15222**

Figure 1

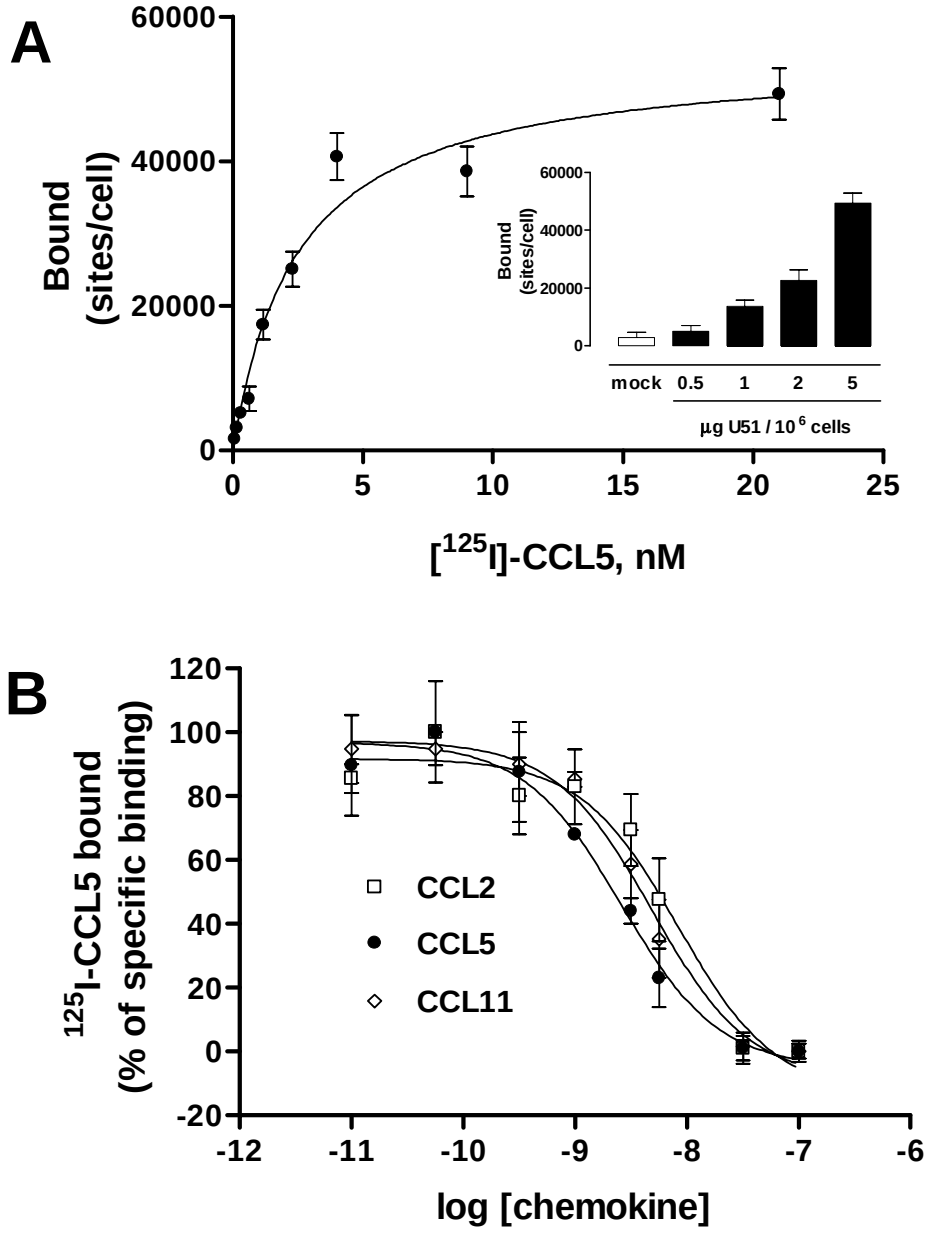

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Figure 2

