New dimensions in CXCR4 and Rac1 regulation

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Summary and concluding remarks
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In multicellular organisms, cells have developed sophisticated mechanisms to cope with the multitude of external cues that they are exposed to. Extracellular stimuli must be thoroughly integrated into specific physiological responses for a proper functioning of the organism. Any disturbances in signal transduction, due to for example aberrant activation or inactivation of a given signaling pathway, could result in serious conditions such as the development of cancer or autoimmune diseases. In many cellular disorders, cells must accumulate different, consecutive abnormalities (i.e. mutations) in order to cause disease. For example cancer and autoimmunity are caused by excessive cellular proliferation combined with targeted cell migration. The aim of this thesis was to gain new insights in the complex regulation and signaling of two important proteins taking part in the signal transduction toward both cell proliferation and migration i.e. CXCR4 and Rac1.

CXCR4 is a GPCR expressed on a multitude of different cells including malignant cells. GPCRs are a large family of seven transmembrane receptors that sense a wide range of extracellular cues, such as hormones, neurotransmitters and cytokines, and therefore represent attractive targets for drug development. For instance, CXCR4 gained much attention after discovering its involvement in HIV pathogenesis. Moreover, this receptor appeared to induce tumor cell proliferation and metastasis and functions at the center of hematopoietic stem cell homing and mobilization. Over the years, the advantage of structure-based drug design has been established. However, three-dimensional structures of GPCRs appeared to be hard to determine due to the seven-transmembrane-spanning nature of the receptor. Therefore, it is clear that more knowledge on the conformations adopted by these receptors is required for the development of efficient therapeutic targeting strategies.

Rac1 is a small GTPase belonging to the Rho family of GTPases. Since its discovery, Rac1 was rapidly established as an important regulator of cytoskeletal dynamics taking part in many physiological processes, including cell polarization, cell migration, cellular attachment to the extracellular matrix and cell division. Consequently, Rac1 signaling takes part in embryonic development, cell differentiation and proliferation and immune responses. Rac1 knock-out mice die at the embryonic stage, underscoring its crucial role. Like CXCR4, Rac1 was identified as a key player in the development and metastasis of cancer. Thus, a detailed understanding of the molecular mechanisms regulating the fine-tuning of CXCR4 and Rac1 activation and downstream signaling is crucial.

Considering the critical role of CXCR4 in many biological processes, cells have developed several highly structured mechanisms to regulate the activation and signaling of this receptor. The levels of CXCR4 expression on the cell surface is an important determinant of the amplitude of CXCR4-mediated signaling. Therefore, CXCR4 regulation begins at the transcriptional level. Next to the basal control of CXCR4 transcription, several stimuli can affect its transcription. Lack of oxygen in the cellular environment, for example, can induce the upregulation of CXCR4 via the transcription factor hypoxia-inducible factor. This mechanism is mostly employed by neoplastic cells to egress areas of low oxygen. In addition, several cytokines and growth factors can modulate the expression of CXCR4. Once expressed, CXCR4 is subjected to posttranslational modifications that contribute to proper expression and function on the
cell surface. The N-terminal extracellular region of CXCR4 is subjected to glycosylation and sulfation. N-linked glycosylation of CXCR4 is important for the cell surface expression of the receptor. In addition, sulfation as well as glycosylation of CXCR4 is necessary for proper ligand binding\textsuperscript{22-25}. Finally, via complex mechanisms involving vesicular trafficking, CXCR4 is transported to the cytoplasmic membrane. However, a pool of CXCR4 is kept into intracellular vesicles constituting a reserve pool, which is ready to translocate to the cell surface when required by the extracellular stimuli. Once on the surface and in steady state conditions, CXCR4 levels can still be modulated by spontaneous internalization and recycling\textsuperscript{26,27} and ligand-independent endocytosis provoked by external factors, such as the cytokines interleukin-4 (IL-4), IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF)\textsuperscript{28}. The control of CXCR4 function from its transcription to its cell surface expression constitutes just one phase of the multiple phases of CXCR4 regulation. The management of CXCR4 signaling upon ligand binding is the second phase of CXCR4 regulation and has been addressed in \textbf{chapter 1} of this thesis.

The ability of CXCR4 to switch conformations after ligand binding is established\textsuperscript{29}. In the absence of CXCL12, CXCR4 is in its inactive conformation. Stimulation with CXCL12 induces the active conformation of CXCR4 that is required for the subsequent activation of G\textsubscript{i}. The existence of antigenically distinct conformations of CXCR4 on the cell surface in steady state conditions added a new level of complexity to CXCR4 regulation\textsuperscript{30-32}. This generated a new hypothesis proposing the existence of a responsive as well as a non-responsive CXCR4 conformation.

