Selective Elimination of High Constitutive Activity or Chemokine Binding in the Human Herpesvirus 8 Encoded Seven Transmembrane Oncogene ORF74*

Mette M. Rosenkilde, Thomas N. Kledal, Peter J. Holst, and Thue W. Schwartz‡

From the Laboratory for Molecular Pharmacology, Department of Pharmacology, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark

Open reading frame 74 (ORF74) encoded by human herpesvirus 8 is a highly constitutively active seven transmembrane (7TM) receptor stimulated by angiogenic chemokines, e.g. growth-related oncogene-α, and inhibited by angiostatic chemokines e.g. interferon-γ-inducible protein. Transgenic mice expressing ORF74 under control of the CD2 promoter develop highly vascularized Kaposi’s sarcoma-like tumors. Through targeted mutagenesis we here create three distinct phenotypes of ORF74: a receptor with normal, high constitutive signaling through the phospholipase C pathway but deprived of binding and action of chemokines obtained through deletion of 22 amino acids from the N-terminal extension; an ORF74 with high constitutive activity but with selective elimination of stimulatory regulation by angiogenic chemokines obtained through substitution of basic residues at the extracellular ends of TM-V or TM-VI; and an ORF74 lacking constitutive activity but with preserved ability to be stimulated by agonist chemokines obtained through introduction of an Asp residue on the hydrophobic, presumed membrane-exposed face of TM-II. It is concluded that careful molecular dissection can selectively eliminate either agonist or inverse agonist modulation as well as high constitutive activity of the virally encoded oncogene ORF74 and that these mutant forms presumably can be used in transgenic animals to identify the molecular mechanism of its transforming activity.

Chemokines are chemotactic cytokines that regulate immunological processes through interaction with 7TM G-protein-coupled receptors expressed mainly on leukocytes. For example, during inflammation chemokines secure appropriate cell recruitment. Chemokines are also involved in tissue sustaining processes such as angiogenesis (2, 3). Genes coding for homologs of mammalian chemokine and chemokine receptors have been found in a number of herpes- and poxviruses (4–7). These molecules have presumably been obtained by the virus through an ancient act of molecular piracy and are structurally optimized for a particular pharmacological phenotype of benefit to the virus. The proposed functional properties of virally encoded chemokines are multiple. Some act as chemokine antagonists, for example vMIP-II from HHV-8 (8, 9) (HHV-8 is also known as Kaposi’s sarcoma-associated herpesvirus) and MC148 from Molluscum contagiosum (9, 10), and some act as agonists, for example UL146 from human cytomegalovirus (11) and vMIP-II (12).

In contrast to the viral chemokines, the function of the virally encoded chemokine receptors is not that clear yet. In general these receptors are not required for viral replication in vitro (13). However gene deletion experiments in both mouse and rat cytomegalovirus have shown that, for example the UL33 receptor is essential for targeting and/or replication of the virus in salivary glands (14). Several γ2-herpesviruses including HHV-8 (15), herpesvirus Saimiri (16), equine herpesvirus 2 (17), and the murine γ-herpesvirus 68 (18) encode homolog versions of a CXC chemokine receptor with highest homology to CXCR2 among mammalian chemokine receptors. In HHV-8 the receptor is known as ORF74, but it is also frequently referred to as Kaposi’s sarcoma-associated herpesvirus-G-protein-coupled receptor (Fig. 1). A prominent pharmacological feature of ORF74 from HHV-8 is its high degree of constitutive, ligand-independent signaling through the phospholipase C (19, 20) as well as the c-Jun N-terminal kinase and the p38 mitogen-activated protein kinase pathways (21). Furthermore ORF74 has angiogenic properties and its signaling is closely coupled to production and secretion of vascular endothelial growth factor and to cellular transformation and formation of highly vascularized tumors in SCID mice (21). In humans, ORF74 is expressed in Kaposi’s sarcoma lesions (15) and body cavity-associated lymphomas (22) and has been proposed to be causatively involved in these malignancies (21). Recently, transgenic mice expressing the ORF74 receptor under control of the CD2 promoter have been reported to develop highly vascularized Kaposi’s sarcoma-like tumors (1), thus supporting the oncogenic potential.

