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Chemokine receptor internalization and intracellular trafficking

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Abstract

The internalization and intracellular trafficking of chemokine receptors have important implications for the cellular responses elicited by chemokine receptors. The major pathway by which chemokine receptors internalize is the clathrin-mediated pathway, but some receptors may utilize lipid rafts/caveolae-dependent internalization routes. This review discusses the current knowledge and controversies regarding these two different routes of endocytosis. The functional consequences of internalization and the regulation of chemokine receptor recycling will also be addressed. Modifications of chemokine receptors, such as palmitoylation, ubiquitination, glycosylation, and sulfation, may also impact trafficking, chemotaxis and signaling. Finally, this review will cover the internalization and trafficking of viral and decoy chemokine receptors.

Keywords

Chemokine receptor; Internalization; Trafficking; Endocytosis; Modifications

1. Introduction

Chemokine receptors undergo a basal level of internalization and degradation or recycling in the absence of ligand. Ligand binding can greatly enhance the internalization and trafficking of these G protein-coupled receptors (GPCRs) and can increase the dynamics of receptor sensitization versus desensitization and of receptor recycling versus degradation. The receptor trafficking pathways may vary depending on the presence or absence of ligand. Two major choices are available for this trafficking: clathrin-mediated endocytosis, versus lipid raft/caveolae-dependent internalization. Some receptors take advantage of both of these pathways, while others may follow one pathway the majority of the time. The cell type in which the receptor is expressed may in part determine the likelihood of utilization of one pathway as compared to another. This may be due to the ratio of specific adaptor proteins, the lipid composition of the membrane in proximity to the domain the receptor is localized in, or other poorly characterized determinates. The fate of the receptor after ligand stimulation (to traffic or not to traffic) may affect the length, strength, or type of intracellular signals generated. Moreover, the type of post-translational modifications of the receptor can also have major effects on ligand mediated signaling. In this review, we will cover four major aspects of chemokine receptor trafficking: clathrin mediated endocytosis; caveolae/lipid raft mediated

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trafficking; effects of receptor trafficking on downstream signal transduction and impact of receptor modifications on receptor trafficking and signaling.

2. Chemokine receptors and the clathrin-mediated endocytic pathway

A major mechanism by which chemokine receptors undergo ligand-induced internalization is through clathrin-mediated endocytosis (Fig. 1) [1–5]. The binding of ligand results in phosphorylation of Ser and Thr residues in the intracellular loops and carboxyl-terminus of the chemokine receptor by G protein-coupled receptor kinases (GRKs) (Table 1) [6–9]. Phosphorylation results in the uncoupling of the G protein subunits from the receptor and receptor desensitization in some cases [8,10]. In addition, the phosphorylation of these residues and/or the presence of di-leucine motifs in the carboxyl-terminal domain of chemokine receptors are important for the recruitment of adaptor molecules that link the receptor to a lattice of clathrin that facilitates receptor internalization. Two adaptor molecules that play important roles in chemokine receptor internalization are adaptin 2 (AP-2) and β -arrestin. β -arrestin binds with higher affinity to the phosphorylated receptor, to the β 2-adaptin subunit of the AP-2 heterotrimeric protein complex, and to clathrin to mediate endocytosis [11–15]. It was originally thought that β -arrestin binding to GPCRs was only mediated through phosphorylated residues in the carboxyl-terminus. However, more recent chemokine receptor studies suggest that binding can also occur through the intracellular loops. Studies on CCR5 demonstrate that phosphorylated Ser residues in the carboxyl-terminus and a conserved Asp-Arg-Tyr sequence motif in the second intracellular loop are necessary for β -arrestin association [16]. Moreover, β -arrestin binds to both the carboxyl-terminus and the third intracellular loop of CXCR4 [17]. AP-2 binds directly to some chemokine receptors, including CXCR2 and CXCR4, through highly conserved Leu-Leu, Ile-Leu, Leu-Ile motifs in the carboxyl-terminus [18,19]. The association of receptors with these adaptor molecules results in recruitment of clathrin and formation of clathrin-coated pits which ‘pinch off’ from the membrane through the action of dynamin and become clathrin-coated vesicles [1,2,4,20–25]. The clathrin-coated vesicle is then uncoated and the receptor-ligand complex enters the early endosomal compartment. Recent findings suggest that β -arrestin not only plays an important role in the desensitization and internalization of chemokine receptors, but also in the intracellular trafficking of chemokine receptors. According to immunofluorescence staining and confocal microscopy, β -arrestin accompanies CXCR4 to the early endosome following CXCL12-induced internalization [24]. However, it remains unclear whether this endosomal colocalization points to an active contribution of β -arrestin to the endosomal trafficking of CXCR4 or whether it is just a consequence of the binding of β -arrestin to both clathrin and CXCR4 during internalization. The chemokine receptor can then either enter the perinuclear recycling compartment and traffic back to the plasma membrane to be reexposed to ligand, or it can enter the late endosomal compartment where it will be sorted to the lysosomal compartment for degradation.

3. Internalization of chemokine receptors via lipid rafts and caveolae

Recent studies suggest that the trafficking and signaling of certain chemokine receptors may, in some instances, be regulated by clathrin-independent pathways. These pathways may be mediated through lipid rafts or through cholesterol-rich structures called caveolae [26–29].

Lipid rafts, also known as membrane rafts, glyco-sphingolipid-enriched microdomains, or detergent-resistant microdomains, are relatively resistant to solubilization with commonly used detergents such as nonylphenyl-polyethylene glycol (NP-40), Triton X-100 or 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), in contrast to the bulk membrane. The lipid rafts are held together mainly by hydrophobic interactions between sphingomyelin and glycosphingolipids, and the stable formation of the rafts is further enhanced

by intercalated cholesterol molecules [30–32]. Several GPCRs have been shown to signal in lipid rafts/caveolae [33–36]. At present, at least two chemokine receptors, CCR5 and CXCR4, have been identified to some degree in raft membranes [37–39]. The observation that CCR5 and CXCR4 are constitutively associated with other raft proteins, such as CD4, provides indirect evidence for their raft localization [40]. Moreover, inhibition of glycosphingolipid synthesis reduces the CCR5 levels in cell membranes, suggesting association of CCR5 with rafts for proper receptor transport [41]. Although CXCR1 and CXCR2 are soluble in Triton X-100 and co-patched with non-raft rather than raft markers, a fraction of CXCR1 and CXCR2 is associated with alternative cholesterol-based rafts [42]. Post-translational modifications of chemokine receptors, such as palmitoylation, are probably crucial for association with specialized lipid domains in the membrane. These modifications are discussed in a later section of this review.

Lipid rafts are likely to contribute to the structure and function of caveolae, which are considered to be a non-planar subfamily of lipid rafts, because caveolae membranes are as highly enriched in cholesterol and glycosphingolipids as rafts. The shape and structural organization of caveolae are due to the presence of a specific set of proteins (caveolin-1, -2, and -3) that self-assemble in high mass oligomers to form a cytoplasmic coat on the membrane invaginations. Once internalized, some GPCR enter a compartment known as the caveosome and then fuse with early endosomes, which are also used in the clathrin-dependent pathway [43]. The mechanism by which this fusion occurs remains to be elucidated. In addition, it is not known whether these clathrin-independent pathways utilize different compartments in intracellular trafficking or whether lipid raft compartments regulate the events that occur in intracellular compartments. It appears that caveolae/lipid raft-dependent trafficking pathways may play an important role in recycling because recycling compartments are highly enriched in cholesterol and other raft components [44].

So far, the information regarding the caveolae/lipid raft-dependent internalization of chemokine receptors is very scarce. Venkatesan et al. report that CCR5 is internalized to vesicles that partially colocalized with endogenous caveolin [3], but Signoret et al. conclude that CCR5 endocytosis was completely independent of caveolae [5]. The mechanism by which CCR5 internalizes is controversial and needs to be investigated further. This review will refer to studies that suggest a role for lipid rafts/caveolae in CCR5 internalization as well as studies that suggest that CCR5 internalization is completely clathrin-dependent. It has also been demonstrated that CCR4 internalization depends on lipid raft integrity and functionality of clathrin-coated pits [45]. These various results may reflect differences in cell type and experimental approach. The cell type is especially important because caveolin-1 is not expressed in all cell types (reviewed in [46]). A number of studies utilize cholesterol depletion or cholesterol oxidation to assess the role of these pathways. However, this may not be a definitive way to explore the role of these alternative pathways since these cholesterol-depleting agents may have a number of nonspecific effects. For example, disrupting cholesterol in the cells may potentially alter chemokine receptor conformation and affect ligand binding. Currently, there is no other method to assess the role of lipid rafts. It will be necessary to develop more targeted approaches to interfere with lipid rafts before a role can be established. The ability of RNA-interference to knockdown endogenous caveolin-1 is a useful technique to assess caveolin-dependent internalization pathways. Astrocytes exhibiting caveolin-1 knockdown showed diminished ability to down-modulate and internalize CCR2 in response to ligand stimulation [47]. Moreover, there may be cell type differences in utilization of lipid rafts for chemokine receptor internalization. For example, extraction of cholesterol by hydroxypropyl-beta-cyclodextrin inhibits CXCR4 internalization in T cell lines [39], although CXCR4 only internalizes by the clathrin-dependent pathway in HeLa cells [3]. All these data suggest that lipid rafts/caveolae could play a role in the internalization of at least some chemokine receptors. However, lipid raft integrity and cholesterol dependency for ligand

binding and internalization does not automatically reflect the necessity of raft association of chemokine receptors for these processes to occur. Furthermore, lipid rafts do not appear to be involved in the internalization of several other chemokine receptors such as CXCR1 and CXCR2, based upon studies showing that cholesterol depletion does not significantly alter the steady-state cell surface densities, trafficking, and signaling potential of CXCR1 and CXCR2 in neutrophils [42]. However, nonspecific effects of cholesterol depletion cannot be ruled out.