While exploring CXCR4 signaling toward Rac1 in the context of cell migration, we found that inhibition of Rac1 decreased CXCR4 cell surface expression. The decrease of CXCR4 signal at the cell surface was not caused by enhanced internalization of the receptor, but rather caused by impaired binding of the conformation-sensitive antibody that we used. Our hypothesis that Rac1 inhibition changes the conformation adopted by CXCR4 was confirmed by the use of a conformation-independent antibody against CXCR4, the binding capacity of which was unaffected by Rac1 inhibition. These findings, described in \textbf{chapter 2}, identify Rac1 as an allosteric modulator of CXCR4 that dictates the conformation of the receptor (Figure 1).

A few studies that investigated CXCR4 conformational changes in different contexts used bioluminescence resonance energy transfer (BRET) technology\textsuperscript{29,33,34}. In this assay CXCR4 DNA constructs are fused to a bioluminescent energy donor (usually renilla luciferase) or a fluorescent acceptor (usually YFP) and co-transfected into a cell. Assuming that CXCR4 can form homodimers that are expressed on the cell surface, this will result in a BRET signal. Any conformational change in CXCR4 is presumed to drive the CXCR4 couple away from each other or closer to each other causing a decrease or an increase of the BRET signal, respectively. This assay is usually performed in Hek293T or HeLa cells, because they are easy to transfect and to monitor by confocal microscopy. However, these cells do not express endogenous CXCR4 and ectopically expressed CXCR4 may not reflect the physiological behavior of this receptor. In this context, the use of conformation-(un)sensitive antibodies is a useful tool to monitor CXCR4 conformational changes in different cell lines or primary cells expressing endogenous CXCR4. A recent paper has reported the crystal structure of CXCR4 thereby providing the three
dimensional structure of the first chemokine receptor\textsuperscript{35}. This represented a powerful tool for us in order to ‘visualize’ the conformational change of CXCR4 upon Rac1 inhibition. Nevertheless, our results in chapter 2 and 3 demonstrate that inhibition of Rac1 induces the non-responsive conformation of CXCR4 that impairs CXCL12-mediated signaling. Thus, we show for the first time that Rac1 can act as a positive allosteric modulator of a GPCR. Some allosteric modulators can affect receptor signaling without interfering with the ligand binding capacity\textsuperscript{36,37}. However, we show that Rac1 inhibition blocks CXCL12 binding, suggesting that the conformational change affects the ligand binding pocket of CXCR4, which is formed, in part, by the 2\textsuperscript{nd} extracellular loop (ECL2)\textsuperscript{35,38,39}. The fact that the conformation-dependent antibodies recognizing an epitope on the ECL2 both lose their reactivity upon Rac1 inhibition makes it highly probable that Rac1 activity regulates CXCR4 responsiveness by stabilizing a ‘ready to go’ ECL2 conformation.

An important question remains: what is the extracellular signal that regulates and directs Rac1 activity towards CXCR4 under physiological conditions? It is possible that CXCL12-induced activation of Rac1 initiates a positive feed-back loop responsible for increasing and maintaining the CXCL12-responsive CXCR4 conformation. This hypothesis is hard to verify since CXCL12 stimulation is already causing a conformational change in CXCR4, which is followed by receptor internalization. A second mechanism could involve “cross-conformational regulation”\textsuperscript{40}. Like

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\caption{Rac1 regulates CXCR4 conformation. (A) Rac1 activity keeps CXCR4 in its responsive conformation that allows for CXCL12 association as well as HIV binding and viral entry into the cell. We propose a model wherein Rac1 recruits CXCR4 to lipid raft microdomains, thereby promoting conversion to the responsive conformation of CXCR4. (B) (1) Inhibition of Rac1 causes a conformational change of CXCR4 turning the receptor into the non-responsive conformation. (2) This non-responsive conformation impairs ligand association and also blocks HIV binding and entry into the cell.}
\end{figure}
receptor cross-activation, it could be possible that a specific receptor signals through Rac1 to induce the responsive conformation of CXCR4 and enhance its activity. In the context of cell migration, this receptor could be a chemokine receptor, a growth factor receptor or a receptor involved in cell adhesion.

