Heterodimerization of CCR2 Chemokines and Regulation by Glycosaminoglycan Binding*§

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†The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S5.

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§The abbreviations used are: CCL2, MCP-1; CCL8, MCP-2; CCL7, MCP-3; CCL13, MCP-4; CCL11, eotaxin; CCL4, MCP-1β; CCL5, MIP-1α; CCL3, RANTES (regulated on activation normal T cell expressed and secreted); CCL1, I-309; CCL4, PF4; CCL8, interleukin-8; CCL21, SLC; CCL13, BCA-1; CCL19, ELC; CCR, CC-chemokine receptor; GAG, glycosaminoglycan; ESI-FTICR, electrospray ionization Fourier transform ion cyclotron resonance; MS, mass spectrometry; HSQC, heteronuclear single quantum correlation.

Despite the wide range of sequence diversity among chemokines, their tertiary structures are remarkably similar. Furthermore, many chemokines form dimers or higher order oligomers, but all characterized oligomeric structures are based primarily on two dimerization motifs represented by CC-chemokine or CXC-chemokine dimer interfaces. These observations raise the possibility that some chemokines could form unique hetero-oligomers using the same oligomerization motifs. Such interactions could modulate the overall signaling response of the receptors, thereby providing a general mechanism for regulating chemokine function. For some chemokines, homo-oligomerization has also been shown to be coupled to glycosaminoglycan (GAG)-binding. However, the effect of GAG binding on chemokine hetero-oligomerization has not yet been demonstrated. In this report, we characterized the heterodimerization of the CCR2 ligands MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13), and eotaxin (CCL11), as well as the effects of GAG binding, using electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometry. Strong heterodimerization was observed between CCL2 and CCL8 at the expense of homodimer formation. Using NMR, we showed that the heterodimer is predominant in solution and forms a specific CC chemokine-like dimer. By contrast, only moderate heterodimer formation was observed between CCL2-CCL13, CCL2-CCL11 and CCL8-CCL13, and no heterodimerization was observed when any other CCR2 ligand was added to CCL7. To investigate the effect of a highly sulfated GAG on the formation of heterodimers, each chemokine pair was mixed with the heparin pentasaccharide, Arixtra, and assayed by ESI-FTICR mass spectrometry. Although no CCL8-CCL11 heterodimer was observed in the absence of GAG, abundant ions corresponding to the ternary complex, CCL8-CCL11-Arixtra, were observed upon addition of Arixtra. Heterodimerization between CCL2 and CCL11 was also enhanced in the presence of Arixtra. In summary, these results indicate that some CCR2 ligands can form stable heterodimers in preference to homodimers and that these interactions, like those of homo-oligomers, can be influenced by some GAGs.

Chemokines are small (8–12 kDa), secreted proteins that have critical roles in many biological processes, including lymphoid trafficking and inflammation, angiogenesis/angiostasis, and development, as well as in many disease states (1, 2). Based on the pattern of N-terminal cysteine residues, they have been categorized into CC, CXC, C, and CX3C families. However, despite the fact that sequence identity between chemokines can range from <20% to >90%, their tertiary structures are highly conserved and consist primarily of a disordered N terminus, a long irregular loop ending in a 310 helix, three anti-parallel β-strands, and a C-terminal α-helix (1, 3, 4). In addition, many chemokines dimerize, some form higher order oligomers, and in many cases, oligomerization is critical for their in vivo function. For example, although the monomeric mutants of MCP-1 (CCL2),4 MIP-1β (CCL4), and RANTES (CCL5) have wild-type chemotactic activity in vitro, they are inactive in vivo (5). In contrast, some chemokines, specifically eotaxin (CCL11), MCP-3 (CCL7), and I-309 (CCL1), cause cell migration in vivo; however, there is no reported evidence that they oligomerize in solution by themselves (6–8).