4. Regulation of chemokine receptor trafficking by Rab GTPases

Rabs are small GTPases that cycle between GDP-bound (inactive) and GTP-bound (active) states and regulate a number of cellular trafficking events. The exchange of GDP for GTP, GTP hydrolysis, and GDP displacement are regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP dissociation inhibitors (GDIs), respectively. Rabs are post-translationally modified with geranyl-geranyl groups at their carboxyl-termini [48,49]. This modification allows Rabs to associate with intracellular membrane-bound compartments. Interestingly, individual Rab family members associate with particular endocytic compartments. For example, Rab4 and Rab5 associate with the early endocytic compartment [50–52] and Rab11 associates with the perinuclear recycling compartment [53–55] (Fig. 1).

Rab5 is an important mediator of the early endocytic response. The fusion of early endosomes in vitro requires Rab5 [50] and expression of a dominant negative Rab5 (S34N mutant) results in a decrease of transferrin receptor endocytosis (transferrin co-localization is a marker for early endosomes) [56]. Rab5 binds to type I phosphatidylinositol 3-kinase (PI3K) and promotes production of phosphatidylinositol 3-phosphate (PI(3)P) [57]. Rab5 and PI(3)P then recruit EEA-1 (early endosomal antigen-1) and other proteins that stimulate fusion with early endosomes [58]. Rab5 appears to be important in chemokine receptor endocytosis and trafficking as well. CXCR2 localizes to Rab5-positive endosomes during early time points of ligand stimulation. Moreover, ligand-stimulated CXCR2 internalization requires Rab5 GTP hydrolysis. Expression of a Rab5 dominant negative mutant significantly attenuates CXCR2 internalization [59]. In addition, internalization of CXCR4 and CCR5 is also inhibited by expression of a dominant-negative Rab5 mutant [3].

There are two main endosomal recycling pathways, a slow and a rapid recycling process to which Rab11a and Rab4 can contribute, respectively. Rab11a localizes to the perinuclear recycling compartment and plays a prominent role in the slow recycling process [60]. The recycling pathway involving Rab11a is important in the intracellular trafficking of and the responses mediated by chemokine receptors. Following ligand stimulation, CXCR2 localizes to the Rab11a-positive compartment. The expression of a dominant negative (Rab11a-S25N) mutant results in significantly reduced CXCR2 recycling [59]. Two proteins that interact with Rab11a and play a role in recycling are myosin Vb [61] and Rab11-Family Interacting Protein 2 (FIP2) [62]. Expression of the myosin Vb tail (mutant that lacks the motor domain) and Rab11-FIP2(129–512) truncated mutant inhibits recycling of CXCR2 and impairs its resensitization. In addition, expression of the myosin Vb tail impairs CXCR2- and CXCR4-mediated chemotaxis [59]. These studies demonstrate the potential importance of recycling in chemokine receptor function.

The second pathway bypasses the Rab11a-positive perinuclear endosomes and mediates rapid recycling of receptors through Rab4-positive endosomes [63,64]. This occurs in a PI3K-dependent manner [65]. It remains unclear what mechanisms mediate these different recycling pathways. Recent studies suggest that the low-affinity N-formyl peptide receptor (FPR) utilizes both the rapid and slow recycling pathways. The receptor shows extensive co-localization with

Rab4 and partial co-localization with Rab11, suggesting that the receptor primarily recycles through the rapid pathway, but also utilizes the slower recycling pathway [66].

Rab7 mediates the movement of late endosomes to the lysosome by interacting with microtubule motor proteins [67]. Prolonged exposure of chemokine receptors to ligand results in their lysosomal degradation [1,68,69]. Rab7 appears to be involved in the lysosomal sorting of chemokine receptors. Expression of a dominant negative mutant of Rab7 (Rab7-T22N) results in decreased localization of CXCR2 to the lysosomal compartment (LAMP-1-positive) after prolonged ligand treatment. CXCR2 localization to Rab5- and Rab11a-positive endosomes increased with expression of Rab7-T22N. These data suggest that Rab7 regulates the transfer of CXCR2 to the lysosome and blocking its activity results in accumulation of CXCR2 in early and recycling endosomes [59].

5. Regulation and functional consequences of internalization

The internalization of chemokine receptors occurs after ligand binds to the receptor. Depending on the percentage of receptors being activated, this process may dramatically reduce the level of membrane expression of the receptor and therefore change functionality. This ligand-triggered internalization is most likely the reason for the down regulation of most chemokine receptors, if not all of them. The rate of chemokine receptor internalization may be determined by several factors, such as the carboxyl-terminus of the receptor, the type of ligand, the cell type and the phosphorylation status (Table 2).

5.1. Importance of the carboxyl terminus

Upon ligand binding chemokine receptors become phosphorylated on Ser and Thr residues in the carboxyl-terminal domain and intracellular loops leading to receptor desensitization prior to internalization. Furthermore, the presence of di-leucine motifs in the carboxyl-terminus is necessary for internalization of some chemokine receptors (Table 1). These di-leucine sequences can bind AP-2, an important member of the clathrin-adaptor complex [18]. These signals and other potential unidentified motifs in the carboxyl-termini of chemokine receptors are important for receptor internalization.

Mutation of the LLKIL motif in the carboxyl-terminus of CXCR2 results in decreased binding of CXCR2 to AP-2 α and β subunits without affecting β -arrestin association with the receptor in HEK293 cells. This mutation leads to a markedly decreased CXCR2 internalization and deficient CXCR2-mediated chemotaxis [70]. Further studies on CXCR2 in which the LLKIL motif is mutated, reveal that this motif is not only involved in AP-2 binding but also plays a role in mediating polarization of intracellular signals in transfected HEK293 cells. PH-Akt/protein kinase B (PKB)-GFP, a probe for phosphatidylinositol-3,4,5-trisphosphate (PIP3), localizes to the leading edge of cells expressing wild type CXCR2. This localization is impaired in cells expressing the LLKIL CXCR2 mutant. In addition, Rac1 and cdc42 do not recruit to the leading edge in cells expressing this mutant receptor [71]. Mutational analysis of the carboxyl-terminus of CXCR4 demonstrates that several residues in the tail of the receptor are required for internalization of the receptor, including the di-leucine motif and a number of Ser residues that can be phosphorylated [24,72]. The di-leucine motif in the carboxyl-terminus of CCR5 affects internalization of the receptor in a phosphorylation-independent manner [73]. In contrast, internalization of CXCR3 induced by any of its three ligands CXCL9, CXCL10, or CXCL11 is not affected by mutation of the LLLRL motif located in the carboxyl-terminus. However, mutation of Ser and Thr residues in the carboxyl-terminus results in reduced internalization of CXCR3 by CXCL9 and CXCL10 in 300-19 cells. These studies also show that the third intracellular loop of CXCR3 contributes to CXCL11-mediated internalization [22]. Additional reports indicate that phosphorylation of residues in the carboxyl-terminal

domains of CXCR1 and CXCR2 is also necessary for internalization in rat basophilic leukemia (RBL)-2H3 cells [74].

5.2. Additional regulatory regions and proteins

It is likely that additional motifs that mediate internalization reside in the domains other than the carboxyl-termini of chemokine receptors. This is demonstrated by studying the ligand-induced internalization of chimeric receptors in which the carboxyl-termini of the slowly internalizing CXCR1 and rapidly internalizing CXCR2 are exchanged. The internalization rates of these chimeras did not follow the expectation based on the concept that only carboxyl-terminal domains encode motifs responsible for regulation of slow versus fast endocytosis [74].

A potential role for protein chaperone molecules in the regulation of receptor trafficking was introduced when it was shown that the heat shock cognate 70 (Hsc-70)-interacting protein (Hip) binds CXCR2 in two hybrid screen and immunoprecipitation experiments. Overexpression of a Hip deletion mutant unable to bind the ATPase domain of Hsc-70 results in decreased ligand-dependent internalization of CXCR2 and CXCR4 and inhibits CXCR2-mediated chemotaxis [75]. These data are supported by studies on the transferrin receptor which demonstrate that internalization and recycling of the transferrin receptor are inhibited by overexpression of an ATPase-deficient Hsc-70 [76]. Hsc/heat shock protein (Hsp)-70 may modulate clathrin dynamics and the interaction of Hip with CXCR2 may help in the uncoating of the clathrin-coated vesicle [77]. Alternatively, Hip may be involved in the conformational changes in the receptor that mediate receptor internalization.

The presence of other motifs and whether these motifs and phosphorylations regulate the intracellular trafficking of chemokine receptors is largely unknown. For example, a screen identifying interactions between various post-endocytic sorting molecules and carboxyl-termini of various seven transmembrane receptors revealed an interaction between CXCR2 and the G protein-coupled receptor-associated sorting protein (GASP) [78]. The GASP family of proteins regulate lysosomal sorting and may be involved in directing intracellular trafficking.

5.3. Type of ligands

The rate of receptor internalization is also affected by the types of ligand (Table 2). Most chemokine receptors bind more than one chemokine with high affinity and these various ligands may differentially affect internalization of the receptor. CXCL6 and CXCL8 are two of the chemokines that bind and activate CXCR2. Although CXCL6 and CXCL8 have similar binding affinities for CXCR2, they trigger different rates of receptor internalization. A 60% reduction in membrane CXCR2 requires treatment with 1 µg/ml of CXCL6 for 2 h at 37 °C, while 1 h treatment with CXCL8 under the same conditions causes an 80% reduction in membrane associated CXCR2 using fluorescence activated cell sorting (FACS) analysis [79]. CC chemokine receptor 4 (CCR4) plays an important role in T cell trafficking in immunity and inflammation. Although CCL17 and CCL22 can both activate CCR4, they induce CCR4 internalization with strikingly different efficiencies. Treatment with 100 ng/ml CCL22 for 30 min causes 90% of CCR4 to internalize in human Th2 cells, while the same amount of CCL17 can only induce a small fraction of CCR4 internalization [45]. Apparently, small differences in ligand-receptor interactions result in different internalization efficiencies.

