New dimensions in CXCR4 and Rac1 regulation

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Role of the 3rd intracellular loop of CXCR4 in regulating the Rac1-mediated active conformation of the receptor

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Abstract

Activation of CXCR4 by its ligand CXCL12 is associated with the migration and/or proliferation of many cell types, including leukocytes, hematopoietic stem cells (HSCs) and tumor cells. Ligand binding to CXCR4 triggers a cascade of signaling pathways most of them originating from Goi, activation. The duration and intensity of CXCR4 signaling are tightly regulated at different levels. We here show in different cell types that inhibition of Rac1 or interference with the 3rd intracellular loop (ICL3) of CXCR4 cause a conformational change in the receptor. Biochemical analysis showed that the active form of Rac1 preferentially associates with ICL3. By using a human glioblastoma cell line (U87) stably expressing different chimeric CXCR4 receptors, we found that Rac1 requires the ICL3 domain of CXCR4 to control the receptor’s conformation. Importantly, the conformational change of CXCR4 upon inhibition of Rac1 or functional competition with ICL3, impaired CXCL12 binding and decreased the receptor’s signaling capacity as measured by a drop in calcium mobilization and low migration rates. Finally, we show that lipid rafts are essential for the maintenance of CXCR4 conformation. In conclusion, this study identifies the ICL3 of CXCR4 as the domain mediating Rac1-dependent active conformation of the receptor, thereby providing new targets for therapeutic CXCR4 manipulation.
Introduction

The chemokine receptor CXCR4 belongs to the large family of G protein-coupled receptors and is one of the best studied chemokine receptors because of its role in HIV infection\(^1\) and tumor cell growth and metastasis\(^2\). CXCR4 and its ligand CXCL12 are also regulating multiple biological processes involving cellular migration, such as angiogenesis, neuronal development and HSC and immune cell trafficking. CXCL12 or CXCR4 deficiency in mice leads to embryonic lethality, underscoring the importance of this chemokine/receptor pair in mediating crucial physiological processes\(^3\);\(^4\).

Ligand binding to CXCR4 induces a conformational change of the receptor, which then can act as a guanine nucleotide exchange factor for the heterotrimeric G\(\alpha_i\) protein, facilitating the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G\(\alpha_i\) subunit\(^5\). These events initiate the activation of multiple signaling pathways leading to the generation of a chemotactic response towards a CXCL12 gradient in various types of cells\(^6\) or to the induction of cellular proliferation\(^2\);\(^6\).

Considering its important role in many vital processes, CXCR4 signaling must be tightly regulated at different levels. Several mechanisms regulating CXCR4 function have been described, among which the control of receptor trafficking and the abundance on the cell surface\(^7\);\(^10\), which determines the amplitude of the signaling response. Tumor cells, for example, often display increased cell surface expression of CXCR4, which in turn promotes their metastasis\(^11\). Thereafter, signal termination is achieved by receptor internalization, desensitization and degradation\(^12\);\(^13\). These steps are controlled by the canonical pathway in which G protein-coupled receptor kinase phosphorylates serine/threonine residues on the ICL3 and the cytoplasmic tail of CXCR4. Phosphorylation of these residues facilitates the binding of \(\beta\)-arrestins, which uncouple the G\(\alpha_i\) subunit from the receptor and initiate its internalization\(^13\). Another less well understood mechanism controlling CXCR4 responsiveness is heterologous desensitization. Specifically, receptor-specific ligand binding to and activation of its cognate receptor could modify ligand binding and responsiveness of other neighboring chemokine receptors\(^14\);\(^15\). This phenomenon has been observed in cells co-expressing the chemokine receptors CCR2, CCR5 and CXCR4 and is attributed to their ability to form multimeric receptor complexes\(^16\).