ORF74 from HHV-8 binds various human CXC chemokines (19, 20). The properties of these chemokines on ORF74 signaling cover the whole pharmacological spectrum: GROa, GROβ and GROγ are agonists, IP-10, stromal cell-derived factor-1α, granulocyte colony stimulating factor-2, and vMIP-II are inverse agonists, whereas the inflammatory CXC chemokines IL-8, neutrophil-activating peptide-2, and epithelial cell-derived activating peptide-78 are neutral ligands, which despite high affinity binding do not affect signaling of the receptor (20). Interestingly, the chemokines, which act as agonists on ORF74, are normally angiogenic chemokines in the host, whereas the chemokines that act as inverse agonists are normally angiosta-
tic or angiomodulatory messengers (23). Constitutive activity has been described in many 7TM receptors, for instance the adrenergic (24–27), the angiotensin and bradykinin (28, 29), and the glucagon receptors (30).

The present study is aimed at trying to characterize, in the ORF74 receptor from HHV-8, the structural basis for its broad spectrum binding profile of chemokines as well as the structural basis for its high constitutive activity based on knowledge on ligand binding and signaling properties in other 7TM receptors. Thereby, we created specific ORF74 mutants in which certain elements of the pharmacological repertoire have been selectively eliminated; to exploit these mutants in future transgenic studies to identify the molecular mechanism that HHV-8 has exploited in ORF74 to precipitate the different clinical features of Kaposi’s sarcoma and other HHV-8-related malignancies.

**EXPERIMENTAL PROCEDURES**

**Materials**—The human chemokines were purchased from R&D systems (epithelial cell-derived activating peptide-78, GROβ, and GROγ) or kindly provided by Kuldeep Neote, Pfizer (IP-10, granulocyte colony stimulating factor-α, and GROα) by N. C. Wells, Scandinavian Pharmaceutical Research Group, Ares-Serono, Geneva (neotrophil-activating peptide-2 and vMIP-II), Mikael Luther, Glaxo Wellcome (Met stromal cell-derived factor-1α), or Thomas P. Boesen at this laboratory (IL-8). The ORF74 (GenBank access number U24275) was cloned from a biopsy taken from a Kaposi’s sarcoma skin lesion from an human immunodeficiency virus, type I-infected patient (8). Monomiodinated [125I]-IL-8, [125I]-GROα, and myo-[125I]inositol (PT6–271) and Bolton-Hunter reagent for iodination of IP-10 were purchased from Amersham Pharmacia Biotech. AG 1-X8 anion-exchange resin was from Bio-Rad.

**Iodination of IP-10**—The Bolton-Hunter reagent was dried by a gentle stream of nitrogen for 30–60 min. 5–10 μg of IP-10 was incubated on ice with 1.5 mcI of Bolton-Hunter reagent in a total volume of 50 μl of 0.1 m NaCl buffer, pH 8.5, for 1 h, and the reaction was terminated by the addition of 0.5 ml of H2O supplemented with 0.1% v/v trifluoroacetic acid. The iodinated chemokines were purified by reverse phase high pressure liquid chromatography.

**Construction of Mutant Receptors**—The cDNA encoding the ORF74 receptor was cloned into the eukaryotic expression vector pTeJ-8 (31). Mutations were constructed by polymerase chain reaction using either the exon shuffling method (32) for mutations located internal in the receptor or flanking-extended primers for the N-terminal truncated receptor. The polymerase chain reaction products were digested by the receptor or flanking-extended primers for the N-terminal truncated receptor was cloned into the eukaryotic expression vector pTEJ-8 (31).

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**RESULTS**

The N-terminal region of the ORF74 receptor is characterized by the occurrence of many acidic residues, whereas multiple basic residues are found at the extracellular ends of TM-V and TM-VI and in extracellular loops 2 and 3 as shown in Fig. 1. These two regions were initially targeted for mutagenesis to try to selectively alter the ligand binding without affecting signaling of the virally encoded receptor.

**N-terminal Truncation of ORF74, a Phenotype with Eliminated Chemokine Binding and Activity but Preserved High Constitutive Activity**—Gene dosage experiments showed that deletion of the N-terminal 22 amino acids, including 7 acidic residues (Δ22-N-terminal), did not affect the basal signaling activity of the receptor, i.e. as determined by PI turnover (Fig. 2A). However, neither the agonist GROα nor the inverse agonist IP-10 could affect the high constitutive signaling activity of the mutated receptor (Fig. 2C). A whole panel of human CXC chemokines were tested on the Δ22-N-terminal mutant form of ORF74, but none of them were found to have any influence on its signaling (Fig. 2F), although several of these chemokines act either as agonists or inverse agonists on the wild type receptor (20). The three radioligands, [125I]-GROα, [125I]-IP-10, and [125I]-IL-8, which all bind with high affinity to the wild type receptor did not show any binding to the Δ22-N-terminal mutation (Table I).