5.4. Cell type specificity

The rate of chemokine receptor internalization may also depend on the specific cell type (Table 2). CXCR2 internalization occurs faster in leukocytes than in HEK293 and 3AsubE cells [42,74,80]. The availability and/or abundance of various endocytic machinery components, such as β-arrestin, other adaptor proteins, and caveolae components may determine the rate of

internalization and in some extreme cases, even the pathways through which they internalize. Some chemokine receptors, like CCR5, are reported by some groups to utilize both the clathrin-dependent endocytic and the caveolae-dependent vesicular pathway. However, in some cell types, one pathway may play a more dominant role over the other in mediating internalization of the receptor. For example, in chinese hamster ovary (CHO) and mink lung endothelial cells, CCR5 internalizes mainly through the clathrin-dependent pathway [5], while in primary T cells or HEK293 cells, CCR5 predominantly internalizes through caveoli [3]. The result is that receptors undergo a much faster receptor internalization in CHO and mink lung endothelial cells than those in primary T cells and HEK293 cells [3,5].

5.5. Phosphorylation-dependency of internalization: importance for chemotaxis and signaling

Inhibition of the phosphorylation of the receptor by mutation of Ser and Thr residues or deletion of the carboxyl-terminus can dramatically reduce or completely inhibit ligand-dependent internalization of some chemokine receptors [19,74,81] (Table 1). Alternatively, inhibition of phosphorylation of these residues by mutation or truncation does not appear to be crucial for other receptors. The studies on the effects of inhibiting receptor carboxyl-terminus phosphorylation on receptor-mediated intracellular signaling and biological function show different results depending on the receptor, cell type, and methodology used. Theoretically, non-phosphorylated receptors may not desensitize and this may generate more sustained and stronger signaling. Based upon this concept, receptors that show deficient internalization due to inhibition of phosphorylation in the carboxyl-terminus may exhibit enhanced signaling. This seems to be true for receptor-mediated calcium mobilization for most chemokine receptors. Reports have shown that sustained calcium mobilization is associated with expression of mutant CXCR2 that weakly internalizes in 3AsubE cells [68], RBL-2H3 cells [81,82] and HEK293 cells [19]. Sustained calcium mobilization was also found for a CCR2B receptor that is deficient in internalization in murine pre-B lymphocyte cells [83], for CXCR4 in RBL-2H3 cells [84], for CCR5 in RBL-2H3 cells [73] and for protease-activated receptor-1 (PAR-1) in murine hematopoietic progenitor cells [85].

The role of chemokine receptor internalization in the mediation of signaling pathways other than mobilization of intracellular free calcium are much more complicated. A phosphorylation-deficient mutant of CXCR1 that does not internalize upon ligand stimulation results in increased coupling to G proteins and increased phosphoinositide hydrolysis. In addition, RBL-2H3 cells expressing this mutant receptor demonstrate enhanced cAMP activation, exocytosis, and phospholipase D activation. This mutant receptor displayed deficient chemotaxis and calcium mobilization. Interestingly, activation of phospholipase C-beta3 was unaffected. These studies suggest that phosphorylation of the receptor is necessary for optimal internalization and chemotaxis but not for activation of a number of other signaling cascades [81]. In some studies, more heterotrimeric G-protein activity was found in cells expressing internalization-compromised CXCR2 [81] or CXCR4 [84]. RBL-2H3 cells expressing a carboxyl-terminal truncated CXCR4 mutant show lower levels of ligand-dependent internalization but exhibit enhanced G protein activation and sustained calcium mobilization. Phospholipase C-beta 3 activation is not affected in cells expressing the truncated mutant of CXCR4 [84]. Others report that in absence of internalization, the receptor coupling to G-proteins and activation of extracellular signal-related kinase (ERK) are not enhanced but the calcium mobilization is increased [83,85]. These data suggest that calcium mobilization and ERK signaling are activated through different pathways. In HEK293 cells, expression of mutant CXCR2 which was compromised in internalization does not alter the level of ERK, Akt and Rho GTPase activity, but fails to induce the polarized redistribution of PIP3, cdc42 and Rac1 in response to gradient delivery of ligand [71,86].

The functional role of receptor internalization in receptor-mediated chemotaxis and signaling is controversial. For example, studies conducted on CXCR1 and CXCR2 in primary human neutrophils demonstrate that ligand concentrations necessary for internalization are 10-fold higher than concentrations that induce chemotaxis [42]. Data from other reports on CXCR2 transfected into HEK293 and RBL cells show that internalization-impaired receptors also mediate weak chemotaxis compared to wild type receptors. Interestingly, expression of chemokine mutant receptors in RBL cells causes sustained calcium mobilization and enhanced G-protein coupling [70,74,81,87]. In the case of CXCR4, the intracellular carboxyl-terminally truncated CXCR4 receptor maintains normal calcium mobilization and ERK activation, but exhibits reduced ligand-triggered receptor internalization and chemotaxis [88]. CCR3 internalization is critical for eosinophil functional responses, such as cell shape change and actin polymerization [89].

Mutation studies on CXCR3 demonstrate that the carboxyl-terminus and the DRY motif in the third transmembrane domain are required for chemotaxis, ERK1/2 phosphorylation, and calcium mobilization in 300-19 cells. Interestingly, the CXCL11-mediated internalization of CXCR3 is not impaired in mutants that contain deletions in these regions, even though the carboxyl-terminus is necessary for CXCL9- and CXCL10-mediated internalization of CXCR3 [22]. Studies on CCR2B demonstrate that the non-phosphorylated mutant of the CCR2B receptor has compromised receptor internalization and impaired chemotaxis but more sustained calcium mobilization and normal ERK activation [83]. In cases where internalization is necessary for chemotaxis and signaling, the question of how much internalization is required for these processes to occur arises. Whether there exists a threshold for the number of receptors necessary to internalize in order to initiate the polarization of intracellular signals required for chemotaxis remains to be investigated. Many of the conflicting observations may be due to differences in cell type, ligand concentration, and internalization detection methods (Table 2). Furthermore, the studies completed thus far suggest that chemokine receptor internalization and chemotaxis may share common signal transduction steps, making it difficult to determine whether chemotaxis is dependent on internalization.

6. Regulation of chemokine receptor recycling

Little is known about chemokine receptor recycling and what factors mediate the fate of the chemokine receptor once it is internalized. It is likely that many factors contribute to differential recycling of chemokine receptors (Table 3). These factors may include the duration and concentration of ligand stimulation as well as sorting motifs located in the intracellular domains of the receptor. It does appear that the length of stimulation with ligand plays a role in the recycling/degradation sorting decision. For example, CCR5 exhibits plasma membrane and recycling endosome localization at early time points of ligand stimulation and localization to the late endosomal compartments at later time points [90]. At early time periods after CXCL8 stimulation of CXCR2, the receptor enters the recycling compartment. Following extended periods of stimulation, the receptor enters the late endosomal and lysosomal compartments [59]. The ability of internalized CXCR2 to recycle is crucial for continued gradient sensing and chemotactic response to ligand. When CXCR2 recycling is inhibited, chemotaxis and signaling are impaired [59,91]. It is not yet known what other sorting molecules bind to chemokine receptors and how they may mediate trafficking. The factors that have been shown to regulate the recycling of chemokine receptors will be discussed in the following sections and are summarized in Table 3. In summary, whether chemokine receptor internalization and recycling are necessary for optimal chemotaxis as a general rule for chemokine receptors remains controversial.

6.1. Chemokine receptor recycling dynamics mediated by different ligands

The factors that contribute to the dynamics of chemokine receptor recycling remain elusive. Evidence from promiscuous receptors that bind multiple ligands with high affinity suggests that various ligands can differentially regulate the recycling dynamics of the receptor. For example, studies on the CCR3 receptor show different internalization and recycling patterns for the two ligands CCL5 and CCL11. CCL11 stimulation of cells expressing CCR3 results in an increase in CCR3 degradation and prolonged desensitization to the ligand [92,93]. In contrast, stimulation with CCL5 results in slow recycling of the receptor [92]. Similarly CCL22 causes a more rapid internalization and recycling of the CCR4 receptor when compared to CCL17 [45].

6.2. Actin-dependency of chemokine receptor recycling

Studies on the chemokine receptors CXCR1 and CXCR2 also show that recycling occurs through actin filaments and is regulated by actin-related kinases. Treatment of cells expressing these receptors with cytochalasin D, an actin depolymerizing agent, results in decreased recycling of CXCR1 and CXCR2. Recycling of a carboxyl-terminally truncated mutant of CXCR2 is not affected by cytochalasin D [94]. Further investigation into actin-dependent recycling of CXCR1 and CXCR2 reveals that actin-related kinases, which are wortmannin-sensitive, regulate the recycling of these receptors. The kinase-dependent recycling of CXCR2 requires the phosphorylation sites in the carboxyl-terminus of CXCR2. This phosphorylation is most likely associated with alterations in actin rearrangements, which are required for the movement of endocytic compartments [95]. These results implicate the importance of the carboxyl-terminus in the regulation of chemokine receptor recycling. Furthermore, expression of the myosin Vb tail, a mutant form of an actin-associated motor protein that is missing the motor domain, inhibits CXCR2 recycling [91]. However, it is difficult to be absolutely certain that other processes in addition to receptor trafficking are not affected by the expression of the myosin Vb tail, which blocks receptor recycling. The recycling of CCR5 is also inhibited by cytochalasin D, suggesting that CCR5 recycling may also be dependent on actin [96].