It is known that cells display a heterogeneous set of CXCR4 conformations\(^17\) that have been associated with different G\(\alpha_i\) activation patterns. Moreover, certain conformations generated inactive receptors\(^18\). Thus, the conformation adopted by CXCR4 determines ligand binding and/or signaling capacity, thereby adding a new level of complexity to the regulation of CXCR4 signaling. Little is known about the factors regulating the different CXCR4 conformational states. It has been proposed that some post-translational modifications as well as ubiquitylation\(^19\) could account for CXCR4 heterogeneity. In addition, allosteric modulators also affect the conformation adopted by the receptor, thereby enhancing or reducing its biological activity\(^20\);\(^21\). We recently identified Rac1 as a positive allosteric modulator of CXCR4, which stabilizes its responsive conformation\(^22\).

Rac1 is a prominent member of the Rho family of GTPases, which control cytoskeletal dynamics. Rac1 lies at the center of actin polymerization in migrating cells\(^23\) and acts as a molecular switch, cycling between an active GTP-bound state and an inactive GDP-bound state.
In this study, we show that Rac1 requires the ICL3 of CXCR4 to regulate the receptor’s conformation. Interfering with this domain alters the conformation adopted by CXCR4. Furthermore, we demonstrate that Rac1 and the ICL3 are both responsible for controlling the responsive conformation of the receptor, which allows ligand binding and efficient signaling. Finally, we demonstrate that lipid raft integrity, but not actin polymerization or Gαi coupling plays a role in the maintenance of CXCR4 conformation.

In summary, our data demonstrate that Rac1 regulates CXCR4 conformation via the ICL3 thereby promoting ligand binding and receptor signaling.

Materials and methods

Cell lines and cell culture

The Jurkat T-lymphocyte, the HL60 and the U937 leukemic cell lines (from the ATCC, Rockville, MO, USA) and HeLa and HEK293T cells were maintained at 37°C and 5% CO2 in Iscove’s Modified Dulbecco’s Medium (IMDM; Lonza, Basel, Switzerland) containing 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (all purchased from PAA laboratories, Pasching, Austria) and 10% v/v FCS (Bodinco, Alkmaar, The Netherlands). U87 glioblastoma cells stably expressing CXCR4 chimeras were maintained in the same culture medium supplemented with 0.5 µg/ml puromycin and 300 µg/ml G418 (both from Invitrogen, Carlsbad, CA, USA). Hela cells were passaged using trypsin (PAA laboratories) and U87 cells were passaged using 10 µM ethylene-diamine-tetra-acetic acid (EDTA; R&D Systems, Abingdon, United Kingdom). HeLa cells were transiently transfected using Transit (Mirus, Madison, WI, USA) according to the manufacturer’s recommendations.

Antibodies, flow cytometry

Antibody staining was performed in phosphate-buffered saline (PBS; Fresenius Kabi’s Hertogenbosch, The Netherlands) containing 0.5% w/v Bovine Serum Albumin (BSA) (Sigma-Aldrich, Steinheim, Germany) named FACS medium to reduce aspecific binding. CXCR4 expression was determined by two different allophycocyanin (APC)-labeled conformation-dependent antibody clones, i.e. clone 12G5 (BD Biosciences, Breda, The Netherlands) and clone 44717 (R&D Systems) or by the conformation-independent 4G10 clone followed by anti-mouse IgG F(ab’)2- fluorescein isothiocyanate (FITC) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA). CXCR7 and CCR5 expression was determined by using mouse anti-human CXCR7- phycoerythrin (PE) (clone 358426, R&D Systems) and mouse anti-human CCR5-APC (conformation-dependent clone 2D725, BD Biosciences) respectively. The isotype controls used were: mouse anti-human IgG2A-APC (BD Biosciences) for the CXCR4 antibody clone 12G5 and for the CCR5 antibody, mouse IgG2B-APC (R&D systems) for the CXCR4 antibody clone 44717, mouse IgG2A-PE (R&D systems) for the CXCR7 antibody and mouse IgG1 (Sanquin Reagents, Amsterdam, The Netherlands) for the CXCR4 antibody clone 4G10.

