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Chemokine Receptor Oligomerization and Allostery

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Abstract

Oligomerization of chemokine receptors has been reported to influence many aspects of receptor function through allosteric communication between receptor protomers. Allosteric interactions within chemokine receptor hetero-oligomers have been shown to cause negative cooperativity in the binding of chemokines and to inhibit receptor activation in the case of some receptor pairs. Other receptor pairs can cause enhanced signaling and even activate entirely new, hetero-oligomer-specific signaling complexes and responses downstream of receptor activation. Many mechanisms contribute to these effects including direct allosteric coupling between the receptors, G protein mediated allostery, G protein stealing, ligand sequestration and recruitment of new intracellular proteins by exposing unique binding interfaces on the oligomerized receptors. These effects present both challenges as well as exciting opportunities for drug discovery. One of the most difficult challenges will involve determining if and when hetero-oligomers versus homo-oligomers are involved in specific disease states.

Keywords

G protein-coupled receptor; chemokine receptor; oligomerization; dimerization; allostery; CCR2; CCR5; CXCR2; CXCR4; CXCR7; DARC

I. Introduction

The chemokine family of G protein-coupled receptors (GPCRs) and their protein ligands control the migration, activation, differentiation and survival of leukocytes in many normal physiological contexts including development, hematopoiesis, immune surveillance and inflammation. However, inappropriate expression, regulation or exploitation of these proteins contributes to a wide spectrum of inflammatory and autoimmune diseases, cancer, heart disease, and HIV, making chemokine receptors prime targets for therapeutic intervention [1–4]. Understanding the molecular details that control chemokine receptor interactions with ligands and their signaling responses should contribute to drug discovery efforts, and add to the list of approved therapeutics, which now include the CCR5 HIV entry inhibitor, Maraviroc, and the CXCR4 targeted stem cell mobilizer, Mozobil.

Approximately 45 chemokines have been identified in humans and are classified into 4 families (CC, CXC, XC and CX3C) on the basis of the pattern of conserved cysteine

residues [4]. The majority of the ligands are secreted in response to inflammatory signals while others are constitutively produced and involved in homeostatic processes such as lymphopoiesis and immune cell patrol of abnormal physiology. There are 22 known human receptors, most of which couple to heterotrimeric G α i protein complexes. Four of the receptors (D6, DARC, CCX-CCR1, and CXCR7) are classified as “atypical receptors” that lack canonical DRY boxes and consequently do not signal through G α i. Instead they have scavenging, decoy, transport, presentation and other accessory functions [5, 6]. CXCR7, has also been reported to be a β -arrestin-biased signaling receptor [7, 8] although a recent report suggests some signaling through G α i in astrocytes and glioma cells [9].

A subset of the receptors (CXCR4, CXCR6, CCR6, CCR8 and CCR9) have only one known ligand while most have multiple ligands (~11 in the case of CCR1, ~12 for CCR3). The atypical receptors (DARC, D6) and virally encoded receptors (e.g. US28) tend to be particularly promiscuous with respect to ligand recognition. Similarly, many of the ligands bind multiple receptors making for a complex network of interactions, just considering the receptors and ligands alone (see [4, 10] for an up-to-date matrix of the chemokine receptors and the ligands that they bind). This promiscuous pairing of ligands and receptors initially gave rise to the notion that there is significant redundancy built into the chemokine system for robustness of the immune response [11, 12]. Furthermore, redundancy has been used as a potential explanation for the failure of drug candidates targeting a given receptor for the treatment of specific diseases [10, 13]. However there are reasons to believe that the system is not as redundant as initially believed [14], and mechanisms for regulation and fine tuning of signaling responses are beginning to emerge.

Initially, different spatial and temporal patterns of expression of chemokines and receptors were hypothesized to impose some level of functional non-redundancy [14]. However there is now extensive evidence for homo- and hetero-oligomerization of chemokine receptors, as well as oligomerization of chemokine receptors with GPCRs outside the chemokine family and with non-GPCR receptors, which can modulate aspects of signaling and cause diverse functional responses, even with the same ligand. In this review we provide examples of the pharmacological effects that these oligomeric interactions have been reported to have on the function of chemokine receptors compared to the receptors in (apparent) isolation. Note that we primarily use the term oligomer rather than dimer to refer to these complexes since it is not known whether they are predominantly dimers or higher order assemblies.

II. Background: Chemokine Structure and Interactions with Receptors

Before delving into oligomerization and allostery, it is useful to review concepts regarding chemokine:receptor structure and interactions that prevailed prior to knowledge that they form homo- and hetero-oligomers. Much is known about chemokine structure and function from a wealth of NMR, X-ray and mutagenesis studies, and recently, the first structure of a chemokine receptor was solved.

II.A. Chemokines have conserved tertiary structures but diverse oligomerization states

Despite their functional diversity, chemokines are small 8–12 kDa proteins with remarkably conserved tertiary structures stabilized by 1–3 disulfide bonds [15]. The basic ~70 residue

chemokine module generally consists of a disordered N-terminus which is a critical signaling domain tethered to a folded α/β core domain (Figure 1). Some chemokines (e.g. SDF-1 γ /CXCL12 γ and SLC/CCL21) also have extended C-terminal domains which are thought to function in binding to glycosaminoglycans (GAGs). The two most unique chemokines (CXCL16 and fractalkine/CX3CL1) are fused to the N-terminus of a large mucin-like stalk that tethers them to the cell membrane and allows them to function as adhesion molecules when membrane bound, and as canonical chemokines after proteolytic release from the transmembrane domain.

In solution, different chemokines adopt a broad range of oligomerization states with some forming stable monomers (e.g. MCP-3/CCL7, SLC/CCL21), while others form reversible dimers (MCP-1/CCL2, IL-8/CXCL8, SDF-1/CXCL12), tetramers (PF-4/CXCL4) and polymers (MIP-1 α /CCL3, MIP-1 β /CCL4, RANTES/CCL5) [2, 16]. There are basically two types of dimer structures -- CC dimers that are characteristic of the CC chemokine family and CXC-type dimers that are formed predominantly by CXC chemokines (Figure 1). These dimers form the basic substructure of the higher order oligomers and polymers [17, 18]. Furthermore, interaction of chemokines with GAGs promotes or stabilizes further oligomerization of many if not all chemokines [19, 20]. Oligomerization and interactions with GAGs are important for locally sequestering chemokines on cell surfaces to prevent diffusion and facilitate the formation of chemokine gradients that help guide cell movement. GAG interactions have also been shown to facilitate transcytosis of chemokines across cells, chemokine mediated signaling, and they can act as cofactors in promoting receptor interactions [21–23]. Nevertheless, as demonstrated using monomeric variants in bare filter transwell migration assays, the reversibility of chemokine oligomerization is necessary because it is the monomeric form that binds to the receptor with highest affinity and promotes cell migration [24, 25].

II.B. Evidence for the two site model of chemokine:receptor binding and activation

Early mutagenesis studies of IL-8/CXCL8 from Clark-Lewis revealed that the chemokine N-terminus is a critical signaling domain, and more specifically, the prominent signaling role of the N-terminal ELR motif in a subset of CXC chemokines [26]. Subsequent mutagenesis studies of many chemokines revealed that if the N-terminus is mutated, deleted, or extended, the signaling properties of a given chemokine can be dramatically altered without significantly affecting receptor binding affinity. For example, deletion of 7 residues from MCP-1/CCL2 converts it from an agonist into a high affinity antagonist [27], as does addition of methionine to RANTES/CCL5 or CCL2 [28, 29], or the introduction of a Pro2Gly or Lys1Arg mutation into CXCL12 [30]. By contrast, deletion of the first eight residues of HCC-1/CCL14 produces a more potent agonist [31], and deletion of 4 and 15 residues from the precursor CTAP-III generates the chemokines β -thromboglobulin and NAP-II/CXCL7, respectively, which have distinct biological activities [32]. Chemical and genetic modification of the N-terminus of RANTES/CCL5 has yielded superagonists and antagonists with more potent abilities than the WT chemokine to internalize the receptor CCR5, making these modified chemokines more effective in inhibiting HIV entry into cells [33, 34]. Thus, the N-termini of chemokines are thought to interact with receptor binding pockets formed primarily by the receptor transmembrane (TM) domains (referred to as

chemokine recognition site 2, CRS2 in [4]), perhaps mimicking the binding and activation of other GPCRs by small molecules ligands. In other words this small domain seems to have the largest influence on the conformational state of the receptor and thus the signaling response.

By contrast mutations of the chemokine core domain (everything beyond the first cysteine) generally modulate binding affinity and signaling to a proportional extent but without producing dramatic switches in pharmacology such as the conversion of agonists into antagonists. Numerous studies including the structure of a sulfated N-terminal peptide from CXCR4 in complex with SDF-1/CXCL12 [35] have demonstrated that the core domain interacts with the N-terminus of the receptor (referred to as chemokine recognition site 1, CRS1 in [4]), which is largely unstructured in the absence of ligand engagement. Together, these data along with evidence that the monomeric forms of chemokines promote cell migration, has give rise to the concept of a two-site model of receptor activation [36]. In this model, the CRS1 binds to the chemokine core domain in an initial docking interaction. This interaction then orients the chemokine N-terminal signaling domain into the receptor CRS2, which triggers the requisite conformational change (Figure 2).