Similar to the corresponding features in their tertiary structures, the dimerization motifs of chemokines tend to be structurally conserved and fall primarily into one of two types represented by CC and CXC chemokines. In general, chemokines in the CC family dimerize by formation of a two-stranded anti-parallel β-sheet involving residues near the N termini, whereas CXC family members dimerize through the first stand of the β-sheet, which is preformed in the monomer structure (9). Recent structural studies have also revealed tetrameric complexes in which both interfaces occur (10, 11). The facts that...
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Each chemokine has a similar fold and they utilize a limited set of dimer interface motifs raise the possibility that some chemokines, particularly those with high sequence homology around the dimer interface residues, may be able to form specific heterodimeric or higher order hetero-oligomeric structures. Indeed, it has been shown that the CXC chemokine, PF4 (CXCL4), binds to another CXC chemokine, IL-8 (CXCL8) with a dissociation constant of 42 nM (12). Furthermore, the presence of both chemokines together was shown to have functional consequences, including attenuation of CXCL8-induced signaling in CD34+ human hematopoietic progenitor cells, enhanced CXCL8-induced chemotaxis of CXCR2-transfected cells, and enhanced anti-proliferative activity of CXCL4 on endothelial cells (12, 13). In another example, it was shown that the CCR7 ligand, SLC (CCL21), interacts with the CXC chemokine, IL-8 (CXCL8), which is not an agonist of CCR7, to form hetero-oligomeric complexes. Furthermore, the combination of CXCL13 in concert with either CCL21 or another CCR7 ligand, ELC (CCL19), triggered CCR7-mediated responses at lower concentrations than the agonists alone, again demonstrating synergy (14). These results indicate that the formation of hetero-oligomers may be a more general property of chemokines and suggest another mechanism to modulate their in vivo function.

It is well established that chemokines bind to GAGs, and there is accumulating evidence that this interaction is critical for their function (5, 15). For example, it has been demonstrated that GAG binding-deficient mutants of CCL2, CCL4, and CCL5 cannot induce cell recruitment in vivo even though they are functional in vitro (5). Additionally, mice engineered to have attenuated N-sulfation of the heparan sulfate chains on their endothelial cells show reduced chemokine transcytosis and presentation and impaired neutrophil migration (16). There is also increasing evidence that the oligomerization of chemokines is in some cases related to their GAG-binding properties. For example, in the presence of low molecular weight heparin, the apparent molecular weights of CCL5, MIP-1α (CCL3), and CCL2 are significantly elevated, consistent with the formation of higher order oligomers (17, 18). CXCL8, CCL2, CCL3, and CCL5 have also been shown to oligomerize on immobilized heparin and on human umbilical vein endothelial cells, and in the case of human umbilical vein endothelial cells, binding is reduced by deglycosylation enzymes (17). Given the correlation between homo-oligomerization and GAG binding, it is not unreasonable to postulate that GAG binding may also modulate chemokine hetero-oligomerization. However, such interactions have yet to be systematically explored.

We have recently shown that ESI-FTICR mass spectrometry is a valuable tool with which to characterize chemokine-GAG noncovalent interactions (19), and as shown in previous studies (19, 20) the results are in good agreement with solution studies by NMR, analytical ultracentrifugation, and isothermal titration calorimetry. This process is facilitated by electrospray ionization, which enables transfer of intact noncovalent complexes from solution into the gas phase (21). Analysis of the ion populations can then yield information about molecular weight, abundance, binding stoichiometry, and relative binding affinity of the complexes (19). This previous mass spectrometric study indicated that, under the conditions used, the CCR2 ligands can be divided into two groups based on their multimeric states. CCL2 and MCP-2 (CCL8) exist as monomers in equilibrium with dimer, the latter being preferentially bound by the highly sulfated heparin octasaccharides. Conversely, CCL7, MCP-4 (CCL13), and CCL11 are monomers and only form 1:1 complexes with heparin octasaccharides.

Because of the significant sequence homology among the CCR2 ligands in the residues involved in CC dimerization (supplemental Fig. S1), we set out to investigate whether any or all of these chemokines are capable of heterodimerizing. In fact, of the ten positions in the first shell of the CC-dimer interface (residues 1–3, 8–12, and 18–19) (22, 23), five are completely conserved within this set of chemokines. Of special note is a proline in position 8, which is essential for dimerization of CCL2 (24) and is present in each of these chemokines except CCL7. If differences in the ability of the chemokines to heterodimerize were observed, the results would not only suggest another mechanism for regulating chemokine function, but it would provide evidence for nonredundant behavior of the CCR2 ligands. Redundancy refers to the fact that many chem-

5 E. K. Lau, S. E. Crown, and T. M. Handel, additional unpublished AUC data.
kines bind the same receptor. For example, CCL2, CCL7, CCL8, CCL11, and CCL13 all bind and signal through CCR2, although CCL11 functions as an antagonist (25–30). However, it is unclear whether physiologically they have overlapping or separate functions.