A key finding in chapter 2 is that CXCR4 conformational change upon Rac1 inhibition blocks cellular infection by the CXCR4-using HIV-1 strain (X4-HIV-1), by preventing virus binding and entry into the cell. In a parallel experiment, we showed that inhibition of Rac1 does not affect the infection caused by a CCR5-dependent HIV-1 strain. This finding demonstrates that in our experimental settings, early steps of HIV-1 infection do not require a physiological role of Rac1, including actin polymerization. Accordingly, it has recently been shown that cortical actin in resting CD4 T cells acts as a barrier for viral post entry migration. To overcome this restriction the virus signals via CXCR4 to activate the actin-depolymerizing machinery.

Since the ECL2 is an important determinant on CXCR4 for binding of X4-HIV-1, we suggest that the virus can not properly bind to CXCR4 following its conformational change. Our findings open new possibilities for therapeutic manipulation of CXCR4. Our group previously reported that the cell permeable peptides encoding the C-terminus of Rac1 could efficiently be employed as Rac1 inhibitors in vivo. In a rheumatoid arthritis mouse model, treatment with these peptides to block immune cell trafficking, reduced lymphocyte infiltration and swelling of the inflamed joints. However, inhibition of Rac1 as a strategy to block X4-HIV-1 infection is not possible, because this would affect the wide range of biological processes controlled by Rac1 including immune responses and integrity of epithelial and endothelial monolayers. It is therefore important to better understand how Rac1 can stabilize the responsive (HIV-1 permissive) conformation of CXCR4 and to determine the proteins via which Rac1 acts. This approach could eventually lead to targets that allow for therapeutic intervention via regulation of CXCR4 conformation. Recently, allosteric agonist peptides of CXCR4 have been developed. These peptides do not depend on the binding pocket of CXCL12 and can induce CXCR4-mediated signaling. Therefore, a combination of a drug that alters CXCR4 conformation with these peptides represents a potentially successful approach to prevent HIV-1 entry, while preserving CXCR4 physiological responses (Figure 2).

In an attempt to better characterize the Rac1-CXCR4 axis, we identify in chapter 3 the domain of CXCR4 responsible for mediating the Rac1 effect on the receptor’s conformation. The 3rd intracellular loop (ICL3) of CXCR4 associated preferentially with active Rac1 and was responsible for mediating the effect of Rac1 on the conformation of CXCR4. The use of cell-permeable peptides encoding the amino acid sequence of ICL3 as a dominant negative competitor was sufficient to induce a conformational change of CXCR4 that did not support CXCL12-mediated signaling. The ICL3 has been shown to mediate Gα signaling responses, such as calcium mobilization and the activation of the MAPK pathway. Thus, it appears that this domain functions at the center of CXCR4 function. While ICL3 has a preference for binding to active Rac1, the full length receptor bound to Rac1 independently of its GTP-bound state. We still do not know the exact function of inactive GDP-Rac1 association to CXCR4. However, there is increasing evidence that GDP-bound Rac1 fulfills signaling functions. Moreover, our group showed that ubiquitylation and degradation is one of the mechanisms that control the prevalence
and signaling of GDP-Rac1. This contradicts the central dogma stating that only GTP-Rac1 is signaling competent. In chapter 3, we explored several mechanisms that could possibly control the conformation of CXCR4. We excluded Gαi coupling to the receptor or actin polymerization as possible mechanisms governed by Rac1 and responsible for stabilizing CXCR4 conformation on the cell surface. However, we found that the disruption of lipid raft microdomains alters CXCR4 conformation. As CXCR4 localization in lipid raft-rich membranes has been shown to positively affect its function, we suggest that CXCR4 presence in lipid rafts induces its responsive conformation. There is evidence that both Rac1 and CXCR4 co-exist in the same lipid raft microdomains. Moreover, Rac1 signaling has been shown to regulate the subcellular localization of many proteins. Thus, we propose that Rac1 promotes CXCR4 incorporation into lipid rafts as a possible model explaining how Rac1 regulates CXCR4 conformation. This hypothesis is further supported by the work of Lin et al. showing that attenuation of lipid raft-associated Rac1 activity in esophageal carcinoma cells abrogates CXCR4 localization at lipid raft microdomains and inhibits CXCL12-induced invasion of these malignant cells.