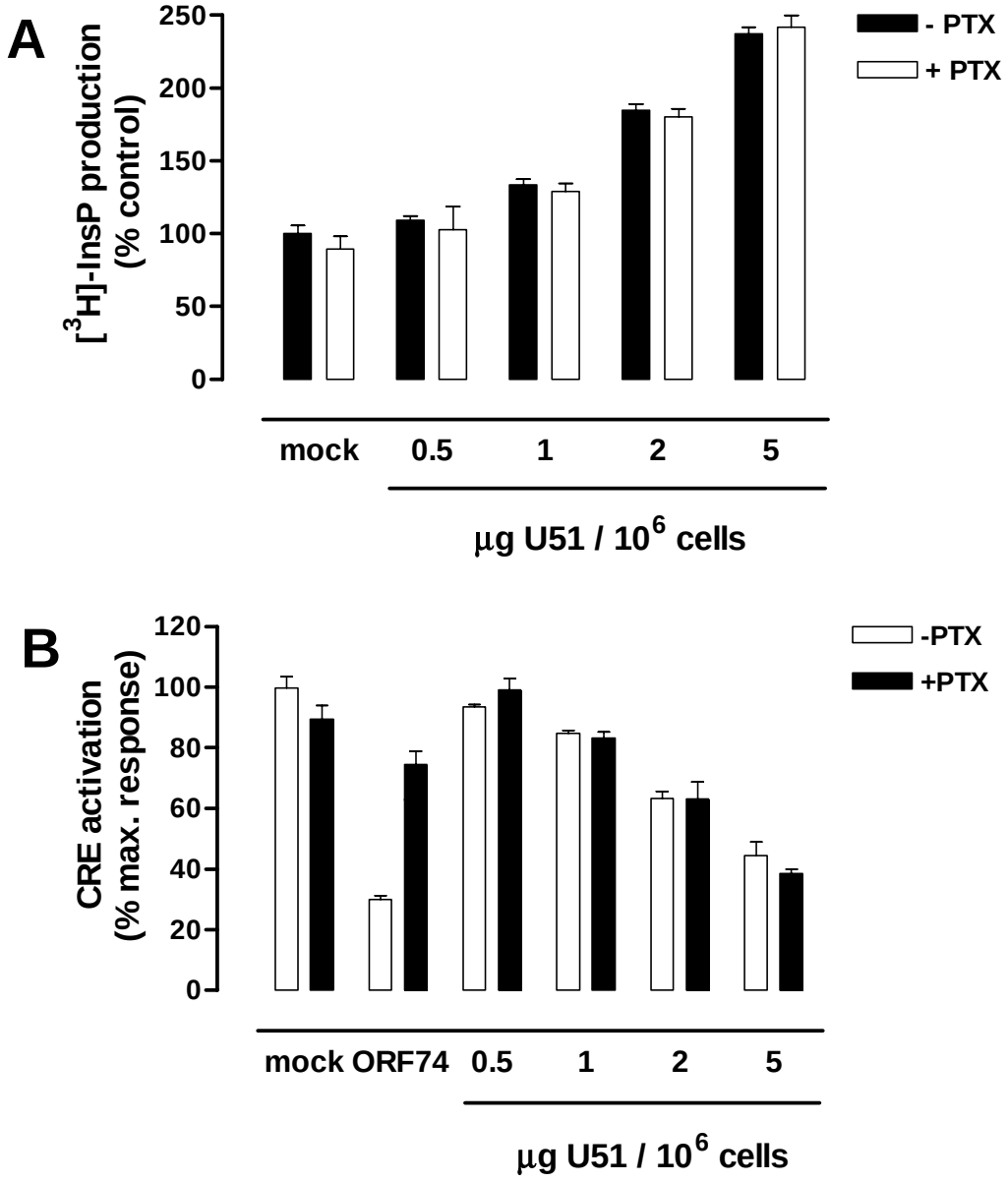

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Figure 3

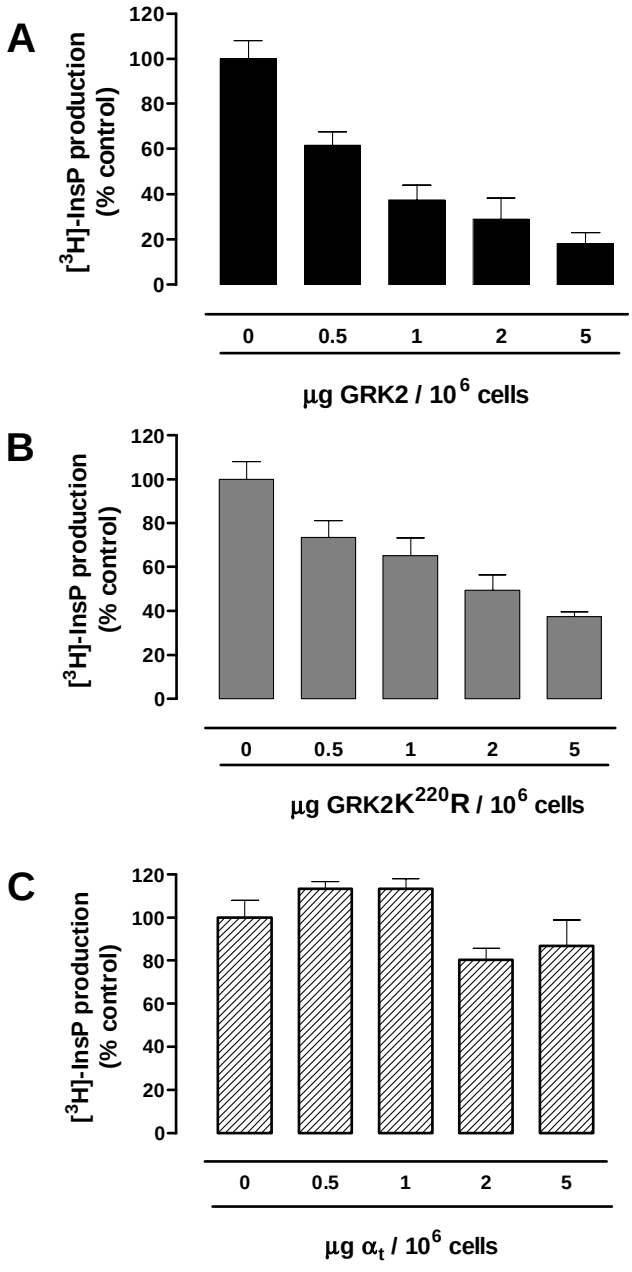

