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The activation mechanism of chemokine receptor CCR5 involves common structural changes but a different network of interhelical interactions relative to rhodopsin

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Abstract

In G protein-coupled receptors (GPCRs), the interaction between the cytosolic ends of transmembrane helix 3 (TM3) and TM6 was shown to play an important role in the transition from inactive to active states. According to the currently prevailing model, constructed for rhodopsin and structurally related receptors, the arginine of the conserved "DRY" motif located at the cytosolic end of TM3 (R3.50) would interact with acidic residues in TM3 (D/E3.49) and TM6 (D/E6.30) at the resting state and shift out of this polar pocket upon agonist stimulation. However, 30% of GPCRs, including all chemokine receptors, contain a positively charged residue at position 6.30 which does not support an interaction with R3.50. We have investigated the role of R6.30 in this receptor family by using CCR5 as a model. R6.30D and R6.30E substitutions, which allow an ionic interaction with R3.50, resulted in an almost silent receptor devoid of constitutive activity and strongly impaired in its ability to bind chemokines but still able to internalize. R6.30A and R6.30Q substitutions, allowing weaker interactions with R3.50, preserved chemokine binding but reduced the constitutive activity and the functional response to chemokines. These results indicate that the constitutive and ligand-promoted activity of CCR5 can be modified by modulating the interaction between the DRY motif in TM3 and residues in TM6 suggesting that the overall structure and activation mechanism are well conserved in GPCRs. However, the molecular interactions locking the inactive state must be different in receptors devoid of D/E6.30.

Keywords: Activation; CCR5; Constitutive activity

1. Introduction

The CC chemokine receptor CCR5 is expressed on memory T lymphocytes and the monocyte-macrophage lineage [1–3] and responds to nanomolar concentrations of MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, MCP-2/CCL8 and a truncated form of HCC-1/CCL14 [4–6]. When expressed in recombinant systems, CCR5 displays a constitutive activity that is inhibited by inverse agonists such as TAK-779 [7]. The physiological significance of this constitutive activity in vivo remains however to be determined. In addition to its role as a chemokine receptor involved in the recruitment of leukocytes in a number of physiological and pathological situations (such as rheumatoid arthritis, graft rejection, neurodegenerative diseases and asthma), CCR5 constitutes the major co-receptor for macrophage-tropic strains of human immunodeficiency virus (HIV). It allows, together with CD4, the binding of the viral particles to the cell surface through the envelope protein gp120, and this interaction triggers the subsequent membrane fusion process [8,9]. CCR5 forms homodimers, but also heterodimers with its closest homologue CCR2, in a ligand-independent manner [10–16]. This oligomeric organization was demonstrated in native cells, and has functional consequences, as negative binding cooperativity was demonstrated between the binding pockets of each protomer, resulting in the binding of a single chemokine molecule per receptor dimer [15,16].

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In the absence of experimentally determined tridimensional structures for any other member of the GPCR family,[17] the bovine rhodopsin crystal structure is used as a template for homology modeling of rhodopsin-like GPCRs and as a common support for structure–function relationship studies [18]. Biochemical and biophysical approaches, supported by modeling studies, have identified key structural motifs involved in the activation mechanism of GPCRs [19–22]. Labeling of receptors with fluorescent probes sensitive to the changes in biophysical environment provided evidence that activation of selected GPCRs involved relative movements of their TM3 and TM6, suggesting that the activation mechanisms would require the disruption of intramolecular interactions that stabilize the receptor in its inactive conformation [23]. One such constrain is the ionic lock between the arginine of the highly conserved (D/E)RY motif in TM3 with its adjacent Asp/Glu residue and an Asp/Glu residue at the cytoplasmic end of TM6, which is conserved in a large subset of GPCRs [24]. Disruption of these constrains can be promoted either by agonists or by mutations affecting the key residues involved and it has been demonstrated that charge-neutralizing mutations of D3.49 or R3.50 in TM3, and D/E6.30 in TM6, results in increased constitutive activity of rhodopsin and a number of structurally-related class A GPCRs [19,25–29]. However, these charged residues are not shared by all GPCRs and different interactions might therefore regulate the equilibrium between inactive and active states in some receptor subfamilies, more distantly related to rhodopsin. Among these, chemokine receptors in general and CCR5 in particular do not share with rhodopsin the negatively charged residue at position 6.30, which is occupied by an arginine. It was also shown previously that mutation of D3.49, within the "DRY box" of CCR5, results in a reduction, rather than an increase, of both the basal and agonist-induced activity of the receptor [7]. We have now extended this study by investigating more specifically the role of the R6.30 residue located at the cytosolic end of TM6. The nature of this amino acid does not allow an ionic interaction with the R3.50 of TM3 and the locking of CCR5 in an inactive state. In addition, chemokine receptors contain also the family-conserved D2.40 in TM2, which is Asn in rhodopsin and in most other members of class A GPCRs. It was shown that mutation of D2.40 increased the constitutive activity of the chemokine-homologous Kaposi's sarcoma-associated herpesvirus GPCR [30]. In order to study the role of these chemokine family-specific residues we replaced by using site-directed mutagenesis R6.30 and D2.40 with various amino-acids to modulate their potential inter-helical interactions and analyzed the properties of the resulting mutants in terms of cell surface expression, receptor dimerization, chemokine binding, basal and chemokine-stimulated activity and receptor internalization.