Dominant negative peptides and pharmacological inhibitors

Cell permeable peptides containing the amino acid sequence of the ICL3 of CXCR4 (NH2-IISKLSHSGNHQKRLKLT-COOH) or of the C-terminal region of Rac1 (NH2-CPVVKKKRKRCOOH) fused to a protein transduction domain (PTD) or a control peptide that only encodes
the PTD (control) were produced by F-moc protein synthesis (Department of Peptide Synthesis, Netherlands Cancer Institute, Amsterdam, The Netherlands).

Cells were harvested in IMDM containing 0.25% w/v BSA; named assay medium and treated with 200 µg/ml or 100 µg/ml Rac1 C-terminal peptide or ICL3 peptides, respectively, for 30 minutes. Pharmacological inhibition of Rac1 was performed with NSC23766 (Calbiochem, Nottingham, United Kingdom) for 1 hour at a concentration of 50 µM in assay medium.

Hydroxypropyl-β-cyclodextrin (Sigma-Aldrich) was used at a concentration of 3 mM for 1 hour at 37°C. Cytochalasin B (Sigma-Aldrich) was used at a concentration of 10 ng/ml for 1 hour at 37°C. Pertussis toxin (Sigma-Aldrich) was used at a concentration of 20 ng/ml overnight at 37°C. After treatment with the different inhibitors, cells were washed with FACS medium. The DNA-binding dye, ToPro3 (Invitrogen, Carlsbad, CA, USA), was used to determine cytotoxicity. CXCL12 (PeproTech, Rocky Hill, USA) was used at a final concentration of 100 ng/ml for 30 minutes as a positive control for responsiveness of the CXCR4 receptor on the cell surface.

Peptide pull-down assay

HeLa cells were transiently transfected with the indicated constructs and maintained for 24 hours. The cells were washed twice with ice-cold PBS (supplemented with 1 mM CaCl2 and 0.5 mM MgCl2) and lysed in NP-40 lysis buffer (i.e. 50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM MgCl2, 10% glycerol and 1% NP-40) supplemented with protease inhibitors (Complete mini EDTA, Roche, Mannheim, Germany), centrifuged at 20,000 g for 10 minutes at 4°C. The supernatant was pre-cleared by incubation with streptavidin-coated beads (Sigma-Aldrich) for 1 hour at 4°C. The pre-cleared lysate was then incubated with the ICL3 peptide (6 µg) or a control peptide (6 µg) in the presence of streptavidin-coated beads at 4°C for 1 hour with rotation. Initial lysates and pull-down samples were analyzed by SDS-PAGE and immunoblotting using rabbit anti-myc (Sigma-Aldrich) and HRP-coupled secondary antibodies.

Calcium mobilization assay.

The assay was performed using 6x10^6 Jurkat cells per condition. Cells were washed twice with pre-warmed PBS and once with pre-warmed Hepes buffer (132 mM NaCl, 20 mM HEPES, 6 mM KCl, 1mM MgSO4·7H2O, K2HPO4·3H2O) supplemented with 1 mg/ml glucose, 1 µM/ml calcium and 0.5% human albumin. Cells were harvested in 1 ml Hepes buffer and the calcium sensitive fluorescent dye, Indo-1 (Molecular Probes, Oregon, USA) was added (5 µM). Cells were incubated for 30 minutes in the dark in a water bath (37°C), then washed twice and resuspended in 1 ml Hepes medium. 0.5 ml of the suspension was transferred to a tube. During the measurement CXCL12 was added at a concentration of 200 ng/ml. Ionomycin (4 µM) and EGTA (1mM) were used as a positive and negative control, respectively. Indo-1 is exited using UV light (355 nm) and has a peak emission at 405 nm (representing calcium-bound Indo-1) and 485 nm (representing free Indo-1). The FlowJo software was used to calculate the ratio of free Indo-1/bound Indo-1 and make the graphs representing calcium mobilization.