An ORF74 Phenotypes with Preserved High Constitutive Signaling and Preserved Inverse Agonist Activity but with Impaired Agonist Activity—Based on the observation that IL-8 binding to CXCR2 is highly dependent on the presence of two arginine residues located at the extracellular end of TM-V (35) and that they are among the few residues shared between CXCR2 and ORF74, we substituted these residues as well as two arginine residues located in the corresponding region at the extracellular end of the neighboring TM-VI in ORF74 (Fig. 1).

The two arginine-substituted ORF74 mutants, like the wild type receptor, displayed high constitutive signaling as demonstrated by both gene-dosing experiments and by the suppressive effect of the inverse agonist, IP-10 (Fig. 2, A, D, and E). However, GROα, which acts as an agonist on the wild type ORF74, did not stimulate signaling detectably above the high basal level in the arginine-substituted ORF74 mutants (Fig. 2, D and E). This is surprising, because GROα did bind with high normal affinity, albeit with a diminished Bmax value, to the mutant receptors (4.5 and 8.3 fmol/105 cells) for the TM-V mutant (R208H/R212H)-ORF74 and the TM-VI mutant (R278A/R279A)-ORF74, respectively, versus 44 fmol/105 cells for the wild type receptor (Table 1). The binding of the neutral ligand IL-8 was totally eliminated in (R208H/R212H)-ORF74, whereas in (R278A/R279A)-ORF74, IL-8 binding was not affected in respect of affinity (Kd = 0.88 nm versus 1.5 nm for wild type receptor) but was severely decreased in respect of Bmax (2.5 fmol/105 cells as compared with 42 fmol/105 cells for the wild type ORF74) (Table 1). In contrast, for the inverse agonist, IP-10 increased Bmax values were observed in both of the arginine-substituted ORF74 mutants (46 and 170 fmol/105 cells for (R208H/R212H)-ORF74 and (R278A/R279A)-ORF74, respec-
Amino acid sequence of ORF74 from HHV-8. Serpentine diagram of ORF74 with residues of potential importance for the binding and signal transduction marked as white characters in colored circles, whereas other residues are marked with black characters in white circles. Δ22-N-terminal truncation is marked by a line at the point of the truncation. The acidic residues in the N terminus (Glu4, Asp5, Asp12, Asp13, Asp14, Glu15, and Glu19) are marked by red circles. Basic residues in top of TM-V (Arg208 and Arg212) and TM-VI (Arg278 and Arg279) are marked as blue circles, and mutated residues in TM-II (Leu91, Asn92, Ser93, and Leu94), TM-III (Tyr128 and Val142), and TM-VII (Val310) are marked as green circles. Inset, helical wheel model of the ORF74 receptor, based on the crystal structure of bacterial rhodopsin (43) as interpreted by Baldwin (44).
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In the present study three distinct phenotypes of ORF74 are generated through targeted mutagenesis directed toward either presumed ligand binding epitopes in the extracellular domains or toward the seven helical bundle, which is responsible for the signal transduction: 1) a receptor with eliminated constitutive activity, but with preserved inverse agonist action, IP-10, and also with preserved high constitutive activity; and 2) a receptor with eliminated constitutive activity but with preserved ligand binding and importantly with maintained ability to be stimulated by agonists.

General and Selective Elimination of Chemokine Binding and Action in ORF74—The elimination of all chemokine binding through truncation of the N terminus of the receptor is in agreement with the observation that peptide and protein ligands of the 7TM receptors in general often have important interactions with the extracellular segments (36). Especially in chemokine receptors, ligand-receptor interactions have often been located to their N-terminal segment (37–39). Most significantly, Handel and co-workers (40) have recently by NMR demonstrated the precise molecular binding mode of a peptide from the N-terminal segment of the CCR2 receptor to a structurally complementary groove on the main agonist for this receptor, MCP-1 (monocyte chemotactic protein-1). In the case of ORF74, Gershengorn and co-workers (41) recently found in analogy with the observation in the present study that truncation of the N terminus eliminated agonist binding. Importantly, in both studies it was found that the high constitutive activity of the N-terminally truncated ORF74 receptor was similar to that of the wild type receptor (Fig. 2).