In our group we are particularly focused on understanding the role of the hypervariable region of Rac1, which appears to mediate a central role in GTPase function in contrast to

**Figure 2. Therapeutic manipulation of CXCR4.** (1) To induce the conformational change of CXCR4, we intend to inhibit one of the elusive, intermediary proteins that are regulated by Rac1 and may act to convert CXCR4 conformation. This will induce the non-responsive receptor that blocks CXCR4 signaling and HIV infection. (2) As in this situation, CXCL12 binding is also inhibited, stimulation of the receptor with agonistic peptides that bind to CXCR4 through other, allosteric, sites than the binding pocket of CXCL12, would allow maintenance of CXCR4 biological functions. RSVM and ASLW: two peptides with RSVM and ASLW amino acid sequences at their N termini.
what was previously proposed. This region mediates the function of many effector as well as regulatory proteins, regardless of the GDP/GTP-bound state of the GTPase. We identified novel Rac1 interactors, all using the C-terminus of the GTPase, including CXCR4, caveolin-1, PACSIN2 and the nuclear proto-oncogene SET/I2PP2. These proteins act independently of each other and reside in different subcellular compartments and depending on the type of signal, they fulfill different cellular functions.

In chapter 4, we identify the nucleocytoplasmic oncogene nucleophosmin 1 (NPM1) as a novel regulator of Rac1 activation and signaling. NPM1 is a multifunctional phosphoprotein protein taking part in various cellular processes, such as ribosome biogenesis, the maintenance of genomic stability and the inhibition of pro-apoptotic pathways. NPM1 is an oncogene frequently mutated in acute myeloid leukemia (AML) characterized by aberrant NPM1 accumulation in the cytoplasm. In this study, we show that NPM1 interacts with the hypervariable C-terminal domain of Rac1 and negatively regulates its activation resulting in a decrease in cell spreading capacity. NPM1 is a shuttling protein containing both a nuclear localization signal (NLS) as well as a nuclear export signal (NES). However, despite the NES motif, NPM1 is mainly localized in nucleoli. Thus, under physiological conditions, nuclear import of NPM1 dominates its export. Since Rac1 also contains a NLS in its C-terminal tail and is believed to participate in signal transduction inside the nucleus, it is possible that NPM1 is responsible for modulating Rac1 nuclear signaling. Another explanation is that NPM1 senses high Rac1 activity and rapidly moves into the cytosol to dampen Rac1 signaling. This notion is supported by our finding that constitutive active Rac1 drives NPM1 out of the nucleus into the cytoplasm. It is not clear yet how NPM1 decreases Rac1 activity. We show that NPM1 does not target Rac1 for degradation. Thus, NPM1 may enhance GTP hydrolysis or disturb GEF activity. Next to SET, NPM1 is the second nuclear oncogene we identified in our group as a binding partner of Rac1. Like SET, NPM1 nuclear exit is induced by Rac1 activity. As NPM1 cytoplasmic localization is associated with AML, this suggests a possible involvement of Rac1 in malignant transformation of myeloid stem cells and requires further attention.

Zhang et al. identified NPM1 as a novel protein responsible for suppressing CXCR4 signaling and CXCL12-mediated chemotaxis. Like Rac1, NPM1 associates with CXCR4 via the ICL3. As NPM1 inhibits Rac1 activity, we anticipated that NPM1 overexpression would cause a conformational change in CXCR4 via a mechanism involving Rac1 inhibition. However, using our set of conformation-(in)dependent antibodies, we did not observe a conformational change in CXCR4 upon NPM1 overexpression as compared to control conditions (data not shown). This suggests that NPM1 acts as negative regulator of CXCR4 independently of Rac1 and does not induce the non-functional conformation of CXCR4 obtained after Rac1 inhibition.

Our research on the regulation of CXCR4 conformation and the dynamics of NPM1 highlights the importance of the hypervariable C-terminus of Rac1. Indeed, both CXCR4 and NPM1 bind to Rac1 via its C-terminus. Basic knowledge of the Rac1 C-terminus is summarized in chapter 1. In chapter 5, we aimed to provide increased understanding of the role of the hypervariable C-terminus of Rac1 in regulating the function of this GTPase. We previously established that the Rac1 C-terminus contains two binding motifs for signaling proteins that mediate differential signaling events in a non-redundant fashion. The specialized domains
composing the C-terminus of Rac1 are a proline-rich stretch (i.e. three consecutive prolines PPP) and a polybasic domain (i.e. six consecutive basic residues KKRKRK). While this work relied on cell-permeable peptides encoding the amino acid sequence of wild-type Rac1 C-terminus, a proline-mutant C-terminus (i.e. PPP-AAA) or a polybasic mutant C-terminus (i.e. RKR-AAA), in the current study we made use of full-length Rac1 constructs encoding these C-terminal mutations to further dissect the role of these separate regions in Rac1 regulation. First, we examined the subcellular localization of the different Rac1 constructs. As expected, the constitutive active Rac1 was targeted to focal adhesions where it is known to participate in the formation of adhesive structures and to mediate integrin-dependent cell adhesion to the underlying extracellular matrix. The proline-rich domain of Rac1 is mostly involved in binding effector and regulatory proteins and contributes to the NLS. Here we show that mutating this region does not impair the subcellular targeting of active Rac1.