Transmigration assay

Migration assays were performed as previously described28. In brief, Transwells of 6.5 mm diameter, with 5 µm pore size filters (Costar, Cambridge, USA) were coated with 20 µg/ml
fibronectin (sigma-Aldrich). Before use, cells were treated or not with the control peptide or the ICL3 peptide, both at a final concentration of 100 µg/ml. At the start of the assay, 10⁵ cells were harvested in assay medium and placed in the upper compartment of the Transwells and allowed to migrate for 3 hours at 37°C to chemokine containing medium at a final concentration of 100 ng/ml added to the lower compartment. As input control, 10⁵ cells were kept separately.

Migrated cells were collected from the lower compartment and quantified by flow cytometric analysis in the presence of a fixed number of flow-count beads (Beckman Coulter, Fullerton, CA, USA). The percentage of migrated cells was calculated as follows: % of migrated cells = [(number of beads/number of input cells)] * [(number of migrated cells/number of beads)] * 100

**Jurkat whole cell CXCL12 binding assay**

Jurkat cells were washed with PBS, counted and resuspended at 2x10⁶ cells per ml in PBS containing 5 mM EDTA and 0.5% BSA. Cells were incubated on ice for 30 min without or with the Rac1 inhibitor. Subsequently, cells were incubated for an additional 3 hours at 4°C with 75 pM of [¹²⁵I]-CXCL12/SDF-1α (Perkin-Elmer, Waltham, MA, USA) and the indicated concentration of unlabeled CXCL12 (Peprotech) in the absence or presence of the Rac1 inhibitor. Cells were then harvested on polyethylenimine (0.5%)-treated GF/C filter plates (Whatman, Maidstone, UK) and washed three times with ice-cold PBS. Plates were counted by liquid scintillation using a MicroBeta plate counter (Perkin-Elmer).

**Calculations and statistical analysis**

The percentage of CXCR4 surface expression was calculated by normalizing the fluorescence intensity measurements on the mean of the untreated condition measurements. Measurements of both treated and untreated conditions were divided by the mean of the untreated conditions (x100%), giving rise to a mean of 100% ±SEM for the control. The isotype control background was not subtracted from the specific fluorescence intensity signal.

Significance of differences was determined in all experiments with a two-sided unpaired Student’s t test. Two-sided p-values smaller than 0.05 were considered significant.

**Results**

**Rac1 regulates CXCR4 conformation via the 3rd ICL of the receptor**

Previously, we showed that Rac1 acts as an intracellular positive allosteric modulator of CXCR4, which stabilizes the responsive conformation of the receptor. We set out to determine via which domain(s) in CXCR4 Rac1 exerted its function. To this end, we used a cell line stably expressing various chimeric receptors, in which increasing portions of CXCR4 were replaced with the corresponding parts of CCR5 (Figure 1A). We opted for the use of CCR5 domains because Rac1 is selective for CXCR4 and does not interfere with the conformation of CCR5.