In an attempt to affect the binding and action of various types of chemokine ligands selectively, we focused on the extracellular ends of TM-V and -VI, because these epitopes are known often to be involved in both agonist and antagonist binding in 7TM receptors (36). Specifically in CXCR1 and -2, which are the endogenous receptors with ligand binding profiles most similar to that of ORF74, two Arg residues are located at positions V-01 and V-05 facing the main ligand binding crevice and are known to be crucially involved in the binding of IL-8 (35). These residues are conserved in ORF74, and we find here that substitution of the two Arg with His residues in ORF74 also eliminated binding of IL-8, which is a neutral ligand in this virally encoded receptor. However, from a functional point of view this double mutation was even more interesting than expected, because it surprisingly eliminated the action of the agonist chemokine GROα without affecting the binding or action of the inverse agonist chemokine, IP-10 (Fig. 2). GROα still bound to the mutated receptor, albeit with a 5-fold reduced affinity and a 10-fold reduced $B_{\text{max}}$. The inability of the R208H/R212H mutation to be stimulated by GROα cannot be explained simply by the low $B_{\text{max}}$ for the agonist, because, for example the L91D and L94D mutants cannot be explained simply by the low $B_{\text{max}}$ for the agonist, because, for example the L91D and L94D mutants display even lower $B_{\text{max}}$ values for GROα both in total numbers and relative to the $B_{\text{max}}$ values for the inverse agonist IP-10 (Table I). Instead, an explanation could be that the double mutation eliminates a ligand-receptor interaction, which although being essential for the binding of the neutral ligand, IL-8, is only of limited importance for the binding of the agonist GROα but is an interaction that is essential for the function of GROα as an agonist. Importantly, the mutated receptor signals with high constitutive activity and this activity can be blocked normally by the inverse agonist peptide IP-10. Thus this mutation provides a unique tool to determine the relative importance of agonist versus inverse agonist regulation of ORF74 activity for the angiogenic property of the virally encoded receptor to be determined, for example in transgenic animals (1).

“Normalization” of the Basal Signaling of ORF74 without Affecting Ligand Binding—The virally encoded receptor differs significantly from the classical 7TM pattern of conserved residues. Based on these differences, a number of substitutions were made in the transmembrane helical bundle to normalize the receptor structurally and thereby try to nor...
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Homologous competition binding in transiently transfected COS-7 cells with three different radioligands, the agonist 125I-IP10, and the neutral ligand 125I-IL8. The table shows IC50 ± S.E. for the indicated number of experiments (n), followed by Bmax ± S.E. Bmax ratio indicates the ratio between Bmax measured with 125I-GROα compared to Bmax measured with 125I-IP-10. NB indicates no specific binding.