Thus, in the present study, we used ESI-FTICR MS to characterize the heterodimerization of the CCR2 ligands. We also used NMR to determine if the interaction is specific and to characterize the nature of the heterodimer interface of CCL2-CCL8. Finally, we used MS to investigate the effect of GAG binding on the ability of the chemokines to heterodimerize.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arixtra™ (fondaparinux sodium) was purchased from GlaxoSmithKline (Research Triangle Park, NC) and was desalted and quantified for experiments as described previously (20). Heparinase I (EC 4.2.2.7) was purchased from Seikagaku (Falmouth, MA). All the other chemicals were purchased from Fisher Scientific and used without further purification. The CCR2 ligands were expressed and purified as previously described (19). Heparinase I (EC 4.2.2.7) was purchased from GlaxoSmithKline (Research Triangle Park, NC) and was desalted and quantified for experiments as described

**Preparation of Chemokine Heterodimers**—The binary interactions between the CCR2 ligands CCL2, CCL7, CCL8, CCL11, and CCL13 were investigated. In each experiment, two chemokines, each at a concentration of 10 μM, were mixed in 100 mM NH₄OAc (pH 6.8) and incubated at room temperature for 10 min with gentle rocking. The resulting solution was analyzed by ESI-FTICR MS. For characterization of the effect of GAG on heterodimerization, two chemokines (each at 10 μM) were mixed with 10 μM Arixtra in 100 mM NH₄OAc (pH 6.8). The solution was incubated as above and analyzed by ESI-FTICR MS.

**Fourier Transform Ion Cyclotron Mass Spectrometry**—Mass spectra were acquired on a Bruker APEX II 7-tesla FTICR mass spectrometer (Billerica, MA) equipped with an Apollo (Bruker, Billerica, MA) electrospray ion source. Samples were infused into the mass spectrometer at 1 μl/min using a syringe pump (Harvard Apparatus, Holliston, MA). The pressures of the nebulizing and drying gases were maintained at 50 and 25 p.s.i., respectively. The capillary exit voltage was adjusted to 140 V (positive mode) for ion desolvation (19, 31). Ions were externally accumulated in a radiofrequency-only hexapole for 0.5 s and were trapped in the ICR cell using gated trapping. Between 8 and 100 broadband time domain transients containing 1024 k data points were averaged and subjected to zero fill, Gaussian multiplication, and fast Fourier transform analysis. The parameters of the ESI source, ion optics, and cell were optimized for the best ion intensity. All the data were acquired and processed using Xmass version 6.0.0 (Bruker, Billerica, MA).

**NMR**—The 15N-labeled CCL2 was produced as previously described (24). All NMR samples were prepared with 200 μM CCL2 in 5% D₂O (pH 5.42). CCL2-CCL8 samples were prepared with 0, 25, 50, 100, 125, 200, and 800 μM unlabeled CCL8 in addition to the labeled CCL2. Heterodimer formation was as described previously at 26 °C on a 600-MHz Bruker Avance spectrometer. The data were processed in Azara (Wayne Boucher, University of Cambridge). Assignments were derived from a previous study on the structure of CCL2 (22).

**RESULTS**

Both CCL2 and CCL8 exist in equilibrium between monomer and dimer forms. As shown in Fig. 1 (A and C), the 5+ monomer and 9+ dimer ions were clearly observed when 10 μM CCL2 or CCL8 was sprayed from a solution in 100 mM NH₄OAc (pH 6.8). When an equimolar mixture (10 μM each) of CCL2 and CCL8 was analyzed, a distinctly different dimer profile was obtained (Fig. 1B), with only small amounts of CCL2 or CCL8 homodimers. Conversely, an intense peak was observed, corresponding to the 9+ ion of the CCL2-CCL8 heterodimer. Summing the intensities of both 8+ and 9+ charge states, the normalized abundances of the CCL2 homodimer, CCL2-CCL8 heterodimer, and the CCL8 homodimer were calculated to be ~7.4, 83.6 and 9.0%, respectively, indicating almost complete depletion of the homodimers.

The experiments were then repeated for the other binary combinations between the CCR2 ligands and showed very different heterodimerization characteristics. Moderate heterodimer formation was observed between CCL2-CCL13, CCL2-CCL11, and CCL8-CCL13. In these cases, the heterodimers are less abundant than the corresponding homodimers. For example, although CCL13 alone is a monomer (supplemental Fig. S2C), ions corresponding to both
CCL2 homodimer and CCL2-CCL13 heterodimer were observed in the mixed sample, with the latter being less abundant (supplemental Fig. S2). In contrast, heterodimerization was not observed when other CCR2 ligands were mixed with CCL7 or in mixtures of CCL11 with CCL8 or CCL13. For example, the profile of a mixture of CCL2 and CCL7 is shown in Fig. 2. Monomer and dimer CCL2 as well as monomer CCL7 were observed, but no heterodimer was detected.