The two site model has been supported by recent NMR studies of an *in vitro* reconstituted CXCL12: CXCR4 complex [37]. In this study, NMR signals from isotopically labeled CXCL12 were broadened beyond detection when in complex with CXCR4; however when the small molecule antagonist AMD3100 was added, signals from the chemokine N-terminus but not the core domain became visible, presumably because the N-terminus became mobile after being displaced from the receptor by AMD3100, while the core remained bound to CRS1 (Figure 3A). The fact that AMD3100 binds in the TM region of the receptor is consistent with the N-terminus of CXCL12 also binding in this region, although allosteric mechanisms of displacement cannot be ruled out.

In the interpretation of the above studies it was assumed that the receptor would be monomeric and thus that the functionally relevant form of the complex is 1:1 chemokine:receptor. This hypothesis may well be valid but the accumulating evidence that chemokine receptors homo- and hetero-oligomerize raises the possibility of alternative stoichiometries and modes of binding of monomeric chemokines to oligomerized receptors.

III. Evidence for Hetero- and Homo-Oligomerization of Chemokine Receptors

As implied above, for quite sometime, GPCRs were assumed to function as monomeric units. Moreover, by reconstituting the β 2-adrenergic receptor, the μ -opioid receptor, and rhodopsin into nanodiscs, it was demonstrated that they can function as monomers with respect to G protein coupling [38–40], and in the case of rhodopsin, the monomer is also sufficient for rhodopsin kinase phosphorylation and arrestin binding [41]. However, a large body of evidence suggests that many GPCRs form dimers and higher order homo- and hetero-oligomers, and chemokine receptors are no exception (Table 1). These oligomers may be required for assembling large functional signaling complexes and for allosteric communication within the complexes. For example, natively expressed CXCR4, CCR5 and

CD4 have been identified in homogeneous microclusters, predominantly on microvilli, in many cells types [42]. Furthermore these microclusters were identified in small trans golgi vesicles, suggesting their assembly shortly after synthesis and prior to transport to the cell membrane. The authors proposed that this localization and clustering might facilitate more precise sensing of the microenvironment during cell migration and noted that selectins and integrins, which are also important for cell migration, are located on microvilli. Whether these microclusters contain stable contact-mediated oligomerized receptors was not determined, but it seems likely given the number of studies that have demonstrated homo- and hetero-oligomerization of CXCR4 and CCR5 (Table 1).

While the validation of oligomerization in native tissues has yet to be convincingly demonstrated for most chemokine receptors, and the functional relevance of oligomerization on chemokine receptor activity/signaling and dynamics/trafficking is far from well understood, the majority of the chemokine receptors have been reported to homo and/or hetero-oligomerize (Table 1). Questions that have been probed in these studies include: (1) What is the affect of ligand binding, the nature of the ligand (agonist vs antagonist) and the activation state of the receptor on oligomerization? (2) What is the functional significance of homo- and hetero-oligomerization? (3) Is oligomerization required for transport to the membrane surface? (4) What factors regulate receptor oligomerization? (5) Is there allosteric communication between receptors and what are the mechanisms? (6) Are G proteins involved? Answering these questions has motivated the development of many methods for investigating receptor oligomerization in living cells as described in the next section.

III.A. Methods used for studying GPCR oligomerization

Many biochemical and biophysical methods have been used to investigate receptor oligomerization, and are summarized in Table 2, along with their pros and cons. These methods include chemical crosslinking followed by co-immunoprecipitation (Co-IP), protein fragment complementation (PFC, PCA), and many variants of fluorescence energy transfer (FRET) and bioluminescence energy transfer (BRET) techniques, including time resolved FRET (tr-FRET, HTRF), bimolecular fluorescence/luminescence complementation BRET (BiFC/BiLC-BRET), and FRET after photobleaching (pbFRET). However one must be cautious in the interpretation of the data and aware of the artifacts that can arise as a consequence of all of these methods. The biggest criticism is that most methods require heterologous expression of modified receptors, e.g. with fluorescent or other tags for detection, or for resonance energy transfer (RET) based experiments, which can lead to apparent oligomerization due to the unnatural high density of the expressed receptors. The tags may also inhibit interactions with intracellular proteins and alter receptor trafficking. Aggregation of receptors during Co-IP experiments can result in apparent but artificial oligomerization, and chemical crosslinking and protein fragment complementation can stabilize otherwise transient interactions between receptors. These issues have been extensively reviewed [1, 3, 43], and it is now broadly appreciated that methods which identify oligomerization of receptors in native tissues are critically needed. To this end, RET assays based on fluorescent labeling of GPCR ligands rather than the receptors have been developed. However, these ligand-based methods also have limitations because the ligand can modulate the basal state of the receptor. Ligands may alter the oligomerization state,

agonists will often cause receptor internalization or modulate receptor trafficking, and allostery between oligomerized receptors can result in transinhibition or cooperative binding of ligands (see section IV), leading to a lack of correlation between results from RET experiments and receptor oligomerization. In the case of chemokine receptors, the use of labeled chemokine ligands is also complicated by their propensity to bind to and oligomerize on cell surface GAGs, although this issue is not relevant to synthetic small molecule ligands.

All of the methods are also fraught with difficulties in quantitative interpretation. For example, although the half maximal BRET signal (BRET₅₀) in saturation experiments has been interpreted as a measure of receptor stability, in reality it is not possible to compare different receptors. This is due to the fact that many factors influence the results, including receptor expression levels and cellular localization, which are difficult to control and properly quantify between experiments. For example, the stabilizing or destabilizing effect of mutations on the oligomerization of a given receptor may be difficult to address if they alter stability to an extent that is outside the detection range of BRET (whatever that is), and if receptor expression levels differ but the amount of transfected DNA (the standard protocol) is used as a measure of receptor density [44]. Most methods generally reflect steady state average views of receptor oligomerization over the whole cell although some studies have focused on the cell membrane and subcellular organelles [42, 45, 46]. Dynamic, reversible association of receptors is also not captured by the most commonly used approaches, although exciting efforts in this direction have been reported [47]. Nevertheless, these approaches have at least provided leads that oligomerization may have functional relevance for specific receptors, allowing for follow-up studies; and despite the caveats and numerous conflicting reports, much has been learned or at least brought on radar about this fundamentally important feature of GPCRs.

III.B. Chemokine receptor homo- and hetero-oligomerization: Evidence for constitutive ligand independent oligomer formation early after biosynthesis

Table 1 summarizes at least the majority of the reports related to chemokine receptor homo- and hetero-oligomerization along with some of the key observations in these publications. CCR2b was the first chemokine receptor that was shown to oligomerize [48]. In these studies, chemical crosslinking coupled with Co-IP and western blotting was initially used to demonstrate oligomerization that was induced by binding of its ligand MCP-1/CCL2. Similarly, SDF-1/CXCL12 and RANTES/CCL5 were shown to induce oligomerization of CXCR4 and CCR5, respectively [49, 50]. Subsequently, many reports using RET-based methods showed constitutive association of many receptors without the requirement of ligand binding, and the current consensus is that receptors probably form in the absence of ligand binding [4, 46, 51]. Whether the ligand has a significant effect on stabilizing receptor oligomers remains to be seen, as the commonly used RET approaches may not be sufficiently sensitive or quantitative to detect relevant changes. However, BRET studies of CCR2 and CXCR4 homo- and hetero-oligomers suggest that chemokine ligands and small molecule inhibitors affect the conformation but not the basal number of associated receptors based on observed ligand-induced changes in the BRET_{max} but not BRET₅₀ [51]. FRET studies of CCR5/CXCR4 also suggest that the heteromers are preformed in the absence of ligand; however in these studies, stabilization of the hetero-oligomer by CCR5 ligands

MIP-1 α /CCL3 and RANTES/CCL5, but destabilization by SDF-1/CXCL12 was reported [52]. These data are consistent with the early chemical crosslinking/Co-IP results which suggested that chemokines can stabilize receptor dimers [48–50, 53, 54]. However, it is difficult to judge whether such changes observed in single expression point FRET studies result from an actual change in the dimerization equilibrium or from conformational changes within constitutive dimers, illustrating a potential advantage of using BRET saturation titration curves.

The presence of basal, ligand-independent formation of chemokine receptor oligomers is consistent with the idea that oligomers form early along the biosynthetic pathway and can be detected during transport through the ER and golgi [55, 56]. The first and most convincing example of this concept was demonstrated with the class C gamma-aminobutyric acid (GABA)_B receptors which form obligate heterodimers: GABA_B-R1 requires dimerization with GABA_B-R2 in the ER in order to traffick to the cell surface, and although GABA_B-R2 can be transported to the cell surface in the absence of GABA_B-R1, it is not functional unless oligomerized with GABA_B-R1 [57, 58]. Along these lines, constitutive homo- and hetero-oligomerization of CXCR1 and CXCR2 was demonstrated by a combination of BRET and Co-IP, and a novel trapping strategy showed interactions between CXCR1 homomers and CXCR1:CXCR2 heteromers in the endoplasmic reticulum (ER). In these experiments, an ER retention signal was added to the C-terminus of CXCR1 and resulted in a significant reduction in the amount of CXCR1 and CXCR2 that was translocated to the cell surface [55]. Similarly, CCR5 has been shown to oligomerize in a ligand-independent fashion, both at the plasma membrane and in ER subfractions [46]. More recent studies using both BRET and FRET show constitutive association of virtually all chemokine receptors studied [1, 3, 4, 43, 45, 51, 56, 59–61], and the assumption is, that if these hetero-oligomers are relevant in native cells, they probably form prior to reaching the cell membrane.