2. Experimental procedures

2.1. Numbering scheme of GPCRs

We use in this work a general numbering scheme identifying residues located at the same position in the transmembrane segments of different receptors [31]. Each residue is numbered according to the helix (1 through 7) in which it is located and to the position relative to the most conserved residue in that helix, arbitrarily assigned to 50. For instance, R6.30 is the arginine in transmembrane helix 6 (TM6) twenty residues before the highly conserved proline P6.50.

2.2. Construction of CCR5 mutants

Plasmids encoding the CCR5 mutants were constructed by site-directed mutagenesis using the QuikChange method (Stratagene). Following sequencing of the constructs, the mutated coding sequences were subcloned into the bicistronic expression vector pEFIN3, as previously described, for the generation of stable cell lines [32]. All constructs were verified by sequencing prior to transfection.

2.3. Expression of mutant receptors in CHO-K1 Cells

CHO-K1 cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum (Invitrogen), 100 units/ml of penicillin and 100 μg/ml of streptomycin (Invitrogen). Constructs encoding wild-type or mutant CCR5 in the pEFIN3 vector were transfected using FusGENE 6 (Roche Molecular Biochemicals) in a CHO-K1 cell line expressing an apoaequorin variant targeted to mitochondria. Selection of transfected cells was made for 14 days with 400 μg/ml G418 (Invitrogen) and 250 μg/ml zeocin (Invitrogen, for maintenance of the apoaequorin encoding plasmid) and the population of mixed cell clones expressing wild-type or mutant receptors was used for binding and functional studies. Cell surface expression of the receptor variants was measured by flow cytometry using monoclonal antibodies recognizing different CCR5 epitopes: 2D7 (phycoerythrin-conjugated, PharMingen) or MC-5 (kindly provided by Mathias Mack, Munich, Germany). Unlabeled monoclonal antibodies were detected by a phycoerythrin-coupled anti-mouse IgG secondary antibody (Sigma).

2.3.1. GTPγS binding assay

Membranes (10–20 μg) of cells expressing CCR5 were incubated for 15 min at room temperature in binding buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 3 mM $MgCl_2$, 3 μM GDP, 10 μg/ml saponine) containing chemokines or mAbs in 96-well microplates (Basic Flashplates, PerkinElmer Life Sciences). GTPγ^{35}S (0.1 nM, Amersham) was added and microplates were incubated for 30 min at 30 °C in the absence or presence of 1 μM TAK-779. Incubation was stopped by centrifugation of the microplates for 10 min at 800 g and 4 °C, followed by supernatant removal. Microplates were counted in a TopCount (Packard Instrument Co.) for 1 min per well.

2.3.2. Binding assay

Cells expressing receptors were grown near to confluence, collected from plates in Ca^{2+}- and Mg^{2+}-free PBS, centrifuged for 5 min at 1500 g and washed with PBS. Cells were then resuspended in buffer A (15 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA) and disrupted in a glass homogenizer. The homogenates were first centrifuged for 5 min at 500 *g* and the resulting supernatants at 40,000 *g* for 30 min at 4 °C. The cell membrane pellet was washed in buffer A, and resuspended in buffer B (75 mM Tris–HCl pH 7.5, 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose) at a protein concentration of approximately 1 mg/ml. Competition binding experiments were performed by using 0.2 nM ^{125}I-MIP-1β as labeled tracer and variable concentrations of chemokines as unlabeled competitors. Samples were incubated for 60 min at 27 °C, and then bound tracer was separated by filtration through GF/B filters presoaked in 0.5% BSA. Filters were counted in a γ-scintillation counter. Binding parameters were determined with the PRISM software (Graphpad Softwares) using nonlinear regression applied to a single site binding model.

2.4. Aequorin-based functional assay

The functional response to chemokines was estimated by an aequorin-based assay [5]. Briefly, cells were harvested from plates with Ca^{2+}- and Mg^{2+}-free DMEM supplemented with 5 mM EDTA and centrifuged for 2 min at 1000 *g*. The pellet was resuspended in DMEM at a density of 5×10^6 cells/ml, and incubated for 4 h in the dark in the presence of 5 μM coelenterazine H (Promega Corporation). Cells were then diluted 5-fold before use. Variable concentrations of chemokines in a volume of 50 μl of DMEM were added to 50 μl of cell suspension (25,000 cells) per well. Luminescence was measured for 30 s in an EG and G Berthold luminometer (PerkinElmer Life Sciences). Half-maximal effective concentrations