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Figure 4

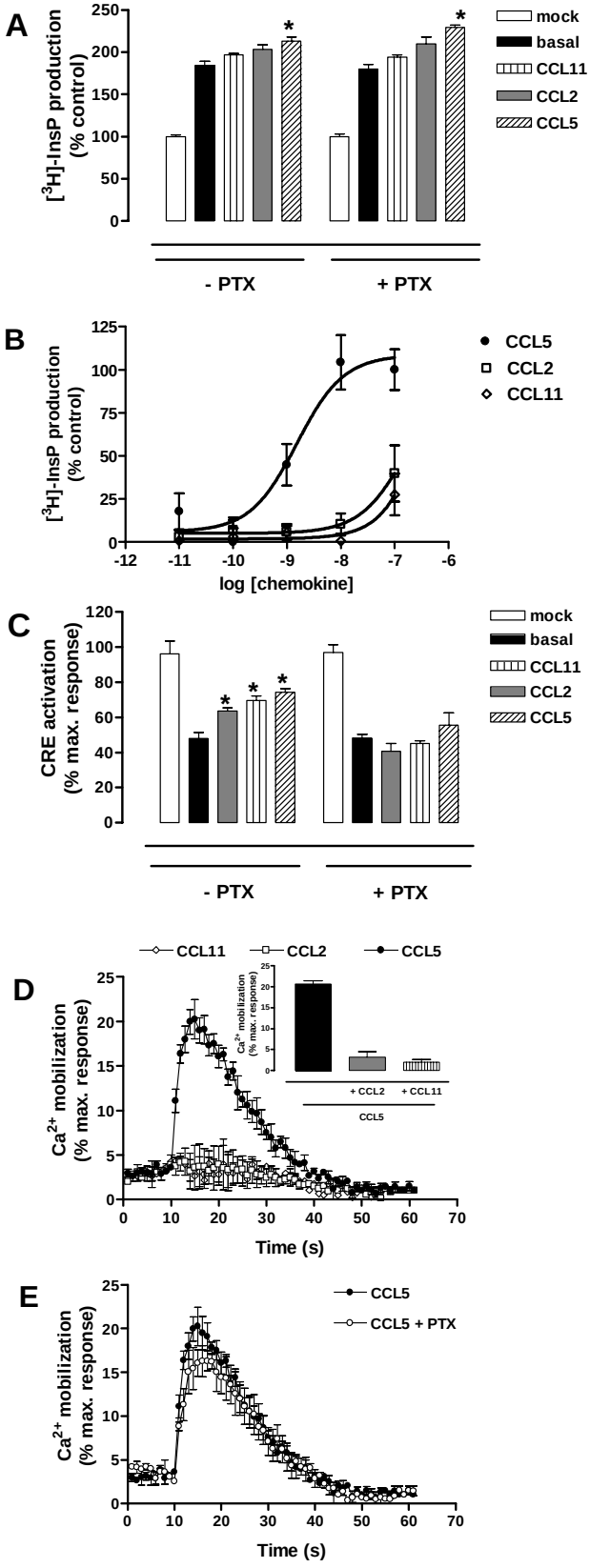