III.C. Crystal structures of CXCR4 reveal homodimers

In keeping with earlier biochemical studies which showed that CXCR4 sedimented as a dimer when purified in nondenaturing detergent [62], recent crystal structures of CXCR4 have provided structural validation to the relevance of contact-mediated receptor dimerization rather than simple clustering within the range detectable by RET. In 2010, five structures were reported and in all cases showed a dimer with subunit interactions primarily between transmembrane (TM) helices V and VI [63]. While one cannot exclude that the dimer was due to crystal contacts, the fact that all five structures showed the same dimer interface despite being in different crystal forms, suggests that they are probably not artifacts of crystallization. This data supports numerous cell-based studies which suggest that CXCR4 forms homo- and hetero-dimers (Table 1).

The structures of CXCR4 were in complex with a small molecule antagonist, It1t, and a 16-residue cyclic peptide inhibitor, CVX15. They revealed a rather large acidic binding pocket and showed that It1t bound in the minor pocket involving TM helices I, II, III and VII while CVX15 bound in the major pocket (TM helices III–VII). These compounds interact with several acidic residues that line the pocket and are known to be involved in binding to

SDF-1/CXCL12. Consistent with the two-site model, the current thinking is that the N-terminus of CXCL12 interacts with the pocket CRS2, formed by the transmembrane helices and ECL2, a β -hairpin structure that helps shape the entry to the pocket, and has been highly implicated in chemokine interactions. No density was observed for the receptor N-terminus up to the first cysteine consistent with the hypothesis that in the absence of chemokine, the CRS1 domain is unstructured.

The observed dimers raise questions about the stoichiometry of chemokine:receptor binding in cells. Originally 1:1 chemokine-monomer:receptor-monomer complexes were assumed, but given the CXCR4 dimer structures, it is possible to envision a 1:2 chemokine-monomer:receptor-dimer complex that still conforms to the two site model (Figure 3B). This issue has yet to be resolved, but it is noteworthy that some biochemical studies suggest that only one chemokine can bind to a receptor dimer at a time (see section IV and [60, 64–66]). Furthermore, as described in section VII, CXCL12 dimers can interact with CXCR4 to produce different downstream signals than those stimulated by CXCL12 monomers [67], suggesting 2:2 chemokine-dimer:receptor-dimer complexes may also be functionally relevant.

Other questions related to the dimer structures include the following: (1) What is the variability and plasticity in chemokine receptor dimer interfaces? Assuming the CXCR4 structures show a relevant dimer interface, is the TM V/VI interface always the interaction surface in CXCR4 dimers? Or are other interfaces used in higher order oligomers or with other heteromeric interactions? (2) Is it possible to engineer non-oligomerizing receptors to answer the functional relevance of chemokine receptor oligomerization? (3) Is there allosteric coupling across the interfaces, what is the functional consequence, and what is the mechanism? (4) Do dimer interfaces make good therapeutic targets?

Answers to these questions are only beginning to emerge. With regard to questions 1, it is worth noting that one of the CXCR4 structures showed an additional interface involving TMS I and II [44, 63]. Furthermore, structures of other GPCRs suggest a great deal of variability in dimer interfaces [68]. Regarding question 2, in order to engineer non-oligomerizing receptors, structural knowledge of the interface is obviously useful but even then, identifying appropriate mutations that destabilize oligomeric receptors without affecting receptor stability, folding and ligand binding may be difficult. Quantifying the effect of mutations is also non-trivial as described above and in [44]. Furthermore, the extent to which the stability of oligomerized receptors is affected by intracellular G or other proteins as well as the lipid environment, is unclear. If the receptors exit in assemblies larger than dimers, as has been suggested [65, 69, 70], then more than one interface may need to be simultaneously disrupted to achieve a monomeric status.

III.D. Attempts to disrupt receptor dimerization

In an early attempt to engineer a non-oligomerizing variant of CCR5, a TM I/TM IV dimer interface was predicted in a bioinformatic analysis [45]. Subsequent mutation of I52V and V150A in TMS I and IV, respectively, were reported to prevent dimerization according to both FRET and crosslinking studies, and TM peptides encompassing Ile52 and Val150 were shown to block dimerization. However this data was subsequently contested based on BRET

analysis by a different group and remains unresolved [61]. Nevertheless, regardless of the affect on dimerization, in contrast to the WT protein, the mutant CCR5 was unable to signal in calcium flux, chemotaxis and JAK-STAT activation assays despite retention of binding affinity for RANTES/CCL5 [45]. Similarly the peptide blocked WT CCR5 receptor function. It therefore remains an open question as to whether the inhibitory affects of the TM peptide and the CCR5 mutant are through the controversial dimer disruption mechanism or through allosteric induction of non-signaling receptor conformations.

More recent BRET studies as well as the report by Lemay tend to argue for allosteric induction of non-signaling receptor conformations. In a 1999 report from Tarasova and coworkers, multiple TM peptides from CXCR4 and CCR5 were shown to block signaling of the receptors and to inhibit HIV replication [71]. Followup studies using BRET suggested that the effects were due to inhibition of ligand-induced conformational changes rather than disruption of receptor dimers, since none of the peptides affected the basal BRET signal but did produce changes in the ligand-induced BRET signals [51]. The authors reasoned that the inhibitory effect of the TM peptide could best be explained by blockade of the allosteric communication between dimerized receptors. Similarly, in 2006, Wang and coworkers reported that a peptide corresponding to TM IV of CXCR4 blocked the migration of monocytes and cancer cells to CXCL12 [72]. In this case, FRET between CXCR4-CFP and CXCR4-YFP was reduced by the peptide, but again changes in FRET efficiency at a single expression level of FRET pairs are difficult to assign to reductions in actual numbers of dimers/oligomers versus conformational changes within stable complexes. Whatever the mechanism, the data overall suggest that targeting TM helices (dimer interfaces or otherwise) can be an effective strategy for chemokine receptor inhibition; the question is whether they can be specifically targeted with small molecules.

IV. Functional Effects of Chemokine Receptor Hetero-Oligomerization on Ligand Binding

In a series of detailed studies, transinhibition of ligand binding between hetero-oligomerized receptors (CCR2/CCR5, CCR2/CXCR4 and CCR2/CCR5/CXCR4) was demonstrated by BRET and ligand binding experiments. These studies made a compelling case for the importance of allostery in controlling the function of chemokine receptors, the potential impact that heterodimerization can have on drug efficacy, and provided insight into the mechanism for allosteric communication between receptor subunits. The first of these reports on the heterodimerization of CCR2 and CCR5 showed that these receptors were basally associated in the absence of chemokine agonists [60]. Furthermore, BRET₅₀ values, which are considered a measure of affinity (with the caveats described in section III.A), were similar for the CCR2 and CCR5 homomers and for the CCR2/CCR5 heteromers suggesting similar propensities for the homo- and hetero-oligomers to form. This is not surprising given the high sequence conservation between CCR2 and CCR5, especially in their transmembrane domains (78.2% identity, 89.4 similarity). Binding of the respective chemokine ligands had no affect on the BRET₅₀ but affected the BRET_{max} signal, suggesting that the ligands induce conformational changes rather than changes in the number of associated receptors, similar to related studies of CCR2/CXCR4 [51]. Binding

of ^{125}I -MCP-1/CCL2 tracer to CCR2 was unaffected by unlabeled CCR5 ligands (MIP-1 α /CCL3, MIP-1 β /CCL4 and RANTES/CCL5) when CCR2 was expressed alone. Similarly, no competitive binding between ^{125}I -MIP-1 β /CCL4 tracer and CCR2 ligands (MCP-1/CCL2 and MCP-2/CCL8) to CCR5 was observed when CCR5 was expressed alone. The surprising finding was that coexpression of the two receptors made the reporter ligands of one receptor susceptible to binding inhibition by the ligands of the other receptor: CCR5 ligands inhibited binding of ^{125}I -MCP-1/CCL2 to cells coexpressing both CCR2 and CCR5, and CCR2 ligands inhibited the binding of ^{125}I -MIP-1 β /CCL4 to the coexpressing cells. Furthermore, the extent of the transinhibition corresponded with the approximate proportion of expressed heterodimers, suggesting some sort of negative binding allostery between the coexpressed receptors. Similar results were observed in both transfected cells as well as T lymphoblasts that naturally express both receptors. These data suggested that there might be allosteric communication between coexpressed and apparently oligomerized CCR2 and CCR5.