The use of a conformation-independent mAb clone 4G10, raised to recognize the N-terminus of CXCR4, revealed that the surface expression levels of WT (wild type) CXCR4 and the three chimeras was very similar (Figure 1B). To investigate whether exchanging CXCR4 domains with the corresponding CCR5 parts would affect CXCR4 conformation, we used two
Figure 1. Rac1 regulates CXCR4 conformation via ICL3. (A) Schematic representation of the different CXCR4 chimeric receptors. WT CXCR4 domains are represented in black and CCR5 domains in orange (B) U87 cells expressing WT CXCR4 or the different chimeric receptors were stained with the conformation-independent anti-CXCR4 mAb 4G10 and mAb signal was subsequently measured by flow cytometry. (n=3) (C) U87 cells expressing WT CXCR4 or the different chimeric receptors were stained with the conformation-dependent anti-CXCR4 mAb 44717 and mAb signal was subsequently measured by flow cytometry. (n=3) (D) U87 cells expressing WT CXCR4 or the different chimeric receptors were treated with 50 µM NSC23766 and the CXCR4 (detected by mAb 44717) surface signal was measured by flow cytometry. (n=3). Bars show the mean fluorescence intensity ± SEM (B-C) and the mean fluorescence intensity expressed as percentage ± SEM compared to untreated conditions (D). **p< 0.01. Isotype controls representing non-specific antibody signal were negligible compared to the specific signal and did not vary between the different conditions and were therefore left out of the graph.
different conformation-dependent mAbs, clone 12G5 and clone 44717. Both mAbs recognize an epitope in the 2nd extracellular loop of CXCR4, which is expressed on the WT as well as on the different CXCR4 chimeras. CXCR4 surface signal of chimera FC-7 (CXCR4 with the C-terminus of CCR5), measured by clone 44717, was similar to WT-CXCR4. However, the surface signal of CXCR4 on chimera FC-6 (CXCR4 with the C-terminus, transmembrane domain 6 and 7 and ECL3 of CCR5) and FC-5 (CXCR4 with the C-terminus, transmembrane domain 6 and 7, ECL3 and ICL3 of CCR5) was drastically decreased as compared to the WT receptor (Figure 1C). The same observations were made using the CXCR4 mAb clone 12G5 (data not shown). The decreased binding of the conformation-dependent antibodies to FC-5 and FC-6, while the signal with the conformation-independent clone was not different between the different chimeras, indicates that both chimeras display a different conformation as compared to the WT receptor.

It has been shown that inhibition of Rac1 alters CXCR4 conformation. We therefore asked which chimeras were sensitive to Rac1 inhibition. Inhibition of Rac1 was performed with the pharmacological inhibitor NSC23766, which does not affect the function of CDC42 or RhoA and did not induce cellular toxicity in our experimental conditions (data not shown). As expected, Rac1 inhibition caused a significant decrease of WT CXCR4 surface signal, measured by the conformation-sensitive mAb clone 44717, which is indicative of a conformational change of the receptor (Figure 1D). The same conformational change was also observed in chimera FC-7, showing that the C-terminus of CXCR4 is not essential for Rac1 to control the receptor’s conformation. The conformation of the chimeric receptor FC-6, which is already different at steady state conditions, was further altered after Rac1 inhibition (Figure 1D). This indicates that none of the replaced domains in FC-6 mediate the effect of Rac1 on the conformation of CXCR4. However, exchanging the 3rd ICL of CXCR4 with the corresponding domain of CCR5, as in FC-5, completely abolished the effect of Rac1 inhibition on CXCR4 conformation (Figure 1D). Together, these results demonstrate that Rac1 regulates CXCR4 conformation via the 3rd ICL of the receptor.

Interfering with Rac1 association to the ICL3 alters CXCR4 conformation

To assess whether Rac1 and ICL3 associate, we performed a streptavidin-based pull-down assay using a biotinylated peptide representing the amino acid sequence of ICL3 in lysates of HeLa cells expressing the myc-tagged WT Rac1 or the myc-tagged active mutant of Rac1 (Q61L). We found that the constitutive active Rac1 mutant clearly interacts with ICL3 of CXCR4 (Figure 2A, upper panel). In contrast, the WT form of Rac1 showed high background staining (Figure 2A, middle panel) comparable to the staining obtained in a pull-down performed with lysates of untransfected cells (Figure 2A, lower panel). This background could mask a band representing Rac1WT binding to ICL3. To further confirm the role of ICL3 in regulating CXCR4 conformation, we used the cell-permeable ICL3 peptide as a dominant negative inhibitor. As shown in figure 2B, treating U937 cells with the ICL3 peptide caused a conformational change of CXCR4, as determined by the loss of conformation-dependent antibody binding without affecting the binding of the conformation-independent 4G10 antibody clone. The cell surface signal of the alternative CXCL12 receptor CXCR7 and the related chemokine receptor CCR5 was not reduced when the cells were treated with the peptide, showing the specificity of the ICL3 peptide for CXCR4 (Figure 2B).
Figure 2. Rac1 associates with ICL3 and Interfering with ICL3 alters CXCR4 conformation. (A) HeLa cells were transfected with myc-Rac1Q61L or myc-Rac1WT. Pull-down (PD) experiment was performed using lysates from transfected HeLa cells with a control peptide or a peptide representing ICL3. Association of myc-Rac1 was detected by immunoblotting with a myc-specific antibody. Data are a representative example out of two independent experiments. kD: kilodalton, TCL: total cell lysates. (B) U937 cells were incubated with a control peptide or the ICL3 peptide and CXCR4 (detected by mAb 44717 and mAb 4G10), CXCR7 and CCR5 surface signals were measured by flow cytometry. (n=3). Bars show the mean fluorescence intensity determined by flow cytometry and expressed as percentage ± SEM compared to untreated conditions. **p < 0.01
Thus, interfering with ICL3 function induces a conformational change in CXCR4 which is probably caused by inhibiting the association of Rac1 with the receptor.