To provide confirmation of the results of the mass spectrometry and to investigate the structure of the strongest heterodimer, we conducted an NMR chemical shift perturbation experiment by titrating different amounts of unlabeled CCL8 into 15N-labeled CCL2, monitoring the 15N HSQC spectrum of the CCL2 component (Fig. 3A). Changes in chemical shift indicate that the chemical environment of the corresponding amide nitrogens and/or protons are altered due to direct contact with CCL8 or from a conformational change due to CCL8 binding and can thus provide evidence of the dimerization interface. Sixty backbone amide peaks from the CCL2-only spectra were unambiguously assigned (supplemental Table S1). Many of the peaks in the CCL2 spectra showed a significant and systematic change in chemical shift with increasing concentrations of CCL8. Several of the peaks in the homodimer spectrum disappear and reappear in a different but nearby location in the heterodimer spectrum. This indicates that the protein is in slow exchange between two stable conformations, the homodimer of CCL2 and the CCL2-CCL8 heterodimer. Peaks in a second set disappear from the spectra at high concentrations of CCL8 but do not reappear near the original location. These peaks change so drastically that they cannot be assigned in the heterodimer spectrum but likely correspond to several new slowly exchanging peaks that appear in the heterodimer spectra. Although small changes are seen scattered throughout the protein, the peaks that migrate significantly (such as those corresponding to residues 3, 4, and 9) are clustered in or around the N terminus, whereas many of the “vanishing” peaks are located in the N terminus (residues 7 and 10–16) and the last β-strand (residues 51–53). Fig. 3B shows sub-spectra of one representative peak in slow exchange.

Fig. 4 shows a plot of the changes in peak intensities as a function of added CCL8 for four of the slowly exchanging amides that can be confidently assigned in both the
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homodimer and heterodimer spectra. With increasing concentrations of CCL8, the intensity of the homodimer peak decreases, while the intensity of the heterodimer peak increases. The intensities of all four peaks were decreased by 50% between 80 and 110 μM CCL8 (approximately a 1:2 molar ratio of CCL8 to CCL2), indicating that half of the CCL2 monomers are in the homodimeric state and half are in the heterodimeric state. Because there are two CCL2 monomers in the CCL2 homodimer while only one is present in each CCL2-CCL8 heterodimer, the concentration of CCL2 homodimer is half of the heterodimer concentration. At 200 μM CCL8 (equimolar to CCL2), all four peaks indicate that there is at most 25% intensity in the homodimer peak, which would correspond to a one homodimer to six heterodimer ratio. This indicates that the heterodimer is predominant, agreeing well with the observations made by mass spectrometry.

The peaks that shift most significantly in the spectra were identified and mapped onto the CCL2 tetramer structure (Fig. 5 and supplemental Table 1). Fig. 5A shows the CC-type dimer interface observed in the CCL2 and CCL8 homodimer structures, whereas Fig. 5B shows the CXC-type interface observed in CXC family chemokines. As indicated above, many of the affected peaks correspond to residues in the N terminus or in the third β-strand, which abuts the N terminus of CCL2. These residues fall near the CC-chemokine interface. In contrast, almost no major changes are indicated at the CXC interface. This strongly suggests that the CCL2-CCL8 heterodimer has the CC-type conformation.

We subsequently investigated the effect of GAG binding on chemokine heterodimerization. For these studies, we chose the heparin pentasaccharide, Arixtra, because it is homogeneous unlike heparin and other natural GAGs (heparan sulfate, chondroitin sulfate, and others) that are heterogeneous in both size and composition. Homogeneity of the GAG greatly simplifies interpretation of the mass spectrometry data, allowing us to distinguish heterodimer-GAG from homodimer-GAG complexes. Arixtra is nearly identical to a highly sulfated heparin pentasaccharide with seven of eight common sulfations present and one rare sulfation site also charged. In a separate study, we demonstrated that its interaction with chemokines is similar to heparin in the context of FTICR MS assays (20). We chose Arixtra because it is longer than other commercially available homogenous GAG homologues. However, it is important to note that, like any model GAG, Arixtra does not capture the potential diversity of protein-GAG interactions or mimic quantitatively the true in vivo interactions.