To further investigate the mechanism and demonstrate the allosteric nature of the binding inhibition caused by CCR2/CCR5 hetero-oligomerization, a subsequent study used “infinite dilution tracer” experiments [66]. These experiments showed that dissociation of the CCR2 specific ligand ^{125}I -MCP-1/CCL2 to cells coexpressing CCR2 and CCR5 was accelerated significantly by the CCR5 specific ligand MIP-1 β /CCL4 compared to the dissociation rate from cells expressing CCR2 alone. Likewise, dissociation of ^{125}I -MIP-1 β /CCL4 from CCR5 was accelerated by CCL2 in cells coexpressing both receptors compared to CCR5 alone. Again, the results were demonstrated in both transfected cells and in T lymphoblasts suggesting the physiological relevance of the observations. Transinhibition of ligand binding was also demonstrated for CCR2/CXCR4 hetero-oligomers in transfected and primary leukocytes [64] and for CCR2/CCR5/CXCR4 multimers in transfected cells as well as primary T cells and monocytes [65].

It has been argued that “G protein stealing” can be the source of transinhibition of ligand binding without the need to invoke heterodimerization [43, 73, 74]. The idea here is that many agonists require G protein coupling for high affinity receptor binding. Thus, independent of receptor hetero-oligomerization, depletion of G protein due to ligation of one receptor with its agonist can result in the apparent lowering of the affinity of the other receptor for its ligand, especially if the pool of G proteins is limiting. However, the partial agonist [10–68]RANTES/CCL5, and the antagonist MET-RANTES/CCL5 were also effective in promoting dissociation of MCP-1/CCL2 from CCR2 in CCR2/CCR5 coexpressing cells. Similarly, the small molecule CXCR4 antagonist AMD3100 and the CCR2 inverse agonist TAK-779 were able to compete off the binding of chemokine from CCR2 and CXCR4, respectively, but only when the two receptors were coexpressed [64]. These data are not consistent with a G protein steal since high affinity binding of antagonists and inverse agonists typically does not require G protein coupling. Along with the results from the infinite dilution tracer experiments, the data suggest that there is direct allosteric communication between hetero-oligomerized receptors (Figure 4).

That is not to say that G proteins don't play a role in the observed transinhibition. Springael and coworkers showed that the addition of pertussis toxin (PTx) or Gpp(NH)p, a nonhydrolyzable analog of GTP, strongly reduced the binding of MIP-1 β /CCL4 to CCR5

[66]. Similarly binding of MIP-1 β /CCL4 was reduced on cells expressing an R126N mutant of CCR5 that does not couple to G proteins, indicating the requirement of G proteins for high affinity binding of CCL4 to CCR5. Although the CCR5-R126N mutant retained the ability to hetero-oligomerize as efficiently with CCR2 as WT CCR5, and CCR5-R126N did not show a dominant negative affect on signaling of coexpressed CCR5 and CCR2 as assessed by calcium flux, MIP-1 β /CCL4 was no longer able to increase the dissociation rate of MCP-1/CCL2 from CCR2 in cells coexpressing both CCR2 and CCR5-R126N. Together the data suggest that the G protein is needed for allosteric communication through the dimer, at least when agonists are involved, and the authors proposed a mechanism for G protein dependent binding transinhibition (Figure 4). However, since antagonists also promote transinhibition, the requirement for G protein is likely to be first and foremost necessary for high affinity binding of agonist, regardless of whether the G protein directly contributes to allostery across the heterodimer.

Other conclusions reached from these studies include the fact that (1) ligand binding does not need to induce the activated state of receptor in order to inhibit ligand binding to the other receptor in the hetero complex [64–66]; (2) that homo and heterodimers likely interact, suggesting larger allosterically coupled arrays of chemokine receptors [65]; (3) that binding transinhibition is not likely due to steric blockade by the competing ligand since small molecules are as effective as chemokines in causing ligand dissociation from the partner receptor; (4) that a receptor heterodimer, and most probably a homodimer, can only bind a single chemokine with high affinity [64–66]. The latter hypothesis is interesting in light of the CXCR4 dimer structure, but further studies are obviously needed to determine chemokine:receptor stoichiometries. Likewise, the stoichiometry between chemokine receptors and downstream signaling partners including G proteins and β -arrestins, may provide key insights that reconcile some of the data.

What is particularly surprising about the results is the fact that ligands with different efficacies and sizes (inverse agonists, antagonists and agonist variants of small both molecules and chemokines) were all capable of ligand binding transinhibition. Whether the effect is common, or specific to the receptors and ligands reported in these studies remains to be seen. However it is worth noting that different ligands have been shown to produce different conformational changes in receptor homo and heterodimers [51, 52]; this suggests that many receptor conformations may produce cross-competition whether it be due to distortions of the binding pocket or perturbations of G protein coupling that affect the ligand affinity of the opposing oligomeric partner. It will be interesting to see if similar findings are observed with other chemokine receptors that have been shown to heterodimerize (Table 1) and in what contexts.

Transinhibition of ligand binding makes sense when there is inhibition of signaling of one receptor by the ligand of the partner receptor as described in the next section. However, there are also examples where the signaling is amplified or completely altered, and how this correlates with ligand binding is an open question. As discussed in section V, DARC heterodimerizes with CCR5 and blocks its downstream signaling without affecting ligand binding and CCR5 internalization. This combination of effects has functional implications consistent with other behaviors of this atypical chemokine receptor. Can chemokine receptor

heteromers bind different chemokines than chemokine homomers? Given the known ligand:receptor promiscuity, and the fact that proteolytic processing of chemokines can cause receptor specificity changes, this scenario would not be difficult to imagine.

V. Effects of Chemokine Hetero- and Homo-Oligomerization on Signaling

V.A. Transinhibition of signaling by ligands in hetero-oligomeric complexes

Early reports suggested synergy in calcium flux due to CCR2/CCR5 heterodimers [75]; however subsequent studies failed to show cooperative calcium signaling with CCR2/CCR5 and with CCR2/CXCR4 and thus this finding remains controversial [64, 65]. Cooperative signaling in cell migration was also not observed with agonists of the coexpressed receptors in transfected as well as native cells. Instead, in both functional assays, small molecule antagonists of one receptor caused inhibition of functional responses of the other receptor to which it does not bind [64, 65]. Thus the CXCR4 antagonist AMD3100 inhibited not only signaling of CXCR4 in response to its ligand SDF-1/CXCL12, but it also blocked signaling of MCP-1/CCL2 to CCR2. Similarly, the CCR2 inverse agonist TAK-779 blocked signaling of SDF-1/CXCL12 in primary CD4⁺ lymphoblasts coexpressing both CCR2 and CXCR4 [64]. These data are in line with the ligand binding transinhibition; however, particularly interesting was the fact that the inhibition of functional responses was stronger than the binding cross competition. The authors proposed that this might reflect allosteric functional effects across larger arrays of receptors than heterodimers [64]. Along these lines, subsequent studies showed cooperative interactions and hetero-oligomerization between CCR2, CCR5 and CXCR4 in T lymphoblasts, and similar inhibition of calcium signaling and migration of receptors by antagonists of the orthogonal receptors. This cross-competition also translated into an *in vivo* air pouch migration model in which the small molecule TAK-779 (which antagonizes both CCR5 and CCR2), blocked migration of cells to CXCL12/CXCR4 [65]. The implications for drug discovery here are quite striking; in principal, it may be possible to inhibit the activity of one chemokine receptor indirectly by targeting another receptor with which it oligomerizes.

V.B. Activation of alternative signaling pathways by hetero-oligomers

In addition to inhibiting signaling, Mellado and coworkers subsequently demonstrated that heterodimerization of CCR2 and CCR5 can produce unique signaling responses compared to the classical G_{αi} signals characteristic of chemokine receptors expressed in isolation [75]. Coexpression of both receptors and stimulation with their respective ligands (RANTES/CCL5 and MCP-1/CCL2) resulted in a PTx-insensitive calcium flux through Gq/11, in contrast to the normal inhibitory effect that PTx has on G_{αi}-mediated calcium flux when the receptors are expressed alone. Furthermore, simultaneous stimulation of the presumed hetero-oligomers by both ligands failed to cause receptor down-regulation and produced a delayed and sustained activation of phosphatidyl inositol 3-kinase (PI3K). The consequence of the altered signaling was linked to more efficient adhesion instead of cell migration. A plausible interpretation was presented in which the homomers and heteromers cooperate to augment the versatility of the signaling responses, with the ligands and their concentrations controlling the formation/stability of the homo- or hetero- complexes. In the Mellado study,

it was suggested that hetero-oligomers might contribute to cell adhesion and “parking” once the cells reach their destination in tissues, while the homo-oligomers promoted migration.

Subsequent studies showed the relevance of the above findings to the recruitment of CXCR4 and CCR5 into the immunological synapse of T cells, and their role in co-stimulation of the T cell receptor during activation through Gq/G11 mediated responses [76]. Similar to the above studies, rather than promoting normal Gai-mediated migration, the recruitment of the receptors resulted in an insensitivity to chemokine gradients, enhanced adhesion to antigen presenting cells and promoted increased proliferation and cytokine production. These results were shown later to be due to the physical association of CXCR4 and CCR5 by BRET and Co-IP; furthermore, it was shown that CXCR4 requires CCR5 for recruitment to the immunological synapse [77]. Together this series of studies demonstrates the ability of chemokine receptor hetero-complexes to differentially signal compared to the homomeric counterparts, in this case due to specificity changes in coupling with G proteins. These studies represent particularly good examples of signaling versatility bestowed by hetero-oligomerization.