6.3. Role for ligand dissociation by vesicular acidification?

It is possible that ligand dissociation from the receptor by a decrease in endosomal pH is important for at least some GPCRs for recycling to occur. The recycling of the β 2-adrenergic receptor is inhibited by raising endosomal pH [97]. This does not seem to be the case for at least some chemokine receptors. Studies suggest that ligand dissociation from CCR5 by vesicular acidification is not necessary for recycling [72]. Ligand dissociation may not be the only effect of vesicular acidification. For example, an increase in endosomal pH suppresses the dephosphorylation of the β 2-adrenergic receptor and alters its association with phosphatases [97]. Studies on CXCR2 show that the protein phosphatase 2A (PP2A) associates with the receptor in a phosphorylation-independent manner [19]. This suggests that some conformational change in the receptor resulting from agonist binding or a change in pH, and not the phosphorylation event itself, may be responsible for PP2A association with the carboxyl-terminus of the receptor. This study also examines the effects of PP2A activity on CXCR2-mediated chemotaxis and calcium mobilization. Inhibition of PP2A activity by treatment with okadaic acid inhibits chemotaxis and calcium mobilization in response to ligand [19]. Vesicular acidification has not been extensively studied for chemokine receptor trafficking but may be an important regulatory mechanism and should be investigated further.

6.4. Impact of dephosphorylation on chemokine receptor recycling?

It is not known whether dephosphorylation is necessary for the recycling of all chemokine receptors. It does appear that recycling of some chemokine receptors may not require dephosphorylation of the carboxyl-terminal residues. An amino-terminally modified CCL5

(AOP-CCL5) inhibits the ability of CCR5 to efficiently recycle to the plasma membrane [98]. Studies into the mechanism responsible for this demonstrate that CCR5 stimulated with AOP-CCL5 shows an improved association with GRK and, β -arrestin, and an increased phosphorylation of Ser and Thr residues in the carboxyl-terminus [2,99]. Further investigation reveals that CCR5 stimulated with AOP-CCL5 does recycle but it rapidly re-internalizes once reaching the membrane because it continues to be recognized by the endocytic machinery [90]. Thus it appears that dephosphorylation may not be required for recycling but is required for establishing functionality of recycled receptor on the plasma membrane.

6.5. A role for β -arrestin in chemokine receptor recycling

The rate of recycling of different chemokine receptors varies and the mechanisms responsible for this discrepancy are poorly understood. Results from studies of other GPCRs suggest that β -arrestin is not only important for GPCR desensitization and internalization, but that the affinity of the interaction between the receptor and β -arrestin corresponds to the rate of recycling of the receptor. Strong affinity interactions result in slow recycling or degradation, while low affinity interactions may result in rapid recycling [100–102]. Interestingly, studies on the chemotactic protease-activated receptor-2 (PAR-2) demonstrate that the receptor utilizes a β -arrestin-dependent mechanism to sequester activated ERK1/2 in the pseudopodia [47]. This raises the possibility that intracellular trafficking of chemokine receptors mediated by β -arrestin may have important implications for localization of signals.

One particular study with FPR suggests an essential role for β -arrestin in FPR recycling. These studies are performed in β -arrestin-1/ β -arrestin-2 double knockout cells and reveal that FPR internalizes in these cells, but then remains sequestered in the Rab11 compartment and cannot recycle to the membrane. This effect is rescued by over-expression of either β -arrestin-1 or-2 [103]. In addition, studies using active mutant forms of β -arrestin to study the effects on FPR trafficking demonstrate that the activation state of β -arrestin is critical for efficient receptor recycling. These studies also suggest that dissociation of β -arrestin from the FPR is necessary for recycling to occur [104]. A protective role for β -arrestin in the control of oxidative burst in neutrophils is suggested by studies conducted in β -arrestin-1/ β -arrestin-2 knockout cells. These neutrophils exhibit enhanced ERK1/2 phosphorylation and activated p38 and c-Jun stress kinases. The stimulation of stress kinases is inhibited by diphenylene iodonium, an inhibitor of NADPH oxidase [105].

7. Role of lipid rafts/caveolae in chemokine receptors functions

One of the most important functions for chemokine receptors is receptor-mediated chemotaxis. Lipid rafts/caveolae have been suggested to play a role in chemotaxis. This is based on the observation that oxidation of cholesterol or enriching the plasma membrane with 22-hydroxycholesterol results in inhibition of CCR5-mediated chemotaxis [106–108]. Moreover, inhibition of lipid raft formation prevents CXCL12-dependent migration [109]. Current evidence indicates that lipid rafts serve as platforms that increase the efficiency of interactions between activated receptors and signal transduction partners. It has been shown that raft-associated CCR5 polarizes to the leading edge in chemotaxing cells [110]. Because pertussis toxin suppresses raft redistribution in directionally stimulated cells, it is suggested that the redistribution of raft-associated CCR5 occurs via an active mechanism that depends on the $G_{\alpha i}$ mediated-chemokine receptor signaling [110]. A similar phenomenon is observed for CXCR4-mediated chemotaxis. CXCR4 co-localizes with lipid rafts in the leading edge of CXCL12-stimulated leukocytes, at the sites of contact with the endothelial surface. Inhibition of lipid raft formation by cholesterol depletion prevents CXCL12-dependent migration and polarization of CXCR4 to the leading edge of the cell, indicating that CXCR4 localized surface expression and signaling requires lipid rafts [109]. However, non-specific effects of cholesterol depletion cannot be ruled out. In addition, studies conducted in astrocytes reveal that the

inhibition of CCL2-triggered down-modulation and internalization of CCR2 by knocking down caveolin-1, a key component for caveolae, significantly inhibits chemotaxis and calcium mobilization [47].

Lipid rafts have also been implicated in diverse membrane processes including the assembly of signaling receptor complexes [111]. This aspect in particular has important implications for the polarization of membrane associated signaling complexes. A number of studies examining the polarization of intracellular signals in neutrophils have been conducted. Whether chemokine receptors actually polarize to the leading edge during response to a gradient of ligand remains debatable. However, some studies have suggested that this does occur and one possible mechanism may be through dynamic lipid rafts. For example, upon activation of FPR, this receptor clusters in the plasma membrane in a cholesterol-dependent manner [112]. In addition, studies using glycosyl phosphatidylinositol (GPI)-anchored GFP demonstrate that CXCL12 and fMLP induce continuous redistribution of the GPI-GFP in Jurkat and HL60 cells [110].

8. Modifications of chemokine receptors

Like other GPCRs, chemokine receptors can undergo various modifications (see Table 4). Facing either the intracellular world or the extracellular space, these molecular ornaments can determine the outlook of the mature receptors and hence drastically impact the physiological interactions with their partners and biological functions [113,114]. The modifications located in the amino-terminal part or extra-cellular loops of the chemokine receptors, like Tyr sulfation and glycosylation, could be expected to affect ligand/receptor interactions and specific signaling cascades. The adornment of the intracellular loops or carboxyl-terminal tail with moieties such as ubiquitin or palmitic acid, may have the potential to influence the membrane association, trafficking, endocytosis, turn-over as well as signaling pathways of the receptor. Some of the known post-translational modifications, like ubiquitination and phosphorylation, are mostly triggered upon ligand-binding and the enzymatic removal of the attached molecules generally follows as part of a dynamic process in one of the later steps in the signaling cascade.

8.1. Ubiquitination of chemokine receptors for proteosomal or endosomal sorting?

Ubiquitination is a tightly regulated process in which ubiquitin (Ub), a 76 amino acid polypeptide, is enzymatically attached to Lys residues in substrates [115–117]. It is well known that labeling of proteins with a poly-Ub chain, due to multiple rounds of ubiquitination on a specific Lys residue on the previously added Ub, can target proteins for proteosomal degradation. More recently, studies in yeast revealed that tagging of Lys residue(s) of GPCRs with one single Ub molecule, called mono-ubiquitination, ensures the endocytosis of GPCRs as well as their subsequent delivery to lysosome-like vacuoles for degradation. Although the mechanisms underlying ligand-dependent internalization are more divergent and more complex in mammals, the involvement of mono-ubiquitination in endocytosis and lysosomal sorting of activated GPCRs seems to be partly conserved from lower to higher eukaryotes.

Weak ubiquitination of endogenous CXCR4 was constitutively detected in monocytes (see Table 5) [118] and could contribute to the heterogeneity observed for CXCR4 in various cell types upon immunoblotting [118–120]. Ligand-stimulation of exogenous CXCR4, co-expressed in HEK293 cells with FLAG-tagged mono-Ub and with the internalization-blocking mutant dynamin-K₄₄A, promoted the ubiquitination of CXCR4 at the plasma membrane [69]. The Lys residues (K₃₂₇, K₃₃₁ and K₃₃₃) within the degradation motif (SSLKILSKGK) discovered in the carboxyl-terminus of CXCR4 likely represented the targets for mono-ubiquitination, since mutation of these three Lys residues to Arg attenuated the rapid CXCL12-mediated ubiquitination of CXCR4. Colocalization studies with lysosomal markers, as well as the inhibition of degradation by lysosomotropic agents but not by proteasomal inhibitors,

demonstrated that ligand-stimulation of wild-type CXCR4 led to lysosomal clearance after endocytosis via clathrin-coated pits. Interestingly, the ubiquitination-deficient CXCR4 mutants displayed normal CXCR4 internalization but defective lysosomal degradation [69]. So far, the trigger for CXCR4 ubiquitination remains unknown, but phosphorylation of the Ser residues in the degradation motif or ligand-dependent exposure of hydrophobic regions are potential candidates [115,121]. The enzyme responsible for the agonist-promoted ubiquitination at the plasma membrane was identified as the Nedd4-like E3 Ub ligase atrophin-1-interacting protein (AIP4) and appeared also indispensable for the degradation of exogenous and endogenous CXCR4 [121]. Indeed, AIP4 additionally mediated the CXCR4-promoted ubiquitination of a conserved, endosomal receptor, called hepatocyte growth factor-regulated tyrosine kinase substrate or Hrs. Hrs was previously reported to bind specifically mono-Ub moieties on cargo (trafficking receptor and associated proteins) and to be crucial for sorting ubiquitinated cargo to multivesicular bodies for lysosomal degradation [115–117]. Hrs and AIP4 colocalized with CXCR4 on endosomes upon CXCL12 stimulation of HeLa cells [121]. Moreover, siRNA against either Hrs or AIP4 blocked agonist-promoted degradation of CXCR4 [121]. Furthermore, the sorting of CXCR4 to the degradative pathway also depended on the AAA-type ATPase vacuolar protein sorting-associated protein 4 (Vps4), although it remains unclear how the latter coordinated the de-ubiquitination of both CXCR4 and Hrs.