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Figure 5

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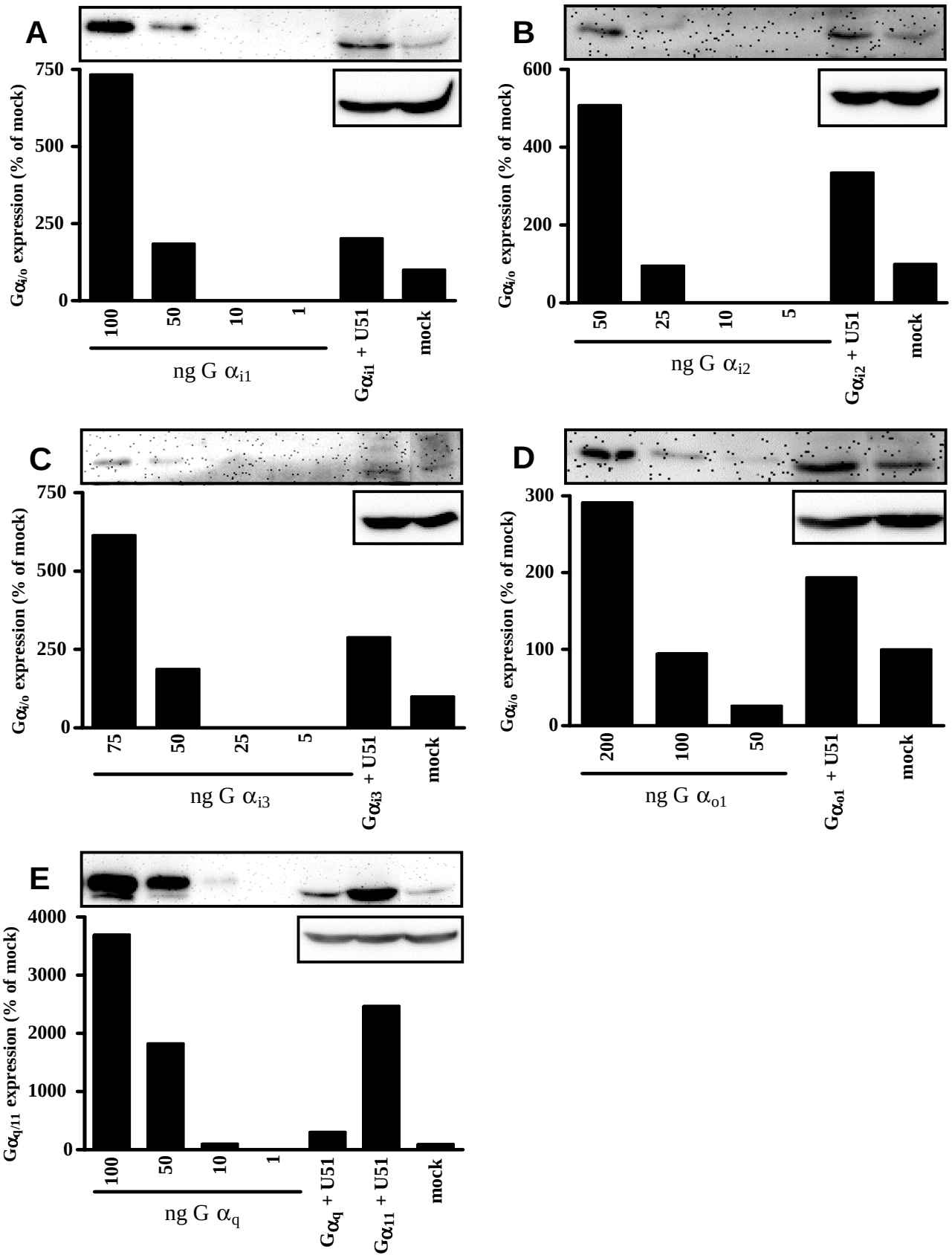