V.C. Modulation of signaling by atypical and virally encoded chemokine receptors

DARC/CCR5—The Duffy antigen for chemokines (DARC), an atypical receptor that does not signal through G proteins, has also been shown to homo-oligomerize and to hetero-oligomerize with CCR5 [78]. Coexpression of CCR5 and DARC showed a marked attenuation of chemotaxis and calcium flux in response to the CCR5 ligand RANTES/CCL5 (which also binds DARC with equal affinity) as well as to the CCR5 specific CCL3 isoform ligand, LD78β/CCL3L1. On the other hand, ligand stimulated CCR5 was internalized to the same extent whether it was coexpressed with DARC or not, even though DARC itself does not internalize upon ligand stimulation or interact with β-arrestin in the cells used in this study. On the basis of these and other data, it was proposed that the DARC/CCR5 interaction inhibits ligand-induced CCR5 signaling by altering the affinity of CCR5 for G proteins or the responsiveness of CCR5 to its ligands, but not by altering its affinity for ligands. One could imagine, for example, that DARC induces a conformation in CCR5 that remains competent for ligand binding and internalization but does not allow CCR5 to adopt conformations required for calcium signaling and chemotaxis. DARC has been suggested to function as a “chemokine rheostat” on endothelial cells by supporting the transport, presentation and concentration of chemokines to balance the inflammatory response [6, 79]. This study suggests a mechanism by which DARC “rheostats” the function of CCR5, turning it down through hetero-oligomerization by blocking signaling [78]. Secondly, the fact that heterodimerization with DARC does not affect CCR5’s high affinity for CCL5 or its ability to internalize (presumably with ligand), adds a second stage to the “dial-down” switch.

CXCR7/CXCR4—It was recently demonstrated that CXCR7 forms hetero-oligomers with CXCR4 [54, 80–82]. In the original report [54], hetero-oligomerization coincided with an increased Ca^{2+} flux response to CXCL12 stimulation. Furthermore, it was shown that the time course of ERK activation was altered when CXCR7 was co-expressed in CXCR4-expressing cells. Specifically, when only CXCR4 was present, ERK was activated in a

biphasic fashion, whereas when the two receptors were co-expressed, only the second delayed peak in ERK activation was observed.

In a later report, however, co-expression of CXCR7 along with CXCR4 decreased the potency of CXCL12-induced Ca^{2+} flux, though the maximal efficacy was unchanged [81]. G protein activation as measured by an ^{35}S -GTP- γS binding assay was similarly reduced in potency when the two receptors were co-expressed, and BRET between CXCR4-YFP and Gai1-Rluc demonstrated that CXCR7 causes a conformational rearrangement within pre-coupled CXCR4 and Gai containing complexes. Finally, this report demonstrated that CXCR7 knockdown in T lymphocytes, which endogenously express both CXCR4 and CXCR7, led to an increased migratory response to a lower concentration (0.3 nM) of CXCL12, which was attributed to the propensity of CXCR7 to scavenge CXCL12 (discussed below). Overall, it was suggested that the effects of CXCR7 on CXCL12:CXCR4-mediated signaling cell migration was due both to allosteric modulation of CXCR4:Gai interactions and hoarding of CXCL12 by CXCR7.

One recent report, which did not directly demonstrate dimerization, nevertheless obtained intriguing results that could involve CXCR4/CXCR7 hetero-oligomerization [83]. In this study it was shown that both CXCL11, a chemokine ligand for CXCR7 but not CXCR4, and CCX771, a small molecule inhibitor of CXCR7, were able to inhibit CXCL12-mediate transendothelial migration (TEM), specifically in the case of migrating cells that endogenously expressed both CXCR4 and CXCR7. Since TEM is driven entirely by CXCR4, the most striking observation was that the CXCR7 ligand CCX771, was substantially more potent than the CXCR4 specific ligand AMD3100 in blocking TEM. These results have tremendous ramifications for drug discovery; for example the authors note that CCX771 might be a particularly potent CXCR4 inhibitor in cells that express both receptors (usually cancer cells) and provide greater selectivity than blocking CXCR4 indiscriminately with CXCR4 antagonists like AMD3100.

Finally, a recent report demonstrated that CXCR7 co-expression along with CXCR4 decreased the Gai-mediated inhibition of cAMP production resulting from CXCL12 stimulation [82]. At the same time, co-expression of the receptors greatly increased the resting and CXCL12-induced β -arrestin recruitment to CXCR7. Interestingly, CXCL11 both reversed the decrease in Gai activity and slightly attenuated the increased arrestin recruitment. This study also showed that CXCR7 co-expression increased ERK, p38 MAPK, and SAPK activation upon CXCL12 stimulation, suggesting a broad change in the signaling response elicited in the case of the heteromer. The authors also found that the CXCL12 response of cells in a transwell migration assay was increased when CXCR7 was coexpressed along with CXCR4. The increase in the activation of downstream signaling proteins as well as the increased chemotactic response were dependent upon β -arrestin expression.

As alluded to above, the allosterically regulated functional effects of CXCR4/CXCR7 hetero-oligomerization may be difficult to dissect from other cooperative interactions between the two receptors. In the interpretation of all of the above studies, for example, it should be noted that CXCR7 has significantly higher affinity than CXCR4 for SDF-1/

CXCL12. Thus some of the effects, for instance any increase in CXCR4's responsiveness to CXCL12 when CXCR7 is blocked, could be attributed to the inhibition of CXCR7 from binding and effectively sequestering (from CXCR4) a large proportion of the SDF-1/CXCL12 present. In this regard, it was recently demonstrated that CXCR7 can aid in the pro-migratory, pro-metastatic effects of CXCR4 by scavenging CXCL12 rather than (or in addition to) allosteric modulation of CXCR4 as suggested in the study by LeVoye [84]. In this intriguing study, CXCR7 was even shown to affect the function of CXCR4 when it was expressed primarily on different populations of malignant cells compared to CXCR4. *In vivo* imaging demonstrated that CXCR7 reduced SDF-1/CXCL12 levels in the primary tumor microenvironment, which in turn reduced CXCR4 internalization and downregulation. While this scavenging function of CXCR7 would potentially limit the effects of CXCL12:CXCR4 on tumor growth, it was proposed that it allows CXCR4 to maintain responsiveness to external CXCL12 gradients that would draw metastatic cells to other tissues [84].

Several aspects of the above observations are in line with emerging principles of chemokine receptor oligomerization. The ability of CXCR7-specific agonists as well as antagonists to alter signaling responses mediated by CXCR4 is especially compelling evidence of allosteric communication within receptor oligomers, as CXCR7 does not activate G proteins and as shown by LeVoye, likely does not interfere with CXCR4 signaling simply by stealing G proteins [81]. Furthermore, the change in CXCR4-G α i interaction as well as the increased activation of β -arrestin mediated signaling proteins (MAPK, ERK, p38, SAPK) suggests that the CXCR4/CXCR7 heteromer is a functionally unique signaling complex. However, not all of the above data can be reconciled with a consistent story, illustrating the dependence on methods and the context dependence of the studies (for example, the effect of the relative densities of the two receptors as well as cell background).

BILF/CXCR4—Many viruses including herpesviruses encode GPCRs with considerable homology to chemokine receptors [85, 86]. Most of these vGPCRs show significant constitutive activity although they also tend to bind numerous ligands. One of the most famous of these receptors is ORF74 from the Kaposi's sarcoma-associated herpesvirus (HHV-8) which was initially identified as being the cause of the highly vascularized Kaposi's sarcoma lesions in AIDS patients, and other proliferative disorders. It binds to at least 12 chemokine ligands whose activities range from inverse agonists to full agonists. On the other end of the spectrum of known ligands and relationship to chemokines, BILF1 is a GPCR encoded by the Epstein-Barr virus (EBV or HHV-4) which persists in B cells following primary infection and contributes to Burkitt's lymphoma and Hodgkin's lymphoma among other oncogenic disorders [87]. It has limited homology to chemokine receptors, and currently is considered an orphan GPCR with no known ligands. This receptor seems to be involved in immune evasion by a number of mechanisms including downregulation of MHC class I receptors and inhibition of RNA-dependent protein kinase activity that would otherwise put a stop to cellular translation and therefore viral replication.