Although the lysosomal sorting of CXCR4 clearly seemed to involve a cascade of tightly regulated ubiquitination and de-ubiquitination steps of the receptor and other proteins, no information is currently available on the ubiquitination status of other chemokine receptors [115–117]. Ligand-dependent ubiquitination of another well studied GPCR, the β_2 -adrenergic receptor, was only necessary for its post-endocytic sorting but not for its internalization. Remarkably, endocytosis of this receptor required the ligand-dependent ubiquitination of the adaptor protein β -arrestin-2 [122]. Furthermore, proteosomal degradation of some chemokine receptors following basal or ligand-induced poly-Ub labeling cannot be excluded, especially not in view of elimination of mis-folded receptors [115].

Either way, it seems likely that the proteasome pathway is in some way involved in the lysosomal degradation of chemokine receptors in an attempt to dampen their signaling [115, 123]. The basal level of association between the cytosolic tail of CCR5 and the proteasome ζ subunit increased upon ligand-activation of CCR5 transfected L1.2 cells [123]. Furthermore, proteasomal inhibitors blocked receptor internalization and chemotaxis in stimulated CCR5 transfected L1.2 cells and Jurkat cells naturally expressing CXCR4, but did not downmodulate mitogen-activated protein kinase (MAPK) signaling [123]. On the other hand, agents affecting lysosomal proteases slightly promoted the basal accumulation of wild type CCR5 in HEK293 transfectants pointing towards a putative lysosomal degradation pathway for CCR5 [124].

8.2. Palmitoylation: effect on life span, signaling, trafficking or raft association?

Protein acylation is another common post-translational modification within the GPCR world [113,125]. Depending on the nature of the fatty acid added, protein acylation can take the form of myristoylation, prenylation or, most frequently, palmitoylation. Palmitoylation occurs when the 16-carbon palmitate is covalently attached, through thioester linkages, on cysteine residues located at the boundary of the seventh transmembrane domain with the cytoplasmic tail of the GPCR. There is evidence suggesting that such palmitoylation could provide a hydrophobic membrane anchor to the carboxyl-terminal domain of the GPCR and hence create a fourth intracellular loop [113,125]. Furthermore, it became clear for some GPCRs that Cys residues in their intracellular loops also represent potential palmitoylation targets. Generally, palmitoylation has been shown to affect various GPCR functions, such as membrane targeting, signaling properties and endocytosis [113,125]. Most of the palmitate moieties are likely to be attached in a post-ER/early Golgi compartment [125]. However, a dynamic palmitoylation/

depalmitoylation interplay in the cytosol or at the plasma membrane could also apply for at least some of the GPCRs. Depalmitoylation could be especially interesting in view of a potential ligand-dependent removal of the membrane anchor palmitate [125].

Most, but not all, chemokine receptors display cysteines in their carboxyl-terminal tails, at positions compatible with palmitoylation [126]. So far, CCR5 has been the only chemokine receptor for which palmitoylation could be demonstrated, namely by metabolic labeling of CCR5 transfected cells with [^{14}C] palmitate (see Table 5) [73,124,126]. Single or combined Ala-replacements of the three Cys residues clustered after the seventh transmembrane segment (C₃₂₁KC₃₂₃C₃₂₄) of CCR5 or incubation of wild-type CCR5 transfectants with a palmitoylation inhibitor prevented incorporation of [^{14}C] palmitate into the receptor [73,124,126]. Although the lack of palmitoylation profoundly reduced the life span of the CCR5 mutants in HEK293T and CHO-K1 cells, as reflected by reduced diffusion in intracellular compartments, enhanced intracellular sequestration, diminished surface expression, decreased half-life and earlier lysosomal degradation [73,124,126], Kraft et al. observed equal expression of wild-type and palmitoylation-deficient CCR5 on the membrane of RBL-2H3 transfectants [73] (see Table 5). In contrast to a specific proteasome inhibitor, a lysosomal targeting agent prevented the early degradation of the CCR5 mutants and promoted their accumulation in lysosomal compartments in HEK293T cells [124]. In general, the non-palmitoylated CCR5 mutants that succeeded in reaching the cell membrane, retained their ligand-binding properties, anti-HIV co-receptor activity as well as ERK stimulatory, cAMP inhibitory and chemotactic capacity [73,124,126]. However, some signaling-mediated effects, such as granule release, GTP γ S binding and receptor phosphorylation, as well as the duration of a functional response, as measured by a microphysiometer, were down-modulated in the absence of palmitoylation [73,126].

Interestingly, contradictory results were obtained concerning the effect of palmitoylation on ligand-mediated calcium mobilization and endocytosis of CCR5 (see Table 5). Palmitoylation deficiency did not affect calcium release or receptor endocytosis as followed by FACS analysis in CHO-K1 transfectants [126]. However, it decreased the internalization of radiolabeled CCR5 ligand, especially at early time points, and caused a retarded but prolonged calcium mobilization in RBL-2H3 cells [73]. The latter is nevertheless also in apparent contrast with the results obtained for a palmitoylation-deficient CCR5 deletion mutant (CCR5₁₋₃₂₀), lacking several other COOH-terminal residues important for endocytosis and CCR5 signaling besides the Cys residues [3]. This truncated CCR5₁₋₃₂₀ mutant was not associated with lipid rafts and underwent rapid endocytosis through a clathrin-mediated pathway in HEK293 and HeLa transfected cells upon ligand treatment. In contrast, the internalization of stimulated wild-type CCR5 only occurred slowly and the palmitoylation of the intact Cys cluster was suggested to be crucial for anchoring wild-type CCR5 to plasma membrane rafts [3]. The lower endocytosis rate of wild-type CCR5 may keep the gate open for receptor resensitization at the plasma membrane. Indeed, an unknown GPCR phosphatase, active at neutral pH and hence different from the endosome-associated phosphatases, was shown to trigger rapid CCR5 dephosphorylation at the plasma membrane [127]. In contrast to the palmitoylation-deficient CCR5 deletion mutant, a considerable fraction of wild-type CCR5 trafficked by clathrin-independent routes into caveolin-containing vesicular structures in HeLa transfectants in response to CCL4 or CCL5 [3]. However, Signoret et al. concluded that the ligand-induced endocytosis of wild-type CCR5 is completely dependent on clathrin, since they found no evidence for caveolae association of internalized CCR5 after CCL5 stimulation of CCR5 transfected RBL, CHO or mink lung endothelial Mv-1-Lu cells [128]. Previously, it was already demonstrated that 11–18% of total CCR5 was constitutively associated with raft membrane domains in unstimulated MCF-7 cells [37], although CCR5 was only localized in non-raft plasma membrane domains in T cells [129]. The latter suggested that raft association of CCR5 seemed dispensable for HIV infection, but that the presence of cholesterol was

required for HIV entry, CCL4 binding and conformational integrity of CCR5 in T-cells [39, 129].

In conclusion, the degree to which palmitoylation will affect surface expression, raft association, signaling efficiency, endocytosis and trafficking of CCR5 will likely depend on the cell type, the natural or exogenous way of expressing CCR5, the duration and the concentration of the stimulus, as well as the utilized detection tools. It remains to be determined whether other chemokine receptors will be targets for this type of acylation.

8.3. Impact of change in receptor outlook by glycosylation and sulfation?

The presence of many Ser, Thr, Asn and Tyr residues in the extracellular loops and especially in the NH₂-terminal tail of most chemokine receptors, suggests that they are likely subjected to some glycosylation or sulfation processes on their way from the ribosomes to the cell surface [114,130,131]. The O-linked glycosylation occurs on the hydroxyl groups on Ser or Thr residues, whereas N-linked glycosylation targets Asn residues within a Asn-Xxx-Ser/Thr consensus motif, where Xxx can be any amino acid except Pro. Tyr residues are common sites for sulfation, although sulfate groups can also be incorporated in the N- or O-linked sugar chains. Theoretically, attached glycans or sulfate groups may not only provide a larger and potentially more flexible binding surface, but may add extra negative charges to the already acidic NH₂-terminus of the chemokine receptors. This may help achieve specific, high-affinity interactions with basic ligands, while maintaining the flexibility of some chemokine receptors to bind different agonists. Previously, glycosylation has also been proven to contribute to the conformational stability, mobilization to the cell surface and resistance to proteolysis of glycoproteins [130].