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Figure 6

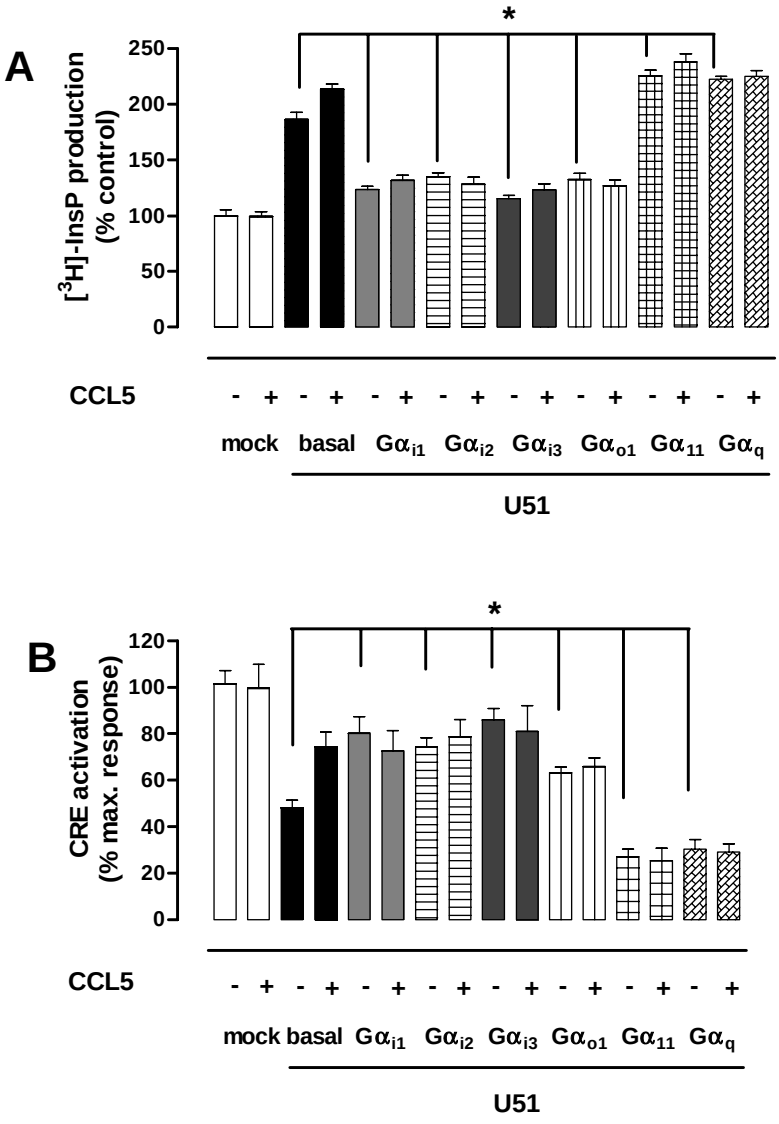

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Figure 7