| Construct          | IC50 (fmol/10^6 cell) | Bmax (fmol/10^6 cell) | IC50 (fmol/10^6 cell) | Bmax (fmol/10^6 cell) | IC50 (fmol/10^6 cell) | Bmax (fmol/10^6 cell) | Bmax ratio |
|--------------------|------------------------|-----------------------|------------------------|-----------------------|------------------------|-----------------------|------------|
| ORF74 wt           | 0.10 ± 0.02 (11)       | 44 ± 10               | 1.5 ± 0.4 (5)          | 42 ± 12               | 0.61 ± 0.19 (13)       | 28 ± 2.9              | 1.57       |
| Δ22-N-terminal     | NB (3)                 | NB                    | NB (3)                 | NB                    | NB (3)                 | NB                    |            |
| Y128A              | 0.53 ± 0.19 (7)        | 4.5 ± 1.9             | NB                    | 0.66 ± 0.05 (3)       | 2.5 ± 0.2              | 0.37 ± 0.10 (9)       | 46 ± 6.2   |
| V142D              | 0.10 ± 0.05 (4)        | 8.3 ± 0.7             | 0.88 ± 0.05 (3)       | 4.4 ± 1.5             | 0.32 ± 0.05 (4)        | 3.2 ± 0.52             | 0.09       |
| Y128A/R279A        | 0.01 ± 0.003 (5)       | 0.28 ± 0.04           | 1.3 ± 0.17 (3)        | 6.6 ± 1.5             | 0.28 ± 0.05 (4)        | 3.2 ± 0.52             | 0.09       |
| L91D               | 0.007 ± 0.000 (3)      | 0.2 ± 0.003           | 0.23 ± 0.003 (3)      | 1.2 ± 0.03 (6)        | 0.72 ± 0.07 (3)        | 11 ± 3.04             | 1.33       |
| N92D               | 0.007 ± 0.000 (3)      | 0.2 ± 0.003           | 0.23 ± 0.003 (3)      | 1.2 ± 0.03 (6)        | 0.72 ± 0.07 (3)        | 11 ± 3.04             | 1.33       |
| S93D               | 0.007 ± 0.000 (3)      | 0.2 ± 0.003           | 0.23 ± 0.003 (3)      | 1.2 ± 0.03 (6)        | 0.72 ± 0.07 (3)        | 11 ± 3.04             | 1.33       |
| L94D               | 0.002 ± 0.002 (5)      | 0.74 ± 0.13           | 1.3 ± 0.17 (5)        | 11 ± 1.7              | 0.38 ± 0.07 (3)        | 7.3 ± 2.7              | 1.66       |
| Y128S              | 0.008 ± 0.003 (4)      | 6.5 ± 1.3             | 1.3 ± 0.17 (5)        | 11 ± 1.7              | 0.38 ± 0.07 (3)        | 7.3 ± 2.7              | 1.66       |
| V142D              | 0.005 ± 0.003 (3)      | 12 ± 2.1              | 1.3 ± 0.17 (5)        | 11 ± 1.7              | 0.38 ± 0.07 (3)        | 7.3 ± 2.7              | 1.66       |
| N92D/V142D         | 0.005 ± 0.001 (3)      | 8.6 ± 0.21            | 0.61 ± 0.07 (6)       | 2.8 ± 0.67            | 0.32 ± 0.04 (4)        | 4.2 ± 0.75             | 1.73       |
| V310N              | 0.09 ± 0.01 (2)        | 17 ± 2.9              | 2.6 ± 0.28 (4)        | 49 ± 5                | 0.87 ± 0.19 (4)        | 22 ± 8.2              | 1.30       |

**Fig. 3. PI turnover in TM-III and TM-VII located mutants.** Biological activity measured as PI turnover in whole COS-7 cells transiently expressing the ORF74 constructs as described under “Experimental Procedures.” A, basal activity, measured as cpm/90 min/5 × 10^5 cells in a gene dosage experiment, using 5, 10, 20, and 40 μg of DNA of following constructs given from the left side: empty expression vector, pTEJ8 (white bars), ORF74 wild type (black bars); TM-III mutants, (Y128A)- (light gray bars), (V142D)- (dark gray bars), and (N92D/V142D)-ORF74 (horizontal line bars); and TM-VII mutants, (V310N)-ORF74 (crossed bars). B, influence on the basal activity (first bar) of IL-8, 10^-4 M (second bar), GROα, 10^-8 M (third bar), and IP-10, 10^-8 M (fourth bar) for mutants presented in A, with the same color code as presented in A, given in percent of basal activity, where the basal activity corresponding to 40 μg of each mutation equals 100%.

...malize the high constitutive activity of ORF74. For example, at the intracellular end of TM-III in most 7TM receptors is found the tri-peptide sequence Asp-Arg-Tyr (DRY) of which the Asp and Arg are highly conserved and crucially involved in receptor G-protein interaction and signaling (26, 27). In ORF74 the Asp in the DRY sequence is substituted with a Val. In the middle of TM-III of ORF74 a large aromatic side chain is located at position III:11 (Tyr128), which in the angiotensin AT1 receptor (28) and in the bradykinin receptor (29) creates a receptor phenotype dominated by high constitutive activity. In TM-VII the highly conserved Asn at position VII:16, which normally is believed to be involved in creating an important interhelical hydrogen bond network, is in ORF74 substituted with a Val lacking the ability to form hydrogen bonds. In TM-II the functionally highly important Asp residue is substituted with either an Asn or a Ser residue depending on how the sequence alignment is performed. However, normalization of ORF74 at these positions did not affect the high constitutive signaling of the receptor (Fig. 4). This was most surprising in the case of the DRY sequence, because substitution of the Asp in several cases, one of which is CXCR2, has created highly constitutively active receptors (26, 27, 42). In the CXCR2 study, it was even argued that this substitution probably was the reason for the high constitutive activity of ORF74 (42); however, the mutation performed here in the ORF74 could not confirm that notion. Thus, the rational approach to normalizing ORF74 signaling...
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was not successful in our hands.