One of the more recent mechanisms discovered for BILF1 is that it heterodimerizes with a number of chemokine receptors including CXCR4 [70, 88]. In fact, by combining bimolecular luminescence complementation and bimolecular fluorescence complementation

with BRET measurements, it was shown that heteromeric complexes between BILF1 and CXCR4 consist of the concurrent interaction of at least four GPCR subunits. BILF1 was shown to inhibit binding of SDF-1/CXCL12 to CXCR4 with the consequence of blocking chemokine-mediated signaling. Since BILF1 is a constitutively active receptor and CXCL12 requires G protein coupling for high affinity, it was hypothesized that this receptor might scavenge G α i protein from CXCR4, forcing it into a low affinity state for its ligand (Figure 5). Indeed, overexpression of G α i1 restored the ability of CXCL12 to bind and signal through CXCR4. Furthermore, a G protein uncoupled mutant of BILF1 was much less effective in inhibiting CXCL12-mediated signaling. Together these data suggest that inhibition of CXCR4 by BILF1 is a consequence of its constitutive activity, and contrasts with the allosteric mechanisms described above for the ligand binding transinhibition between CXCR4, CCR2 and CCR5. In principle, this G protein scavenging mechanism could represent a general mechanism of viral GPCRs for inhibiting the function of chemokine receptors that require G protein coupling for high affinity binding.

VI. Heterodimerization of Chemokine Receptors with Non-Chemokine Receptors

In addition to BILF1, other receptors outside the chemokine receptor family have been shown to hetero-oligomerize with chemokine receptors and impact signaling (Table 1). In particular, members of the opioid family of GPCRs have been shown to form heterodimers with CCR5, CXCR4 and CXCR2 [89–92]. These studies were motivated by the fact that opioid receptors and chemokine receptors are often coexpressed on immune system cells as well as neurons and glial cells in the brain [91], and the fact that opioids have been shown to inhibit migration of leukocytes [93]. In a study of the μ -opioid receptor (MOR) and CCR5 for example, transinhibition of functional responses was observed [89]. Whereas cells coexpressing both receptors were responsive to the MOR agonist (DAMGO) and to the CCR5 agonist (RANTES/CCL5) when they were added individually, pretreatment of the cells with CCL5 inhibited migration to DAMGO, and DAMGO but not the antagonist Naloxone, inhibited migration to CCL5. Similarly DAMGO caused increased phosphorylation of CCR5 and inhibited binding of GTP γ S, while CCL5 treatment caused enhanced phosphorylation and decreased GTP γ S binding to MOR. In contrast to the ligand binding transinhibition described previously for CCR5, CCR2 and CXCR4, there was no significant bi-directional inhibitory effect on the binding affinity of the agonists. Overall, the data suggest that agonist stimulation of each receptor promoted cross-desensitization of the other receptor.

CXCR4 and the δ -opioid receptor (DOR) have also been shown to hetero-oligomerize. In these studies, hetero-oligomerization and simultaneous stimulation with their respective agonist ligands, SDF-1/CXCL12 and [D-Pen2, D-Pen5]enkephalin (DPDPE), inhibited migration of CXCL12-mediated cell migration and adhesion of primary monocytes and monocytic cell lines. These results were also validated with *in vivo* studies of cell migration into the peritoneal cavity of mice [91]. In contrast to the above studies of CCR5 and MOR, the inhibitory effect was not due to heterologous desensitization, nor did DPDPE affect the affinity of CXCL12 for CXCR4. Instead the silencing of CXCR4 was shown to be due to the

inability of the ligand-engaged heterodimers to activate JAK2, which is a pre-requisite for G α i coupling to CXCR4. While FRET studies showed that the receptors formed oligomers independent of ligand binding, additional studies suggested that the receptors function in a ligand-regulated dynamic equilibrium between homo and hetero-oligomers. Specifically, FRET signals from CXCR4 homomers were disrupted with increasing expression of DOR. Furthermore, the heterodimer formation was reversed by DPDPE but not by simultaneous addition of CXCL12 and DPDPE. Overall the results suggest that CXCL12 or DPDPE alone allows signaling by their respective homomeric receptors, but treatment with both ligands stabilizes the hetero-oligomers and blocks signaling presumably by stabilizing the receptors in inactive conformations. This ligand-dependent regulation of the heteromers was proposed to provide a mechanism that might have consequences on physiological processes involving pain and inflammation; for example by increasing sensitivity to pain while simultaneously curtailing migration of cells to sites of inflammation.

Entirely different functional affects were observed in a study of hetero-oligomers between CXCR2 and DOR [90]. In this case, several complementary methods (BRET, FRET, tr-FRET and Co-IP) were used to demonstrate heterodimers in transfected cells. Saturation BRET studies suggested that the heterodimers have a greater tendency to associate than the homodimers. Most importantly, the small molecule CXCR2 antagonist resulted in enhanced signaling responsiveness of DOR to agonists, due to allosteric communication between the receptors while the CXCR2 agonist IL-8/CXCL8 did not. That the nature of the ligand matters makes intuitive sense since agonists and antagonists would likely promote different conformations of the partner receptor in the heterodimer.

In contrast, the Epstein-Barr virus-induced receptor 2 (EBI2) exerts a negative allosteric effect on the function of CXCR5 [94]. In these studies, coexpression of EBI2 lead to decreased responsiveness to CXCL13, as measured by Ca⁺² flux, chemotaxis, and ERK1/2 phosphorylation. These results were attributed to the reduced affinity of CXCR5 for its ligand CXCL13 when the two receptors were co-expressed. Whether the ligand affinity was reduced due to a direct allosteric modulation of EBI2 on the CXCR5 binding pocket, the coupling with G protein, or a combination of both mechanisms was not determined. The cooperation between these two receptors is thought to regulate B cell movement into lymphoid follicles.

CXCR2 has also been shown to associate with the α 1A-andrenoreceptor [95]. In this case, heterodimerization with CXCR2 changes the pharmacology of α 1A such that it strongly recruits β -arrestin upon stimulation with norepinephrine. This effect was inhibited not only by the α 1A antagonist Terazosin, but also by a CXCR2-specific small molecule inverse agonist, SB265610.

VII. Other Sources of Allostery in Chemokine Receptor Signaling: Chemokine Oligomerization

The bulk of this review has been focused on chemokine receptor oligomerization and its functional consequences. However, there are many other sources of allostery related to ligands that are worth noting. Similar to small molecule agonists of GPCRs, functional

selectivity of different chemokine ligands of the same receptor have been reported. One of the best examples involve the ligands of CCR7, SLC/CCL21 and ELC/CCL19, which work together to allow for temporally and spatially distinct responses of CCR7 expressing T cells [96]. Both of these ligands show a similar binding affinity for CCR7 and equipotent ability to activate G proteins and cause calcium flux. However, only CCL19 promotes robust desensitization and phosphorylation of the receptor, as well as β -arrestin recruitment and ERK1/2 activation. This finding and the fact that these ligands are differentially expressed *in vivo* has been used to explain how CCR7 can produce the diverse responses of T cells as they migrate into the T cell zones of peripheral lymph nodes. CCL21 is expressed on the high endothelial venules, and thus it makes sense that it would not cause desensitization, allowing subsequent migration of the cells into T cell zones where CCL19 is expressed.

Another important example is that of synthetic N-terminally modified variants of RANTES/CCL5, which can cause internalization of CCR5 and inhibit it from being recycled back to the cell surface. These studies have provided proof of concept that engineering this type of allosteric functional selectivity can be a powerful approach to inhibiting HIV. There are also many reports of allosteric small molecules of chemokine receptors (for an excellent review see [4]).

All of the above examples of functionally selective ligands fit into the classic view of chemically different variants of related ligands. However, recently in the chemokine field, it has been shown that different oligomerization states of the same ligand can also show biased signaling. Early investigations of RANTES/CCL5 showed that although oligomerization-deficient mutants were equally capable as WT CCL5 in promoting transendothelial cell migration, only the oligomerizing WT chemokine could promote monocyte arrest [97]. More recent studies of an obligate disulfide-locked dimer of SDF-1/CXCL12 has shown that it is capable of binding CXCR4 but has a different signaling profile than WT CXCL12 (which is effectively monomeric below millimolar concentrations). Whereas WT CXCL12 stimulated calcium flux, inhibited cAMP, and promoted cell migration and β -arrestin association, dimeric CXCL12 was impaired in its ability to stimulate cell migration and recruit β -arrestin [67]. Whether dimeric CXCL12 binds and stabilizes monomeric or dimeric CXCR4 remains to be seen, but one can imagine an effect of the oligomerization state of the CXCL12 on the homo or hetero-oligomerization state of CXCR4 and *visa versa*--that the state of CXCR4 affects whether monomeric or dimeric CXCL12 binds to CXCR4.

VIII. Conclusions and Future Perspectives

In the last decade, there has been an explosion in the number of studies focused on demonstrating the presence and functional relevance of chemokine receptor homo- and hetero-oligomerization. Notwithstanding acknowledgement of the fact that there are conflicting reports and perhaps erroneous conclusions because of limitations in methods used, it appears that receptor oligomerization, particularly hetero-oligomerization, can result in many different types of pharmacological responses compared to chemokine receptors in isolation, and as a consequence of many different types of cellular mechanisms. These functional effects and mechanisms include (1) transinhibition of ligand binding whether it be through a G protein steal when the pool of G proteins is limiting, or purely from allosteric

effects because of changes in the conformation of a given receptor (R1) due to the presence of the second receptor (R2) and/or the presence of the agonist or antagonist ligand of R1 or R2; (2) activation of receptor functional responses whether it be through ligand dependent or independent effects; (3) inhibition of functional responses that are ligand independent, or dependent on ligands of one or both of the interacting receptors; (4) ligand regulated formation of receptor heteromers or destabilization of receptor heteromers.