In our previous work we found that the Rac1 C-terminal peptides were targeted to the membrane. The membrane localization of these peptides was dependent on the polybasic RKR motif of the Rac1 C-terminus. These results were surprising since these peptides do not possess a CAAX box and therefore lack a lipid tail, which is responsible for membrane anchoring. However, the positive charge of the polybasic region could be responsible for the association with the membrane that carries a negative charge depending on its phospholipid composition. In contrast to these results, our current analysis shows that the relatively large mutation within the polybasic region of the Rac1 C-terminus (3 out of 6 amino acids) in the context of a full-length activated Rac1 did not hamper its targeting to focal adhesions. This was unexpected because most if not all of the Rac1 regulatory proteins, described by us, require an intact polybasic region in the C-terminus of Rac1 to allow the interaction with Rac1. Moreover, numerous studies describe the importance of this domain in Rac1-targeting specificity. This suggests that Rac1, in its GTP bound active conformation, can bypass the requirement of an intact polybasic region in its C-terminus for proper targeting to specific microdomains.

Next to the Rac1 conformation, other mechanisms could contribute to the targeting of the active Rac1 polybasic-region mutant. The constitutively active Rac1 mutants are known for their decreased sensitivity to RhoGDIs and would therefore be less efficiently retained in the cytoplasm and instead more efficiently targeted to the membrane. In addition, their exposed lipid tail could confer some subcellular targeting specificity. Another explanation is that some effector proteins that bind to Rac1 in its GTP-bound conformation could mediate the translocation of active Rac1 to specific membrane compartments. We also demonstrate that neither the proline-rich region, neither the polybasic region of wild-type Rac1 is required for GEF-mediated activation of the protein. This also suggests that these Rac1 mutants are normally translocating to the plasma membrane, the ‘field of action’ of GEF proteins. Although both Rac1 C-terminal mutants seem to be normally regulated, we showed that they have reduced capacity to mediate physiological responses such as cell spreading. This could be attributed to the lack of binding to regulatory proteins that we and others previously described to require either an intact proline-rich domain or polybasic region in the Rac1 C-terminus.

Our present goal is to use new optical methods that could considerably improve the spatiotemporal control of the different Rac1 mutants of interest. For example, a caged
analog of rapamycin can be locally activated by UV light and induce localized Rac1 activation characterized by spatially confined membrane ruffle formation\(^\text{82}\). Another approach is the use of an active Rac1 mutant with an auto-inhibitory loop, which is released upon application of light at a specific wavelength\(^\text{83}\). These methods restrict the amount of activated Rac1 in the cell and overcome the effects of overexpression and/or compensation for the prolonged presence of the constitutively active GTPase.

A key finding in this chapter is that mis-positioning of an intact hypervariable C-terminus abrogates Rac1 regulation and function, even in the context of a constitutively active form. Indeed, a GFP insertion just before the C-terminus of activated Rac1 inhibits its targeting to focal adhesions and inhibits the induction of cell spreading as compared to the normal activated Rac1 protein. Even the activation of this mutant by a GEF protein was impaired. These findings show that the integrity of Rac1 conformation and a precise position of the C-terminus toward the G domain of the protein is a prerequisite for proper Rac1 function. These data are especially important considering the development of Rac1 biosensors, which contain a fused fluorescent protein and argue against any insertion that would position the Rac1 C-terminus away from the core protein.

In Summary, this chapter provides more knowledge on the complex regulation of Rac1 and describes a previously unrecognized role of the hypervariable C-terminus that intimately cooperates with the G domain to control Rac1 function.

Given the association of CXCR4 and Rac1 to numerous pathological conditions, considerable attention has been paid to these proteins. Yet, we are just beginning to understand the diversity and complexity of the mechanisms regulating Rac1 and CXCR4 signaling. This thesis identifies new mechanisms as well as new players in the regulation of these two proteins. We also show that different proteins, with seemingly unrelated functions and different subcellular localizations, are actually acting in harmony to generate the wide diversity of signaling pathways and responses produced by a cell.

The possible interplay between Rac1, CXCR4 and NPM1 presents new targets for therapeutic manipulation in many pathological conditions, such as hematological diseases, tumor cell pathology and HIV infection and most importantly provides a new topic for exciting research.
References