Nevertheless, introduction of an Asp residue at either position 91 or position 94 in TM-II surprisingly created the desired phenotype, i.e., low constitutive signaling activity combined with the ability to be stimulated by agonists. Because of the poorly conserved primary structure around TM-II, it was unclear how the sequence of ORF74 was best aligned with the consensus 7TM sequences, which basically only dictates the presence of an Asp residue located at position II:10 in an otherwise rather hydrophobic segment. Most likely it is either the Asp<sup>92</sup> or perhaps the Ser<sup>93</sup> residue that corresponds to the TM-II Asp found in almost all 7TM receptors. However, because the introduction of an Asp at these positions had almost no effect on the basal signaling of ORF74, the neighboring positions were also probed and with a surprisingly positive result (Fig. 1). The L91D and L94D substitutions abolished the constitutive activity, without influencing the surface expression of the receptor more than introduction of Asps at the neighboring positions 92 and 93 did; the latter two mutations were displayed as high basal activity as observed in the wild type receptor. From the helical wheel diagram of TM-II, it is predicted that Leu<sup>93</sup> and Leu<sup>94</sup> are both located on a hydrophobic, presumed membrane-exposed face of this helix (Fig. 1). It is unclear what the structural basis for the effect of these substitutions is. It could be envisioned that introduction of the polar, potentially charged Asp residue in the middle of the hydrophobic face would destabilize the receptor structure. However, how this could lead to diminished basal signaling but preserved responsiveness to the agonist is unclear.

Exploiting ORF74 Mutants to Characterize Relationship between HHV-8, Kaposi’s Sarcoma, and Angiogenesis—It has been suggested that ORF74 through its ligand-independent high constitutive activity should be causatively involved in the development of Kaposi’s sarcoma as well as certain types of B-cell lymphomas (19, 21). Recently it was elegantly shown by Lira and co-workers (1) that transgenic mice expressing the wild type ORF74 receptor under control of the CD2 promoter develop Kaposi’s sarcoma-like lesions. This obviously strongly supports a pathogenic connection between ORF74 from HHV-8 and Kaposi’s sarcoma. However, it is still unclear whether it is merely the constitutive activity of the virus-encoded oncogene as such that causes the lesions or whether the carefully evolved control of this activity by angiogenic as well as angiostatic or modulator chemokines is involved in the development of these specially highly vascularized tumors. It should also be noted that Kaposi’s sarcoma predominantly develops in immune compromised individuals and that the function of ORF74 in the life circle of HHV-8 may be more subtle in the normal HHV-8-infected individuals. Nevertheless, expression of the various ORF74 mutants with and without high constitutive activity as well as with and without chemokine or selective angiogenic chemokine regulation in transgenic animals should be able to clarify the molecular mechanism exploited by HHV-8 in cell transformation and angiogenesis.

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FIG. 4. PI turnover in TM-II located mutants. Biological activity measured as PI turnover in whole COS-7 cells transiently expressing the ORF74 constructs as described under “Experimental Procedures.” A, basal activity, measured as cpm/90 min/5 × 10<sup>5</sup> cells in a gene dosage experiment, using 5, 10, 20, and 40 μg of DNA of the following constructs given from the left side: empty expressing vector, pTEJ6 (white bars), ORF74 wild type (black bars), TM-II mutants, (L91D) (green bars), (N92D) (light gray bars), (S93D) (dark gray bars), and (L94D)-ORF74 (green bars). B and C, gene dosage experiments with total activity of unstimulated receptors (filled circles), receptors stimulated with IL-8, 10<sup>−7</sup> M (unfilled triangles) and GRO<sub>α</sub>, 10<sup>−8</sup> M (unfilled circles), and receptors inhibited with IP-10, 10<sup>−6</sup> M (unfilled squares) in the (L91D) (B) and in the (L94D)-ORF74 (C). B and C, gene dosage experiments with total activity of unstimulated receptors (filled circles), receptors stimulated with IL-8, 10<sup>−7</sup> M (unfilled triangles) and GRO<sub>α</sub>, 10<sup>−8</sup> M (unfilled circles), and receptors inhibited with IP-10, 10<sup>−6</sup> M (unfilled squares) for mutants presented in A, with the same color code as presented in A, given in percent of basal activity, where the basal activity corresponding to 40 μg of each mutation equals 100%.
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