In addition to these functional effects, the next level of complexity and a key issues is whether there are cell and tissue dependent effects given differences in receptor expression levels, intracellular signaling partners and intra- and inter-cellular microenvironments. The idea that these effects can impact drug discovery seems indisputable and while it complicates high throughput screening campaigns, in principle there are major opportunities for drug discovery if the biology of the disease and detailed pharmacology of the receptors in question are understood. Technology development to validate the presence of interacting receptors, their functional consequences and mechanisms of action, and determining their role in disease will be required to capitalize on these new insights into the complex function and regulation of chemokine receptors and other GPCRs.

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Figure 1. Ribbon diagrams of: (top left) a typical chemokine monomer (MCP-1/CCL2, PDB ID 1dol); (top right) a CXC chemokine dimer (IL-8/CXCL8, PDB ID 1il8); (bottom) a CC chemokine dimer (MCP-1/CCL2, PDB ID 1dok).

Figure 2.

Two site model of receptor activation. On the left is a surface topology model of CXCL12 bound to the N-terminal CRS1 of CXCR4 (black string with sulfated tyrosines side chains shown). The right illustrates the binding of the chemokine core domain to the N-terminus of the receptor (CRS1, circles represent sulfated tyrosines) and the N-terminus of the chemokine binding into the receptor helical bundle (CRS2).

Figure 3.

Cartoon of the two-site model where the small molecule antagonist AMD3100 (hexagon) binds CXCR4 in the TM domain CRS2, and displaces the N-terminus of SDF-1/CXCL12. Figure A illustrates the hypothetical displacement of the chemokine N-terminus in the context of a 1:1 chemokine-monomer:receptor-monomer interaction. Figure B illustrates the displacement in the context of a 1:2 chemokine-monomer:receptor-dimer interaction. Other stoichiometries are also possible such as 2:2 interactions where two chemokine monomers bind a receptor dimer or one chemokine dimer binds to a receptor dimer.

Figure 4.

A model for the allosteric ligand binding transinhibition of agonists from chemokine heterodimers as suggested by Springael and coworkers [66]. **(1)** Heterodimerization of receptor 1 (R1) and receptor 2 (R2). **(2 and 3)** Ligand 1 (L1) binds with high affinity to R1 when it is coupled to heterotrimeric G α i proteins (G). The arrow illustrates allosteric coupling between the G protein and R1, which allows R1 to adopt a conformation that leads to high affinity binding of L1. **(3 and 4)** Subsequent binding of ligand 2 (L2) to R2 induces a conformational change in R2 that results in the G protein interacting with R2 rather than R1, and subsequently **(4 and 5)** dissociation of L1 from R1 since G protein is required for high affinity interaction of L1/R1. Note that the mechanism for transinhibition of agonist by binding of small molecule antagonists does not need to involve changes in G protein coupling but could be explained simply by allosteric communication between the two receptors [64].

Figure 5.

A model for inhibition of CXCR4 signaling by the EBV viral GPCR, BILF1 [70]. **(1)** CXCR4 (R1), shown here as monomer for simplicity but probably exists as homo-oligomer, is coupled to heterotrimeric G_{ai} proteins (G) and therefore competent for high affinity binding and signaling in response to SDF-1/CXCL12 (L1). BILF1 is represented as R2. **(2)** Hetero-oligomerization of CXCR4 (R1) with BILF1 (R2) scavenges the G protein heterotrimer from CXCR4 due to its constitutive activity. The lack of G protein shifts CXCR4 into a low affinity state and CXCL12 dissociates. **(3)** Uncoupled, ligand-free CXCR4 R1 does not signal.

Table 1

Chemokine receptor oligomers.

Receptors Involved	Interesting Observations	Methods Used	References
<i>Chemokine receptor homomers</i>			
CCR2/CCR2	Homodimerization induced by CCL2	Co-IP with chemical cross-linking, divalent antibody cross-linking	[48, 53, 75]
	Constitutive homodimerization, conformational change caused by CCL2 stimulation	BRET	[51, 98]
	Constitutive, CCL2 stimulation had no effect	BRET	[60]
	Constitutive, negative cooperativity in agonist binding	BRET	[66]
	Simultaneous higher order heteromerization	BiLC-BRET	[65]
CCR5/CCR5	Trafficking-defective CCR5 32 dimerized with WT CCR5 to reduce surface expression	³⁵ S pulse labeling gel analysis IMF Co-IP	[99]
	Homodimerization induced by CCL5	Co-IP with Chemical crosslinking	[49, 75, 100]
	CCR5 32 mutant defect not related to normal trafficking	Flow cytometry	[101]
	Divalent antibodies stabilized dimers and promoted internalization	BRET	[102]
	Constitutive, unaffected by CCL5, BRET signal increased by divalent dimer-stabilizing antibody	BRET	[46]
	Constitutive	Co-IP	[60, 77, 103]
	TM1 and TM4 implicated in homodimer interface	FRET-based mutational analysis	[45]
	Homodimer interface mutations in TM1 and TM4 called into question	BRET	[61]
	GRK-mediated “cross-phosphorylation” across the homodimer interface	BRET	[104]
	Constitutive, negative cooperativity in agonist binding, evidence for G-protein involvement in negative cooperativity	BRET	[66]
	Constitutive, homodimer-specific adaptor protein	BiFC	[105]
	Constitutive, homodimers depend on specific Rabs for cell surface delivery	BiFC	[106]
	Constitutive, homodimer-specific chaperone	BiFC	[107]
CXCR1/CXCR1	Constitutive	Co-IP, tr-FRET, single cell FRET, BRET, ER trapping	[55]
	Homodimers stabilized by CXCL8	FRET	[108]
CXCR2/CXCR2	Constitutive, TM3 and ICL2 implicated in homodimerization	Co-IP	[109]
	Disulfide involvement in homodimerization unclear	WB	[110]
	Constitutive	Co-IP, tr-FRET, single cell FRET, BRET, ER trapping	[55]
	Homodimers stabilized by CXCL8	FRET	[108]
CXCR4/CXCR4	Homodimerization induced by CXCL12	Co-IP with Chemical crosslinking	[50]

Receptors Involved	Interesting Observations	Methods Used	References
	Constitutive	Co-IP, BRET, sucrose gradient centrifugation, BiFC, Bivalent ligand synthesis	[62, 105, 111–114]
	Constitutive, CXCL12 altered FRET signal	FRET	[115]
	Constitutive, CXCL12 caused conformational change in pre-formed homodimers	BRET	[51]
	Constitutive, homodimerization reduced by cholesterol depletion and a TM4 synthetic peptide	Single cell FRET, pbFRET	[72]
	Constitutive, higher order homo-oligomerization	BRET, BiFC-BRET	[69]
	Constitutive, signal altered after CXCL12 incubation	PCA	[80]
	Constitutive, signal modified by ligand stimulation	BRET	[7]
	Constitutive, signal modified by ligand stimulation	BRET	[81]
	Simultaneous higher order heteromerization	BiLC-BRET	[65]
	Homodimers reconstituted into proteoliposomes	Thermal inactivation	[116]
	TM 5 and 6 comprised homodimer interface	X-ray crystallography	[63]
	Constitutive, homodimers depend on specific Rabs for cell surface delivery	BiFC	[106]
	TM 5 and 6 interface supported with minor adjustments	MD simulation	[117]
	Sphingomyelin deficiency increased dimerization and signaling, presumably by causing accumulation of receptor in lipid rafts	FRET	[118]
CXCR7/CXCR7	Constitutive, CXCL12 modulated reporter signals	BRET, PCA	[7, 80, 81]
DARC/DARC	Constitutive	BRET	[78]
<i>Chemokine receptor heteromers</i>			
CCR2/CCR5	Required stimulation with both CCL2 and CCL5	Co-IP with Chemical crosslinking	[53]
	CCR2/CCR5 heterodimerization proposed to result from relatively recent gene duplication and resultant high sequence similarity	Correlated mutation analysis	[119]
	Required co-stimulation with CCL2 and CCR5, cooperative stimulation at lowered chemokine concentrations, distinct signaling proteins recruited and cellular responses elicited	Co-IP with Chemical crosslinking	[75]
	Stimulated by chemokines and divalent antibodies	Co-IP with Chemical crosslinking, FRET	[120]
	No cooperative signaling, negative cooperativity in chemokine binding	BRET	[60]
	Constitutive, negative cooperativity in agonist binding, evidence for G-protein involvement in negative cooperativity	BRET	[66]
	Gene conversion proposed to be the cause of heterodimerization	Comparative and phylogenetic analysis	[121]
	Simultaneous higher order heteromerization and homomerization of CCR2, negative cooperativity in both chemokine and antagonist binding	BiLC-BRET	[65]
	Heterodimer recruited β -arrestin	GPCR-HIT	[122]
CCR2/CXCR4	Required stimulation with both CCL2 and CXCL12	Co-IP with Chemical crosslinking	[53]