Using the mass spectrometry-based filtration trapping assay (9), we observed that Arixtra binds each of the CCR2 ligands. Each possible pair of CCR2 ligands was then mixed with Arix-
observed for the equimolar mixture of these molecules (Fig. 6A). The heterodimer-Arixtra complex was more abundant than the homodimer-Arixtra complex (CCL8-Arixtra). Similarly, although only a low level of CCL2-CCL11 heterodimer was observed in the absence of Arixtra, intense ions corresponding to the heterodimer-GAG complex (CCL2-CCL11-Arixtra) were observed when Arixtra was added (supplemental Fig. S3). These data indicate that binding of Arixtra can significantly drive heterodimer formation (Fig. 7).

Strong ions of heterodimer in complex with Arixtra (CCL2-CCL8-Arixtra) as well as pure CCL2-CCL8 heterodimers were also observed when an equimolar mixture of CCL2, CCL8, and Arixtra was analyzed (Fig. 8). The degree of heterodimerization in the presence of Arixtra is similar to that in the absence of the pentasaccharide. This result indicates that the heterodimer of CCL2-CCL8 has a greater or equal affinity toward Arixtra compared with the homodimer (see Figs. 1B and 8).

Heterodimerization was clearly observed for CCL2-CCL13 and CCL8-CCL13 in the presence of Arixtra, and their relative abundances were compared with those in the absence of GAG (supplemental Figs. S4 and S5). Heterodimerization increased only moderately for CCL8-CCL13 in the presence of Arixtra, and no significant change was observed for CCL2-CCL13.

Results for all combinations of the chemokines in the presence and absence of Arixtra are compiled in Fig. 7.

**DISCUSSION**

As the complexity of chemokine interactions emerge, it is becoming increasingly apparent that understanding their **in vivo** functions represents a significant challenge (33, 34). Chemokines recruit cells by binding to seven-transmembrane G-protein-coupled receptors; however, many chemokine receptors can respond to several different ligands, and many of the ligands signal through more than one receptor. This apparent redundancy may be broken by many regulatory mechanisms that combine to give each chemokine a unique and specific function. It appears that one level of regulation involves the formation of higher order oligomers, an observation supported by the loss of **in vivo** function of monomeric mutants of CCL5, CCL4, and CCL2 (5). It has also been shown that loss of GAG-binding activity abrogates chemokine function **in vivo**, indicating that the presence of GAGs in some way regulates the chemokine network (5). Furthermore, there is evidence that GAG binding and oligomerization are functionally coupled (15, 17, 18, 35, 36). Now it appears that, in addition to these other factors, hetero-oligomerization may also play a role in differentiating between and modulating the activity of specific chemokines.
Chemokine heterodimerization has been observed for CCL3-CCL4, CXCL4-CXCL8, CCL21-CXCL13, and CXCR4-CCL5 (12, 14, 37, 38). In the current study, heterodimerization of several chemokines, which are redundant in that they bind to CCR2, was characterized using ESI-FTICR MS and NMR. The results show that some pairs of CCR2 ligands form heterodimers to varying degrees. Strong heterodimer formation was observed between CCL2 and CCL8, and the corresponding homodimers, it too is suggestive of functional relevance. By comparison, CCL2 and CCL8 have the most identical sequences in their N termini, which may provide some clues. A crystal structure of a CCL5 dimer in complex with heparin disaccharides revealed a pronounced movement of the N-terminal region of one of the monomers when compared with the apoprotein structure (40). The repositioned N-terminal region interacts differently with the other CCL5 monomer, creating two new hydrogen bonds that are thought to stabilize the dimer. Similarly, involvement of the N terminus was also demonstrated in a separate study of CCL4 with disaccharides. In this case, NMR experiments indicated that in addition to the residues constituting the primary GAG-binding site, amino acids at the N terminus of the protein were also perturbed by the presence of disaccharides in the context of the dimer but not the monomer (41). Such chemical shift perturbations can be caused by a direct interaction, or by a conformational change, either of which could be stabilizing. Interestingly, in the context of the dimer, the N-terminal residues are located in close proximity to the primary GAG binding determinants of the other monomer, potentially extending the GAG binding cleft or indirectly stabilizing the dimer by inducing additional contacts between the monomer N termini (41). Similar mechanisms could explain the stabilizing effect of Arixtra on CCL2-CCL11 and CCL8-CCL11, but confirmation requires further structural investigation.