The chemokine receptors known to be glycosylated or sulfated are depicted in Tables 6 and 7, respectively. Glycosylation was generally demonstrated by treatment of cells over-expressing the chemokine receptors with enzymes removing specific sugar chains or with an inhibitor of *N*-glycosylation (tunicamycin) and by subsequently following the size differences of the receptors upon immunoblotting (see Table 6). In order to prove receptor sulfation, in most studies cells transfected with chemokine receptors or control vectors were compared for [³⁵S] sulfate incorporation after metabolic labeling, whether or not in the presence of sulfation inhibitors, such as sodium chloride or arylsulfatase (see Table 7). To corroborate these findings and their biological relevance in vitro and to discover the exact targets for the glycosylation or sulfation, mutant receptors were additionally generated, in which single or multiple residues were replaced by Ala or other amino acids and tested in different experimental settings. However, so far, the occurrence of these modifications in naturally expressed chemokine receptors and their potential biological impact in vivo has only superficially been addressed.

Despite the presence of a potential N-linked glycosylation site in the third extracellular loop of CCR5, only O-linked glycosylation of CCR5 was observed in several transfected cell types on Ser residue S₆ and to a lesser extent S₇ (see Table 6) [132,133]. These *O*-glycans did not influence the surface expression or HIV co-receptor activity of CCR5, but significantly enhanced the binding potency of the CCR5 ligands. The O-linked sugar chains were not sulfated themselves, but contained negatively charged terminal sialic acid residues. Upon enzymatic removal of these sialic acids, the CCR5 binding was almost completely abolished [133]. A similar decrease in ligand binding was also obtained for a CCR5 mutant in which S₆ was replaced by a Pro residue [134]. This decrease, however, was not observed by Blanpain et al. in case of an Ala substitution [135].

In addition, at least two of the four NH₂-terminal Tyr residues of CCR5 were discovered to be sulfated (see Table 7) [132]. Inhibition of CCR5 sulfation did not affect the amount of CCR5 displayed on the cell membrane, but lowered its binding to both CCR5 ligands and complexes

of HIV-1-derived gp120 and CD4 [132]. Transfection of CD4 expressing cells with CCR5 mutants in which one or more of these 4 Tyr residues were substituted, diminished HIV-1 entry [132,136]. Other reports underlined the critical role of the Tyr residues, especially Y₁₀ and Y₁₄, and their sulfation in the recognition of CCR5 by its chemokine ligands and gp120/CD4 complexes and in the co-receptor activity of CCR5 [133–135,137–140]. The relevance of this finding was corroborated by the ability of a Tyr-sulfated peptide based on the NH₂-terminus of CCR5 to inhibit infection of macrophages and peripheral blood mononuclear cells by CCR5-dependent (R5) but not CXCR4-oriented (X4) HIV-1 isolates [138].

In case of CXCR4, sulfation of NH₂-terminally located Tyr residues, especially Y₂₁, considerably improved CXCL12 binding and, to some extent, also enhanced CXCR4-mediated HIV-1 entry (see Table 7) [141]. Indeed, the impact of this Tyr sulfation for CXCR4-mediated HIV-1 infection varied among isolates and was less pronounced than for CCR5-dependent HIV-1 entry [141]. The impaired CXCR4 affinity for CXCL12 and reduced CXCR4 co-receptor activity associated with site-directed mutagenesis of Y₂₁ in CXCR4 was nevertheless confirmed by Brelot et al. [142]. Besides sulfation of Tyr residues, CXCR4 was in some cell lines efficiently modified by a chondroitin sulfate chain at S₁₈ within a consensus glycosaminoglycan attachment site (DGSG) [141]. The attachment of chondroitin sulfate was most prominent in wild-type CXCR4 transfected HeLa cells, less abundant in Cf2Th canine thymocytic and HEK293T transfectants, and absent in U937 or Jurkat cell lines. Enzymatic removal of this sulfated glycosaminoglycan did not alter the HIV-1 infection or CXCL12 affinity for CXCR4 [141].

A more complicated picture was obtained regarding the impact of N-linked glycosylation on CXCR4 (see Table 6) [141,143–146]. Out of the 2 potential *N*-glycosylation sites, only N₁₁ was concluded to be efficiently glycosylated, whereas the likely unglycosylated N₁₇₆ residue might support the three-dimensional structure of CXCR4 [144–147]. In the absence of *N*-glycans, the expression levels of CXCR4 remained mostly unaffected [142,146–150], besides some minor increases [146] or decreases [144,149,150] which might be partly accountable to the detection efficiency of conformation-specific antibodies [135,142,151]. Notably, combined replacement of both potential N-linked glycosylation sites (N₁₁Q/N₁₇₆D) of CXCR4 led to the complete disruption of the epitope for the routinely used 12G5 monoclonal anti-CXCR4 antibody [142].

The N-linked glycosylation of CXCR4 seemed to be required for high-affinity binding of CXCL12, which could explain the observed increase in calcium mobilization (see Table 6) [146]. This glycosylation-dependent binding capacity of CXCL12 was confirmed in insect cells expressing a glycosylation-deficient CXCR4 mutant [147]. More interestingly, however, was the rather common theme that the absence of N-linked glycosylation allowed CXCR4 to serve as a more universal co-receptor (see Table 6) [144–146]. Potempa et al. described how the HIV-2 strain ROD/B suddenly managed to enter CD4-negative cells through CXCR4 when *N*-glycosylation was prevented [145]. Furthermore, removal of N-linked glycosylation of CXCR4 allowed efficient entry of otherwise CCR5 restricted R5 HIV-1 isolates, while the regular co-receptor activity for CXCR4-dependent X4 strains remained unaffected [144]. In addition, Wang et al. demonstrated that glycosylation of the N₁₁ residue in CXCR4 not only facilitated CXCL12 binding, but also inhibited X4 as well as R5 HIV-1 gp120 interaction and virus entry [146]. They argued, however, that non-N-linked-glycosylated CXCR4 mediated R5 HIV-1 entry much less efficiently (100–1000-fold) in comparison to X4 HIV-1. Furthermore, other groups could not detect any alteration in entry of X4 [142,149,150], X4R5 [149] or R5 HIV-1 [150] isolates upon analysis of various CXCR4 mutants with replaced N-linked glycosylation sites, although Thordsen et al. also observed a slight increase in X4R5 infection under these conditions [150]. The apparent discrepancy of these findings might be partly explained by the experimental settings (cells, HIV isolates, infection assays, antibodies

used for CXCR4 detection) as well as by the CXCR4 mutants tested (single N₁₁ versus double N₁₁/N₁₇₆ substitutions and the type of residue replacing the N residue, such as Q, I or A) [146]. It remains unclear whether the low levels of R5 HIV-1 and HIV-2 entry mediated by non-*N*-glycosylated CXCR4 may be of potential value in HIV infection in vivo.

CXCR2 is another CXC chemokine receptor marked by N-linked glycosylation, although the real targeted Asn residues remain undetermined so far (see Table 6) [152]. Remarkably, the N-linked glycosylation protected CXCR2 on the surface of neutrophils against proteolytic attack but did not affect receptor endocytosis, recycling or granule release upon CXCL7 treatment of the cells. Furthermore, the *N*-glycosylation of the CC chemokine receptor CCR2B and the sulfation of its Tyr Y₂₆ residue did not have a major impact on the surface expression of CCR2B (see Tables 6 and 7) [153]. However, the Tyr sulfation remarkably promoted CCL2 binding as well as CCL2-induced calcium mobilization and cAMP inhibition, but did not similarly influence the migratory response and lamellipodia formation of the HEK293/CCR2B transfectants. Upon replacement of the Y₂₆ residue by a Phe instead of a Ala moiety, CCL2 lost even all its affinity for CCR2B, which could explain the dramatic drop in CCL2-mediated calcium release and chemotactic activity [153].

CX₃CL1 is a structurally unique chemokine in that it can occur both in a transmembrane and soluble, shed form. Its role in firm cell adhesion was further evidenced by characterizing the consequences of Tyr sulfation of its receptor CX₃CR1 (see Table 7) [154]. The lack of sulfation of Tyr residue Y₁₄ of CX₃CR1 only resulted in an approximately three-fold decrease in static binding of CX₃CL1, which could be responsible for the small decrease in calcium release. However, sulfation of Y₁₄ considerably enhanced the rapid capture of CX₃CR1 transfected cells flowing over immobilized ligand under physiological flow conditions [154]. Metabolic labeling of HeLa cells transfected with chimeric constructs of the NH₂-termini of various chemokine receptors, suggested that CCR3, CCR8 and CXCR3 represented good candidates to be added to the list of Tyr-sulfated chemokine receptors [132].

D6 and DARC also displayed N-linked glycosylation, which did not influence the binding capacity of the receptors for murine CCL3 and CXCL8, respectively (see Table 6) [155,156]. The potential impact of the observed glycosylation-independent sulfation of D6 was not addressed yet (see Table 7) and the constitutive phosphorylation could fit with the role of D6 as a decoy receptor and its inability to signal upon ligand-binding [156].

In conclusion, the discovery of CXCR4 and CCR5 as the major co-receptors for HIV-1 boosted the research on the characterization of these receptors, whereas the information on modifications of most of the other chemokine receptors is still very limited [119]. Furthermore, the current knowledge on the chemokine receptor modifications is mostly based on over-expressed receptors and the validation with naturally expressed receptors is warranted. In many cases, the impact of the various modifications still needs to be addressed.