**CXCR4 conformational change abrogates ligand binding and impairs cell migration**

To study the functional consequences of the CXCR4 conformational change, we examined whether and how CXCL12 can bind CXCR4 following Rac1 inhibition. Therefore, we performed a homologous competitive binding experiment using radiolabeled $[^{125}\text{I}]$-CXCL12 and Jurkat T-cells. These cells lack the alternative CXCL12 receptor, CXCR7, on the cell surface (data not shown). In the untreated condition, $[^{125}\text{I}]$-CXCL12 bound CXCR4 and was displaced by increasing concentrations of unlabeled CXCL12 (Figure 3A). However, we found that Rac1 inhibition by the pharmacological inhibitor NSC23766 abrogated binding of $[^{125}\text{I}]$-CXCL12 to CXCR4 and only non-specific binding was observed (Figure 3A). Thus, the conformation adopted by CXCR4 upon Rac1 inhibition prevents CXCL12 binding to the receptor.

We further investigated changes in CXCR4 signaling by measuring CXCL12-mediated intracellular calcium mobilization upon Rac1 inhibition. A clear rise in calcium levels was observed in control Jurkat cells (Figure 3B). However, only a minor peak of calcium mobilization was observed after NSC23766 treatment of these cells indicating a decrease of CXCR4 signaling efficiency upon Rac1 inhibition (Figure 3B).

Typically, impaired ligand binding and receptor signaling result in a decrease of cell motility. Therefore, we analyzed the chemotaxis of Jurkat cells displaying an altered CXCR4 conformation. We used the ICL3 peptide to induce a conformational change in CXCR4. Treating the cells with this peptide caused a significant decrease in their migratory capacity compared to untreated or control peptide treated cells (Figure 3C). The same inhibition of cell migration was observed after treating the cells with NSC23766 (data not shown), confirming that Rac1 inhibition suppresses CXCR4 function. These data correlate with the impaired ligand binding and signaling capacity of CXCR4 after adopting a new conformation.

Taken together, these results show that Rac1 and ICL3 are key players in the maintenance of the responsive CXCR4 conformation.