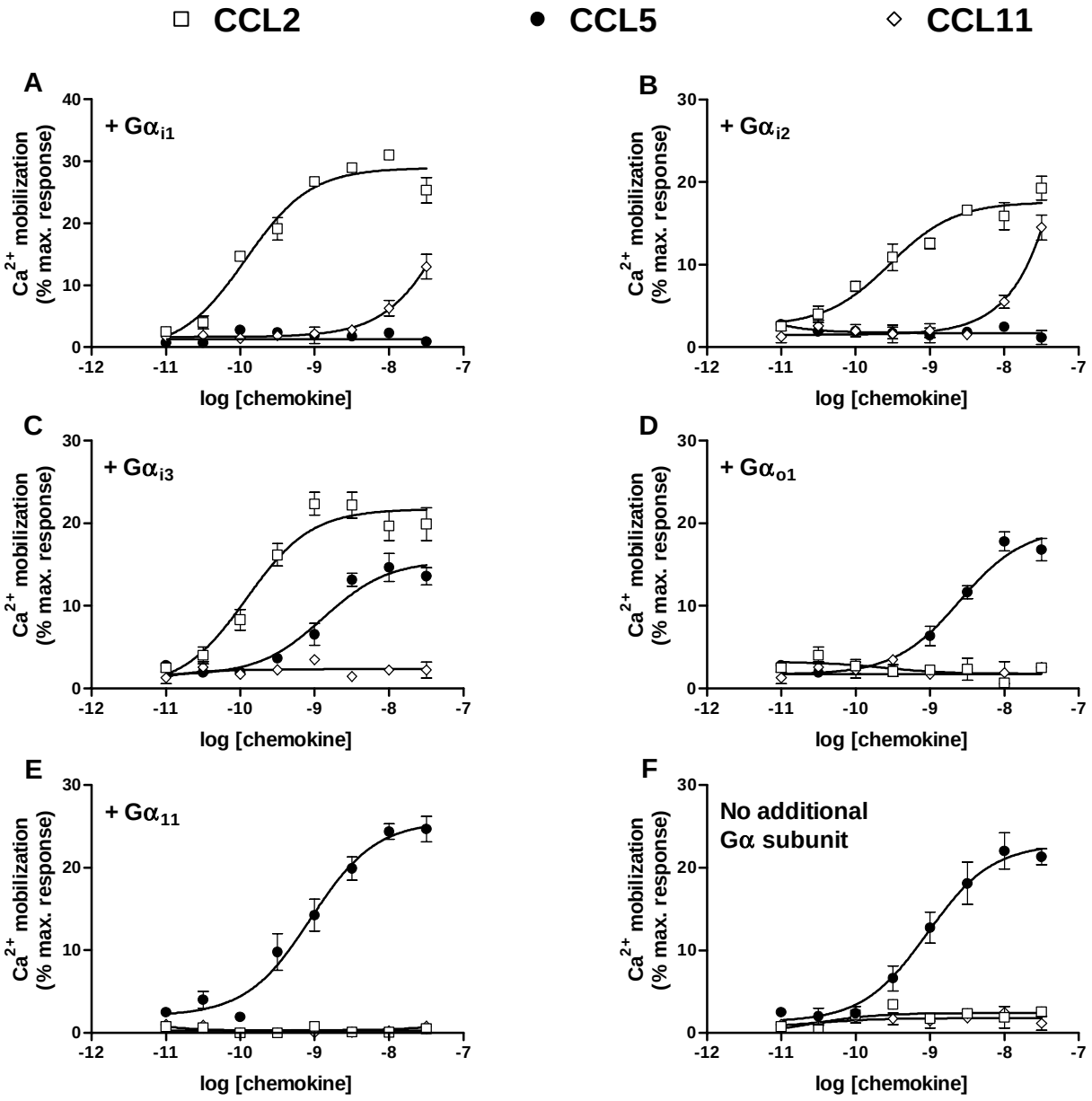

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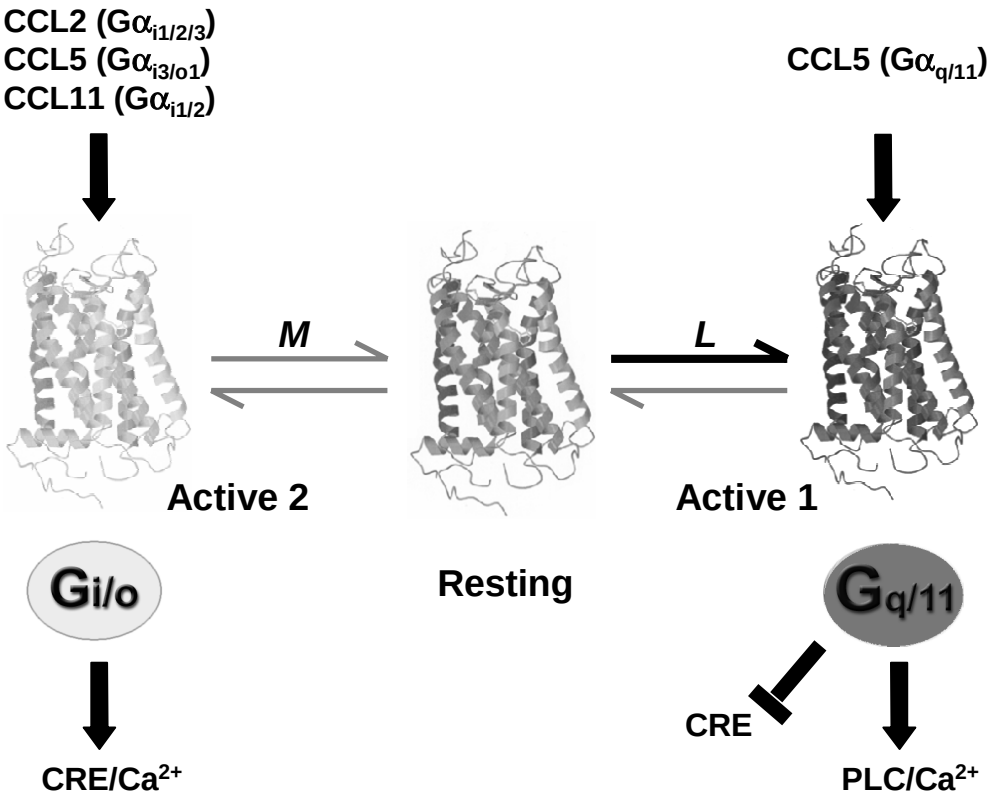
Figure 8



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