9. Viral chemokine and decoy receptors

The human cytomegalovirus genome encodes four seven-transmembrane chemokine-like receptors: UL33, UL78, US28, and US27 [157,158]. The function of these proteins is largely unknown. However, because these receptors can bind with high affinity to chemokines, it has been suggested that they may sequester chemokines in order for the virus to evade the immune response. The US28 receptor binds a number of CC-chemokines and the CX₃C chemokine fractalkine [159–162]. It undergoes rapid and ligand-independent internalization and recycling. Upon internalization US28 co-localizes with recycling and late endosomal markers [163]. Although no ligands have been found for the other three viral receptors, evidence suggests they may have a similar function because they also undergo constitutive endocytosis in the absence of ligand [164]. Interestingly, fractalkine (CX₃CL) binding to US28 reduces its plasma

membrane expression and may inhibit US28 recycling [163]. Studies have shown that US28 is constitutively phosphorylated at its carboxy-terminus by GRK and mutagenesis of these Ser and Thr residues leads to increased membrane expression [165]. Interestingly, studies conducted in β -arrestin deficient cells reveal that CCL5-mediated US28 internalization is not impaired. However, internalization of US28 is impaired when cells are treated with siRNA directed against the μ 2-adaptin subunit [166]. These studies suggest that US28 requires the AP-2 clathrin adaptor and not β -arrestin for internalization. The HHV-8 virus encodes a chemokine receptor known as ORF74 that is constitutively active and internalizes in a ligand-independent manner [167]. This receptor binds almost all CXC chemokines with high affinity [168,169]. ELR+ CXC chemokines serve as agonists for this receptor, while ELR- CXC chemokines act as reverse agonists, a property that may be linked to its pro-angiogenic properties in infected cells [168–172]. Studies show that the carboxy-terminus of ORF74 is critical for its constitutive signaling activity [173].

Decoy receptors bind chemokines with high affinity but do not initiate the signaling pathways that are activated by typical chemokine receptors. In addition, these receptors are often expressed on non-leukocytic cell types. Therefore, it is possible that these receptors have a chemokine-sequestering function (reviewed in [174,175]). This group includes the Duffy antigen receptor for chemokines (DARC), D6, and CXC-CKR [176–179]. Like the viral chemokine receptors, D6 undergoes constitutive ligand-independent internalization and recycling. However, when CCL3 binds to the D6 receptor it becomes trapped in endocytic compartments and degraded. In contrast to other chemokine receptors, extended ligand exposure does not decrease membrane expression of D6, indicating a lack of desensitization [4]. Recent studies show that D6 internalizes through clathrin-coated pits and this process is dependent on β -arrestin. In fact, D6 exhibits constitutive association with β -arrestin [180].

10. Conclusions and perspectives

Chemokines and their receptors play an important role in a variety of biological processes, including host defense, inflammation, HIV infection, angiogenesis, and cancer metastasis. The involvement of chemokine receptors in disease makes them ideal candidates for therapeutic intervention. The elucidation of the mechanisms that regulate the cellular responses mediated by chemokines is crucial for the identification of therapeutic targets. An important aspect of chemokine receptor function is intracellular trafficking. The process of internalization has been studied for a number of receptors.

The necessity of internalization for chemotaxis and signaling remains controversial. The discrepancies in the data may be partly attributed to cell type and the experimental procedures utilized. It must be noted that endosomes are gaining considerable attention as scaffolds for signaling complexes. The assembly of signaling complexes on intracellular endosomal membranes suggests that the intracellular trafficking itinerary of chemokine receptors may have important implications for signaling. These signaling complexes most likely play a crucial role in the polarization of intracellular signals. In addition, several post-translational modifications exist for chemokine receptors and the contribution of these modifications to internalization and intracellular trafficking is largely unknown.

The identification of alternative internalization pathways such as lipid rafts and caveolae suggests that the mechanism of internalization may regulate cellular responses to chemokines. There is a substantial amount of conflicting data on the role of lipid rafts in chemokine receptor internalization. One reason may be that many studies regarding lipid rafts have been conducted using cholesterol-depletion agents such as filipin. These reagents may be too broad and may interfere with aspects of other trafficking pathways. In order to appropriately assess the

relevance of alternative endocytic pathways, more specific and targeted experiments will need to be conducted.

The field of chemokine receptor trafficking is not fully developed and as the data present, it will be necessary to remain flexible in our thinking. For example, β -arrestin was originally considered to be important for internalization and desensitization. However, recent studies on other GPCRs and some chemokine receptors support roles for β -arrestin as an intracellular signaling scaffold and as a mediator of recycling. A great deal of work is needed in this field and will prove to be beneficial for a better understanding of chemokine receptor function.

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Fig. 1. Schematic of Endocytosis. CCP: clathrin-coated pit, CCV: clathrin-coated vesicle, EE: early endosome, LE: late endosome, RE: recycling endosome, RRP: rapid recycling pathway, SRP: slow recycling pathway, EEA-1: early endosomal antigen-1, FIP-2: Rab11-family interacting protein-2, LAMP-1: lysosomal-associated membrane protein-1.

Table 1

Factors that regulate chemokine receptor internalization

Regulatory factors	Receptors	Reference
Carboxyl-terminal residues/phosphorylation sites	CXCR1, CXCR2, CXCR3 [CXCL9, CXCL10], CXCR4, CCR5, US28	[16,22,24,68,72–74,81,165]
di-Leucine motifs	CXCR2, CXCR4, CCR5	[24,70,72,73]
Intracellular loops	CXCR3 [CXCL11], CXCR4, CCR5	[16,17,22]
Dynamin	CXCR1, CXCR2, CXCR3, CXCR4, CCR2, CCR5, D6, US28	[1,2,4,21–25]
Chaperone proteins/Hip	CXCR2	[75]

Table 2
The rates of chemokine receptor internalization and recycling in different cell lines

Receptors	Internalization	Recycling	Cell types	Methods	Reference
CXCR1	$t_{1/2}^a = 8$ min (CXCL8)	60% (90 min)	Neutrophils	FACS	[42]
	$t_{1/2} = 8$ min (CXCL8)	80% (180 min)	HEK293	FACS	[42]
	66.3% (30 min, CXCL8)	81.4% (120 min)	HEK293	FACS	[79,181]
	33.7% (120 min, CXCL6)				
CXCR2	60% (30 min, CXCL8)		RBL-2H3	^{125}I -ligand	[74]
	$t_{1/2} = 2$ min (CXCL8)	30–40% (180 min)	Neutrophils	FACS	[42]
	$t_{1/2} = 5$ min (CXCL8)	80% (180 min)	HEK293	FACS	[42]
	80% (60 min, CXCL8)	63.4% (120 min)	HEK293	FACS	[79,181]
	65% (120 min, CXCL6)				
	60% (20 min, CXCL1)		HEK293	^{125}I -ligand	[11]
CXCR2 342T ^b	95% (30 min, CXCL8)		RBL-2H3	^{125}I -ligand	[74]
	33% (60 min, CXCL1)	35% (90 min)	3AsubE	^{125}I -ligand	[68]
	48% (60 min, CXCL8)		3AsubE		
	8% (60 min, CXCL1)		3AsubE	^{125}I -ligand	[68]
	6% (60 min, CXCL8)		3AsubE		
	62% (120 min, CXCL8)		HEK293	FACS	[181]
CXCR2 325T ^b	75% (20 min, CXCL8)		HEK293	^{125}I -ligand	[70]
CXCR2 331T ^b	<15% (60 min, CXCL8)		RBL-2H3	^{125}I -ligand	[74]
CXCR2 IL/AA ^c	20% (20 min, CXCL8)		HEK293	^{125}I -ligand	[70]
CXCR3	80% (30 min, CXCL11)	60% (60 min)	T cells	FACS	[182,183]
	35% (30 min, CXCL10)				
	30% (30 min, CXCL9)				
	80% (30 min, CXCL11)		B cell line (300-19)	FACS	[22]
	60% (30 min, CXCL10)				
	50% (30 min, CXCL9)				
	92% (30 min, CXCL10)		CHO	FACS	[184]
	80% (30 min, CXCL11)		B cell line (300-19)	FACS	[22]
	50% (30 min, CXCL10)				
	0% (30 min, CXCL9)				
ACXCR3 ^b					

Receptors	Internalization	Recycling	Cell types	Methods	Reference
Δ ST CXCR3 ^b	80% (30 min, CXCL11)		B cell line (300-19)	FACS	[22]
	40% (30 min, CXCL10)				
	20% (30 min, CXCL9)				
i3-CXCR1-CXCR3 ^d	70% (30 min, CXCL11)		B cell line (300-19)		[22]
	60% (30 min, CXCL10)				
	50% (30 min, CXCL9)				
CXCR4	52% (30 min)		RBL-2H3	¹²⁵ I-ligand	[84]
	60–90% (120 min, PMA)	80% (90 min)	T Cells	¹²⁵ I-antibody	[72]
	$t_{1/2}$ = 5 min (CXCL12)				
	$t_{1/2}$ = 8 min (CXCL12)	75% (120 min)	Hela-CD4	FACS	[3]
	Or 80% (30 min)				
	90% (10 min, CXCL12)		PBL(T cells)	FACS	[3]
Δ Cyto CXCR4 ^b	38% (30 min, CXCL12)		RBL-2H3	¹²⁵ I-ligand	[84]
	5–10% (60 min, PMA)				
	0% (20 min, CXCL12)		HEK293	FACS	[3]
CCR2B	70% (30 min, CCL2)				
CCR2B-Ala ^c	50% (30 min, CCL2)		B cell line (300-19)	ELISA	[83]
CCR2B-328T	40% (30 min, CCL2)				
CCR2B-328T/Ala	50% (30 min, CCL2)				
CCR2B-316T	20% (30 min, CCL2)		B cell line (300-19)	ELISA	[83]
CCR5	$t_{1/2}$ = 60 min (CCL5)	40% (120 min)	HEK293	FACS	[3]
	80% (30 min, CCL5)	80% (240 min)	RBL-2H3	¹²⁵ I-ligand	[5]
	70% (30 min, CCL5)		CHO	FACS	[73]
	50% (60 min, CCL3)	80% (60 min)	CHO	FACS	[185]
	60% (60 min, CCL3)	85% (120 min)	Hela-CD4	FACS	[185]
	70% (60 min, CCL3)	88% (60 min)	THP-1	FACS	[96]
CCR5 IL/AA, SSSS/AAA ^c	60% (30 min, CCL5)		RBL-2H3	¹²⁵ I-ligand	[73]
FPR	$t_{1/2}$ = 7 min (fMLP)	75% (40 min)	MEF	FACS	[103]
	Or 60–70% (30 min)				
	60% (60 min, fMLP)		HEK293	FACS	[186]
	$t_{1/2}$ = 4 min		U937	FACS	[187]

Receptors	Internalization	Recycling	Cell types	Methods	Reference
FPR Δ ST ^b	10% (30 min, fMLP)		MEF	FACS	[103]
	0% (60 min, fMLP)		HEK293	FACS	[186]
	0% (30 min, fMLP)		U947	FACS	[187]
PAR1	66% (30 min, SFLLRN)		BaF3	ELISA	[85]
PAR1 Y397Z ^b	35% (30 min, SFLLRN)		BaF3	ELISA	[85]

^aThe time required to induce 50% internalization.