**Mechanisms of CXCR4 conformational change**

The change in CXCR4 conformation upon Rac1 inhibition could be the result of several factors, including CXCR4 exclusion from lipid rafts, impaired actin network organization or changes in $\alpha_i$ coupling. To determine if any of these factors is involved in the conformational change in CXCR4 following Rac1 inhibition, we first examined the effect of hydroxypropyl-$\beta$-cyclodextrin ($\beta$CD), which is a cholesterol-depleting agent disrupting the integrity of lipid rafts and thereby interfering with proper Rac1 localization. In accordance with Nguyen et al., binding of the conformation-dependent mAb 44717 decreased after treating the myeloid HL60 cell line with $\beta$CD while the binding of the conformation-independent mAb 4G10 was not affected (Figure 4A). The same results using $\beta$CD were obtained with the U937 cell line (data not shown). In addition, the use of the second conformation-dependent mAb 12G5 in both cell lines confirmed the results presented above (data not shown). This indicates that lipid raft integrity is important for the maintenance of CXCR4 conformation. We next investigated whether actin polymerization, which is largely mediated by Rac1, could account for the regulation of CXCR4
Figure 3. CXCR4 conformational change abrogates CXCR4 biological activity. (A) Jurkat T-cells were pre-treated with NSC23766 and subsequently incubated with [\(^{125}\)I]-CXCL12 (75 pM) and increasing concentrations of unlabeled CXCL12 in a homologous competitive binding experiment. Binding of radiolabeled [\(^{125}\)I]-CXCL12 was measured as described in the materials and methods section. The graph shows the mean of 3 independent experiments. (B) Jurkat T-cells were pre-treated with the calcium-sensitive dye, Indo-1, in the presence or absence of NSC23766 as indicated in the materials and methods section. Subsequently, Indo-1 biphasic fluorescent signals (calcium-bound: 405 nm and unbound: 485 nm) were measured by flow cytometry. During measurements, cells were stimulated with CXCL12 then ionomycin and finally EGTA. The graph represents the ratio of free Indo-1/bound-Indo1 as calculated by the FlowJo software. (representative example out of five independent experiments is shown). (C) Jurkat T-cells were allowed to migrate for 3 hours to 100 ng/ml CXCL12 in the presence or absence of a control peptide or ICL3 peptide. Bars represent the mean percentage ± SEM (n=3) of cell migration compared to the untreated cells (set at 100%). **p< 0.01.
Figure 4. Mechanisms of CXCR4 conformational change. (A) HL60 cells were incubated with BCD and surface CXCR4 was measured by mAb 44717 or mAb 4G10. (n=3) (B) HL60 cells were incubated with a control peptide, the Rac1 inhibitory peptide or cytochalasin B and surface CXCR4 was measured by mAb 44717. IgG2B was used as isotype control. (representative example out of two independent experiments is shown) Cyto B: cytochalasin B (C) HL60 cells and U937 cells were pre-treated with PTX and stimulated or not with CXCL12. CXCR4 was measured by mAb 44717. IgG2B was used as isotype control. (representative example out of two independent experiments is shown). Bars show the mean fluorescence intensity expressed as percentage ± SEM compared to untreated conditions (A) or the mean fluorescence intensity (B-C).
Conformation. Inhibition of Rac1 with a biotinylated Rac1 C-terminal peptide reduced the binding of mAb 44717. On the other hand, treating the cells with cytochalasin B, which is an inhibitor of actin polymerization, did not affect the binding of this mAb (Figure 4B). This shows that the conformational change in CXCR4 upon Rac1 inhibition is not caused by decreased actin polymerization. Finally, we questioned whether interfering with G$_{\alpha}$i coupling to CXCR4 would affect the receptor’s conformation. The reactivity of the conformation-dependent mAb 44717 was not affected by treatment of HL60 or U937 cells with pertussis toxin (PTX), a bacterial toxin that ADP-ribosylates and thereby inhibits G$_{\alpha}$i coupling to CXCR4 (Figure 4C). Moreover, the decrease of CXCR4 surface expression after CXCL12 stimulation shows that the extent of receptor endocytosis in PTX-treated cells was comparable to control cells (Figure 4C). Thus, CXCL12 binding to CXCR4 after PTX treatment is not altered. These findings demonstrate that G$_{\alpha}$i coupling could not account for the observed CXCR4 conformational change.

To conclude, the integrity of lipid raft microdomains is an important factor for the regulation of CXCR4 conformation.

Discussion

Although the canonical pathways regulating CXCR4 signaling that control receptor trafficking and desensitization are relatively well established, only scant information is available about the mechanisms regulating receptor conformation. Recently, we established Rac1 as a key player in the regulation of CXCR4 conformation\cite{22}. This conformation is not persistently similar, but is a regulated variable that controls the efficiency of ligand binding and downstream signaling. Here, we present data indicating that Rac1 affects the conformation of CXCR4 via the ICL3 of the receptor. We show that the activity of Rac1 as well as the integrity of ICL3 affect CXCR4 conformation and is a prerequisite for efficient ligand binding and normal physiological responses, such as chemokine-induced cell migration.