Receptors Involved	Interesting Observations	Methods Used	References
	Stimulated by chemokines and divalent antibodies	Co-IP with Chemical crosslinking, FRET	[120]
	Constitutive, chemokines caused conformational change	BRET	[51]
	Constitutive, negative cooperativity in both chemokine and antagonist binding	BRET	[64]
	Simultaneous higher order heteromerization and homomerization of CCR2, negative cooperativity in both chemokine and antagonist binding	BiLC-BRET	[65]
	Heterodimer recruited β -arrestin	GPCR-HIT	[122]
CCR5/CXCR4	Transinhibition by both agonists and antagonists	Co-IP, BRET, FRET, BiFC	[52, 53, 59, 65, 77, 105]
	Heterodimerization seemed to be CD4 expression-dependent	IMF, Co-IP	[59]
	Constitutive; Co-recruited into the Immunological Synapse (IS) of T cells; Elicited heterodimer-specific signaling pathways when together in the IS	BRET, BiFC	[77, 107]
	Constitutive, FRET signal modulated by chemokine ligands	FRET	[52]
	Simultaneous higher order heteromerization, negative cooperativity in both chemokine and antagonist binding	BRET, BiLC-BRET	[65]
	Constitutive, distinct adaptor protein from CCR5 homodimer	BiFC	[105]
	Constitutive, heterodimers depend on specific Rabs for cell surface delivery	BiFC	[106]
CXCR1/CXCR2	Constitutive, signal disrupted by CXCL8	Co-IP, tr-FRET, single cell FRET, BRET, ER trapping	[55]
	Constitutive, signal altered by CXCL8	FRET	[108]
CXCR4/CXCR7	Constitutive, CXCR4 signaling was enhanced	Single cell FRET, pbFRET	[54]
	Constitutive	PCA	[80]
	CXCR7 impairs CXCR4-mediated G protein signaling	BRET	[81]
	Increased β -arrestin recruitment, enhanced CXCR4-mediated migration	Co-IP	[82]
CCR2/CCR5/CXCR4 Higher order oligomer	Transinhibition by both agonists and antagonists	BiLC-BRET	[65]
DARC/CCR5	Constitutive, DARC inhibits CCR5 activation	BRET	[78]
Chemokine receptor heteromers with non-chemokine GPCRs			
CXCR2/ α 1A-adrenoceptor	α 1A-AR activation by norepinephrine inhibited by the CXCR2 inverse agonist SB265610; dimerization itself unaffected by ligands	BRET	[95]
CCR5/ μ -OR CCR5/ δ -OR CCR5/ κ -OR	Cooperative ligand effects	Co-IP with Chemical crosslinking	[92]
CCR5/ μ -OR	Constitutive, negative cooperativity between CCR5 and μ -OR agonists; cross-phosphorylation observed in both directions	Co-IP; Bivalent ligand synthesis	[89, 123]
CCR5/C5aR	GRK-mediated "cross-phosphorylation" across the heterodimer interface	BRET	[104]

Receptors Involved	Interesting Observations	Methods Used	References
CXCR2/AMPA Glu 1	Constitutive	Co-IP	[109]
	Dimerization reduces activation by CXCL8; CXCL8 modulates AMPA Glu 1 phosphorylation	BRET	[124]
CXCR2/ δ -OR	CXCR2 antagonists increased δ -OR activation	Co-IP, BRET, FRET	[90]
CXCR4/ μ -OR CXCR4/ κ -OR	Competed for CXCR4 homodimer formation	FRET	[115]
CXCR4/ δ -OR	Heterodimerization proposed to silence receptor functions	FRET	[91]
CCR6/BILF1 CCR7/BILF1 CCR9/BILF1 CCR10/BILF1 CXCR3/BILF1 CXCR4/BILF1 CXCR5/BILF1 CXCR7/BILF1	Constitutive	Co-IP, BRET, tr-FRET	[88]
CXCR4/BILF1	BILF1 inhibits CXCR4 activation	BiFC, BiLC	[70]
CXCR5/EBI2	EBI2 inhibits CXCR5 activation	FRET	[94]
<i>Chemokine receptor heteromers with receptors outside the GPCR family</i>			
CXCR4/CD4	CD4 expression was required for changes in CXCR4 homodimer FRET caused by HIV-1 coat protein gp120IIIB	FRET	[115]
CXCR4/TCR	required CXCL12 stimulation; CXCL12 activated downstream signaling pathways through TCR	FRET, Co-IP	[125]
CCR5/CD4	Constitutive; CCR5 reported to Co-IP with CD4 to a greater extent than CXCR4	Co-IP	[126]

Table 2

Methods used in studying chemokine receptor oligomerization.

Method	Description	Pros & Cons	Other Notes	Reference(s)
Co-IP	Immunoprecipitation, electrophoresis, and immunoblotting of one receptor followed by immunoblotting of a candidate dimer partner receptor	The most well established experimental technique used to study GPCR dimerization; requires the least technologically advanced equipment	Sometimes carried out after chemical and/or antibody crosslinking of dimerized receptors; Co-IP methods were the earliest used to establish chemokine receptor dimerization and tended to suggest that dimerization was agonist-induced	[3, 48]
FRET	Dimerization indicated by fluorescence resonance energy transfer between fluorophores (either fluorescent proteins such as CFP and YFP or small organic fluorophores such as Cy3 and Cy5) coupled to candidate receptors	More sensitive than Co-IP methods; requires equipment capable of FRET detection	This method tends to show constitutive dimerization, but the FRET signal is often increased or reduced upon agonist stimulation, which could indicate either a change in dimerization equilibrium or conformational changes within preformed dimers	[3, 108]
Single cell FRET	Specific type of FRET in which microscopy is used to collect fluorescent signal from a specific region of a single cell chosen by the experimenter	Can be used in combination with microscopy to analyze specific cellular regions (e.g. plasma membrane, ER); requires microscope capable of FRET detection	In one interesting case, this was used to demonstrate an absence of dimerization where a cell population average method, BRET, failed to reach the same conclusions	[45]
tr-FRET	Time-resolved FRET. Specific type of FRET in which a donor with a long fluorescent half-life is used to detect FRET after a time delay (i.e. after the autofluorescence of the cells being assayed has subsided)	Increased sensitivity over simple FRET due to reduced autofluorescence; Often used to detect cell-surface dimerization specifically; requires equipment capable of detecting FRET	This method is promising for the future, as studies with non-chemokine receptors using long lived fluorophores coupled to agonists and antagonists have produced interesting results with respect to ligand:GPCR oligomer stoichiometry. This method can also be used to investigate endogenous receptor oligomers on native cell types of interest	[55, 127]
pbFRET	FRET after photobleaching. This method relies on deducing FRET from recovered donor fluorescence signal after photobleaching the acceptor fluorophore	Can be used in combination with microscopy to analyze specific cellular regions	Usually performed in the context of confocal microscopy-based single cell FRET	[54, 72]
BRET	Similar to FRET, except that a bioluminescent enzyme is used as the donor for resonance energy transfer, so that a chemical substrate is added to produce the observed signal	Increased signal-to-noise ratio over FRET due to the use of a bioluminescent enzyme as a donor rather than a fluorophore that must be excited with light; Requires both luminescence and fluorescence detection capabilities	Tends to show constitutive dimerization; This method allows distinction between agonist-mediated disruption/formation of dimers and conformational changes within pre-formed dimers, and agonists are almost always found to cause conformational changes within dimers without affecting the dimer equilibrium	[3, 51]
BiFC-BRET/BiLC-BRET	Derivative of BRET in which the fluorescent protein (BiFC) and/or bioluminescent enzyme	Allows the detection of higher order multimerization;	These methods have been used to show higher order homomerization and	[65, 69]

Method	Description	Pros & Cons	Other Notes	Reference(s)
	(BiLC) is split, with part of the protein placed on each candidate receptor	Stabilization of split YFP derivatives upon fusion, which will lead to increase in signal unrelated to oligomerization of the actual fused GPCRs, may complicate interpretation of results	heteromerization of chemokine receptors	
GPCR-HIT	BRET-based method in which the fluorescent protein is coupled to one of the candidate GPCRs and the bioluminescent enzyme is fused to β -arrestin	Allows the identification of active, functional heteromers; use is restricted to heterodimer identification	Chemokine receptors were among those used to establish the initial validation of this method	[122]
PFC (BiFC/BiLC)	Either a bioluminescent enzyme or fluorescent protein is split, with part fused to each candidate receptor, and a functional fluorescent or bioluminescent protein is interpreted to result from dimerization of the candidate receptors	Can be used with microscopy to zoom in on single cells/cellular regions; Stabilization of split YFP derivatives upon fusion, which will lead to increase in signal unrelated to oligomerization of the actual fused GPCRs, may complicate interpretation of results	Again, this method tends to show constitutive dimerization, with agonist stimulation often affecting the signal, which could indicate either a change in dimerization equilibrium or conformational changes within pre-formed dimers	[80]
Radio-ligand displacement	Ligand affinity measured by displacement of radio-labeled ligand	Can be used on live cells; Can be used to obtain evidence of allosteric functional effects of receptor oligomerization, such as negative cooperativity; Does not directly establish dimerization	This method has been used several times in recent studies to demonstrate negative cooperativity/transinhibition resulting from chemokine receptor oligomerization	[60, 64–66]