^bThe truncated mutant forms of chemokine receptors.

^cThe point mutation of chemokine receptors in either dileucine motif or phosphorylation sites on intracellular carboxyl-terminal domain.

^dReplacement of the third intracellular loop of CXCR3 with that of CXCR1.

Table 3

Factors that mediate intracellular trafficking of chemokine receptors

Regulatory factors	Receptors	Reference
Multiple ligands	CXCR2, CXCR3, CCR3, CCR4	[22,45,92,93,181]
Cytoskeleton		
Actin	CXCR2, CCR5	[94,96]
Motor proteins	CXCR2	[91]
Length of ligand stimulation	CXCR2, CCR5	[59,90]
Rabs		
Rab4	FPR	[66]
Rab5	CXCR2, CXCR4, CCR5	[3,59]
Rab7	CXCR2	[59]
Rab11	CXCR2, FPR	[59,66]
β -Arrestin/recycling	FPR	[103,104]

Table 4

Overview of known modifications of chemokine receptors and the reported effects

Modification	Site	Receptor	Effect ^a
Ubiquitination	Intra Lys	CXCR4	Lysosomal degradation
Palmitoylation	Intra Cys	CCR5	Life span; trafficking; signaling; raft association; endocytosis
<i>N</i> -glycosylation	Extra Asn ^b	CCR2B, CXCR2, CXCR4, D6, DARC	Binding; surface expression; HIV/protease protection
<i>O</i> -glycosylation	Ser/Thr	CCR5	Binding
Sulfation	Extra Tyr	CCR2B, CCR5, CXCR4, CX ₃ CR1, D6	Binding; signaling; HIV co-receptor activity
	Non-Tyr	CXCR4	Not determined

Intra: intracellular location; Extra: extracellular location.

^aEffect reported for at least one of the chemokine receptors, for more details see Tables 5–7.^b*N*-glycosylation on Asn located within a consensus site Asn-Xxx-Ser/Thr with Xxx any residue except Pro.

Table 5 Ubiquitination and palmitoylation of intracellular chemokine receptor domains

Mod ^a	Receptor	Residues	Cell type	Ligand	Effect	Reference
Ub	CXCR4	K ₃₂₇ ^b , K ₃₃₁ ^b , K ₃₃₃ ^b	HEK293 ^c	CXCL12 ^d	+ Lysosomal degradation = Endocytosis	[69] [69]
			Monocytes	None		
Palmit	CCR5	C ₃₂₁ ^b , C ₃₂₃ ^b , C ₃₂₄ ^b	CHO-K1 ^c	None	+ Intracellular diffusion; Surface expression	[118] [126]
				None	– Intracellular sequestration	[126]
				CCL4-5	+ Duration of response; GTPγS binding	[126]
				CCL4-5; HIV-1	= Binding; endocytosis; Ca; cAMP inhibition; HIV _{co}	[126]
			HEK293T ^c	None	+ Surface expression; Half-life	[124]
					– Intracellular sequestration; Early lysosomal degradation	[124]
				CCL4; HIV-1	= Binding; HIV _{co}	[124]
					= Surface expression	[73]
					+ Phosphorylation; Granulosis; Endocytosis	[73]
					– Retarded/prolonged Ca	[73]
					= Binding; P-ERK; Chemotaxis	[73]

Ca: calcium mobilization; HIV_{co}: HIV co-receptor activity; Ub: ubiquitination; Palmit: palmitoylation; Recept: receptor.

^a Presence of modification is demonstrated after co-transfecting target cells with constructs with tagged moieties (e.g. FLAG-Ub) or after metabolic labeling (e.g. [³H] palmitic acid). These modifications can promote (+), inhibit (-) or not affect the specific effect mentioned.

^b Not determined yet which of the three residues specifically is ubiquitinated.

^c Cells transfected with chemokine receptor.

^d Ligand-dependency = occurrence/increase of modification upon ligand treatment.

Table 6
N- linked and O-linked glycosylation of chemokine receptors

Receptor	Residues ^a	Cell type	Ligand	Effect	Reference ^b
CCR2B	N ₁₄ ^c	HEK293 ^d	None	= Surface expression	[153]
CCR5	S ₆ ^e (S ₇ ^e)	HEK293 ^d ; HeLa ^d ; MF ^d ; Cf2Th-CD4 ^d	None	= Surface expression	[132,133]
		Cf2Th-CD4 ^d	CCL3-4	+ Binding	[133]
CXCR2	N ₁₇ ^c ; N ₁₈₆ ^c ; N ₁₉₇ ^c	Neutrophils	HIV-1; SIV	= HIVco	[133]
			None	+ Proteolytic protection	[152]
			CXCL7	= Granulation, endocytosis, recycling	[152]
CXCR4	N ^c	HEK293T ^d ; HuTK ^{-d}			[141,188]
	N ₁₁₁ ; (N ₁₇₆)	CCC ^d ; Mv-1-lu ^d	None	+ Surface expression	[145]
		BSC-1 ^d	HIV-2	3 HIVco	[145]
		U373-MG-CD4 ⁺ ^d	None	= Surface expression	[144]
		Cf2-Th ^d + sCD4	R5 HIV-1	3 HIVco	[144]
			X4 HIV-1	= HIVco	[144]
			None	= Surface expression	[146]
			X4 HIV-1	3 Binding; HIVco	[146]
			R5 HIV-1	3 Binding; HIVco	[146]
			CXCL12	+ Binding; [Ca]	[146]
D6	N ₁₇	L1.2 ^d	Murine CCL3	= Binding	[156]
DARC	N ₁₆	K562 ^d		= Surface expression	[155]
			CXCL8	= Binding	[155]

HIVco: HIV co-receptor activity; MF: primary rhesus macrophages; sCD4: in the presence of soluble CD4; Ca: calcium mobilization. The term inside the paranthesis indicates glycosylation site effectively used, but to lesser extent or with less biological consequences. [Ca] indicates calcium mobilization likely indirect effect, namely due to enhanced ligand binding.

^aResidues are O-linked or N-linked glycosylated on a specific Ser (S) or Asn (N) residue, respectively. Glycosylation can promote (+), inhibit (−) or not affect the specific effect mentioned.

^bReferences include only those in which glycosylation was really demonstrated.

^cStill unclear whether that particular potential glycosylation site was effectively glycosylated.

^dCells were transfected with the corresponding chemokine receptor.

^e Glycans were sialylated.

Sulfated chemokine receptors

Table 7

Receptor	Residues ^a	Cell type	Ligand	Effect	Reference ^b
CCR2B	Y ₂₆	HEK293 ^c	None	= Surface expression	[153]
			CCL2	+ Binding; Ca; cAMP inhibition	[153]
				= Lamellipodium formation ^d ; Chemo ^d	[153]
CCR5	(Y ₃); Y ₁₀ ; Y ₁₄ ; (Y ₁₅)	CF2Th-CD4 ^c ; HeLa ^c	None	= Surface expression	[132]
		CF2Th-CD4 ^c	CCL3, 4, 5	+ Binding	[132,133]
		CF2Th-CD4 ^c ; HeLa-CD4 ^c	gp120/CD4; HIV-1	+ Binding; HIV co	[132,136]
CXCR4	Y ^e	HEK293 ^c			[132]
	(Y ₇); (Y ₁₂); Y ₂₁	HEK293T ^c	CXCL12	+ Binding	[141]
		CF2Th-CD4 ^c	HIV-1	+ HIV entry	[141]
	S ₁₈ -GAG	HeLa ^c ; CF2Th ^c ; HEK293T ^c			[141]
		HeLa ^c	CXCL12	= Binding	[141]
CX ₃ CR1	Y ₁₄	K562 ^c	None	= Surface expression	[154]
			CX ₃ CL1	+ Binding; [Ca]; cell adhesion under dynamic flow	[154]
D6	Y ^e	L1.2 ^c			[156]

Ca: calcium mobilization; HIVco: HIV co-receptor activity. The term inside the paranthesis indicates sulfation site effectively used, but to lesser extent or with less biological consequences. [Ca] indicates calcium mobilization likely indirect effect, namely due to enhanced ligand binding.

^a Sites of sulfation correspond to sulfotyrosines (Y) or to the glycosaminoglycan chondroitin sulfate attached to Ser residue (S18-GAG). Sulfation can promote (+), inhibit (-) or not affect the specific effects mentioned.

^b References include only those in which sulfation was really demonstrated.

^c Cells were transfected with the corresponding chemokine receptor.

^d Upon substitution of Y26 in CCR2B with a Phe instead of an Ala, the CCL2-induced calcium release and chemotactic activity were dramatically diminished as a result of loss of binding capacity.

^e Still unclear whether a particular sulfation site was effectively glycosylated.