Previously, we found that Rac1 associates with CXCR4 via the Rac1 C-terminus independently of GDP-GTP loading of the GTPase. Furthermore, using different inhibitors of Rac1, we demonstrated that Rac1 activity is required for the regulation of CXCR4 conformation\cite{22}. In this study, we identify the ICL3 of CXCR4 as a binding site for Rac1. Whether this interaction is direct still remains to be definitively established. In contrast to the full-length form of the receptor, ICL3 bound preferentially to the active GTP-loaded form of Rac1. These observations suggest the existence of multiple binding sites on CXCR4 for Rac1 and put forward the possibility that the ICL3 domain is responsible for mediating Rac1 signaling, thereby regulating the receptor’s conformation. This notion was further supported by our finding that Rac1 inhibition did not further affect the receptor’s conformation after exchanging the ICL3 in chimera FC-5, in contrast to chimera FC-6, which still expresses this domain.

In control situations, chimera FC-6 adopted a different conformation compared to WT CXCR4, demonstrating that CXCR4 requires its original domains to preserve its conformation. However, we found that the cytoplasmic tail (chimera FC-7) was not involved in determining CXCR4 conformation. In line with this, it has been shown that only chimera FC-7 was able to elicit a calcium response upon CXCL12 stimulation, in contrast to the FC-6 and FC-5 chimeras\cite{32}. Loss
of the CXCL12 binding pocket, which comprises the N-terminus and the 2nd and 3rd extracellular loops of the receptor, is one explanation for these observations. Our data suggest that the different conformational states of chimeras FC-5 and FC-6 could also contribute to the lack of ligand binding and receptor responsiveness.

A key finding of the current study is that interfering with the ICL3 domain by the use of dominant negative peptides results in a conformational change of CXCR4 probably by hijacking Rac1. The resulting conformation did not support CXCR4 biological activity as measured in a CXCL12 chemotaxis assay. In a similar study, CXCR4 mutants, in which each ICL was exchanged by a scrambled amino acid sequence of ICL1, were constructed to identify the individual roles of these domains. These authors showed that exchanging ICL3 abolished Goi signaling, such as calcium mobilization and MAPK activation. Our data support these findings and suggest a hypothesis that the ICL3 CXCR4 mutant adopts a non-responsive conformation.

The physiological relevance of inactive Rac1 association to CXCR4 is not clear. In a recent paper, Saci et al. showed that Rac1 binds to and targets mTOR to specific membrane domains independently of its GTP-bound state. These findings contradict the central dogma of GTPases stating that only their GTP-bound state is biologically active.

Similarly, Rac1 could target CXCR4 to specific membrane domains where the receptor adopts a responsive conformation. Several reports showed that efficient CXCR4 signaling and HIV co-receptor activity depend on its localization in lipid rafts. Accordingly, we show that disruption of lipid raft integrity affects CXCR4 conformation. Together, these data suggest a model in which Rac1 targets CXCR4 to lipid raft microdomains, thereby promoting its functional conformation.

Moreover, Baribaud et al. described the presence of at least two antigenically distinct conformations of CXCR4 in resting conditions. This heterogeneity in CXCR4 populations was not due to differences in N-linked glycosylation or sulfation of the receptor. We further demonstrate that CXCR4 conformation does not depend on actin polymerization or Goi coupling to the receptor.

In conclusion, our previous and current data establish Rac1 and the ICL3 as important factors controlling the fine-tuning of CXCR4 conformation and activity. Future experiments will be aimed at defining the mechanisms by which Rac1 regulates the conformation of CXCR4. This knowledge may provide new strategies for a more selective and efficient therapeutic interference with CXCR4-mediated signaling.

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Reference list