Here we have presented evidence for the occurrence of heterodimers among the CCR2 ligands and regulation of heterodimerization by GAGs. Further study is needed to determine if these heterodimerizing pairs of chemokines actually exhibit functional differences when compared with the individual heterodimer complex with one bound pentasaccharide was observed when Arixtra was added. Similarly, the heterodimerization of CCL11 and CCL2 was significantly enhanced in the presence of Arixtra. The interaction with Arixtra is not simply a matter of a highly basic protein binding to a highly anionic molecule, because cycloedextrin sulfate, which has 14 sulfates, does not bind CCL2 (20). An R18A/K19A/R24A variant of CCL2, which has mutations of three functionally important heparin-binding residues but retains a basic pI, does not bind heparin octasaccharides (19) or Arixtra (data not shown), indicating specificity.

At this point we can only speculate on the structural or thermodynamic reasons for the heterodimer stabilization by Arixtra, because the primary GAG-binding site on CCL2 is distal to the dimerization interface, and GAG-binding sites for CCL7, CCL11, and CCL13 have not been determined biochemically (15). Furthermore, although we do not have a refined crystal structure, we have observed electron density for Arixtra near this GAG-binding region in co-crystals of CCL8-Arixtra.6 Nevertheless, two studies of CCL4 and CCL5 in the presence of disaccharides may provide some clues. A crystal structure of a CCL5 dimer in complex with heparin disaccharides revealed a pronounced movement of the N-terminal region of one of the monomers when compared with the apoprotein structure (40). The repositioned N-terminal region interacts differently with the other CCL5 monomer, creating two new hydrogen bonds that are thought to stabilize the dimer. Similarly, involvement of the N terminus was also demonstrated in a separate study of CCL4 with disaccharides. In this case, NMR experiments indicated that in addition to the residues constituting the primary GAG-binding site, amino acids at the N terminus of the protein were also perturbed by the presence of disaccharides in the context of the dimer but not the monomer (41). Such chemical shift perturbations can be caused by a direct interaction, or by a conformational change, either of which could be stabilizing. Interestingly, in the context of the dimer, the N-terminal residues are located in close proximity to the primary GAG binding determinants of the other monomer, potentially extending the GAG binding cleft or indirectly stabilizing the dimer by inducing additional contacts between the monomer N termini (41). Similar mechanisms could explain the stabilizing effect of Arixtra on CCL2-CCL11 and CCL8-CCL11, but confirmation requires further structural investigation.

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ual chemokines, as has been shown for other heterodimeric mixtures in vitro (12–14, 38). These functional effects could include cooperative, antagonistic, synergistic, or completely new activities of chemokine-stimulated cells. For example, as an antagonist of CCR2, CCL11 could block the action of CCL2 both by binding to the receptor and by sequestering CCL2 in nonfunctional heterodimers. However, this is mere speculation; many possible scenarios can be envisioned, and they all will require careful investigation. Such studies will not be trivial because the significance of these interactions may be difficult to demonstrate with in vitro experiments, as was the case for proving the functional role of GAG binding and oligomerization of chemokines (5).

At this stage, we can at least consider the requirements for heterodimer formation. In order for it to be an important factor in chemokine regulation, there must be the potential for heterodimers to form in vivo. In other words, two chemokines must overlap in spatial and temporal space. This condition is supported by many reports in the literature (3). Many studies that have identified chemokine genes induced by immunological perturbations of cells or tissues have found evidence for the up-regulation of more than one of these chemokines. In particular, CCL2 and CCL8 are co-induced in certain cultured cells upon activation with cytokines and in disease models such as murine asthma and multiple sclerosis (42–44). CCL2 and CCL8 have also been shown to be regulated so that their expression levels coincide (45). Similar studies can be found for all the CCR2 ligand pairs in this study (42, 46–52).

Another condition for functional relevance requires that the chemokines accumulate at sufficiently high concentrations for their equilibrium distribution to include significant heterodimer. This condition is also supported by several lines of evidence. The NMR data presented here indicate that the CCL2-CCL8 heterodimer forms in preference to the homodimers at equimolar concentrations. Because a monomeric variant of CCL2 is inactive in vivo (5), CCL2 is likely to form dimers at some point during its functional lifetime. This may be facilitated by chemokine binding to GAGs on cell surfaces, concentrating the chemokine due to the GAG interaction and surface effects, and shifting the equilibrium toward dimer and higher order oligomers (17, 53). Therefore, it seems likely that mixtures of CCL2 and CCL8 would also be able to reach concentrations at which heterodimer would form. However, the in vivo state of chemokines is not well understood, and further study is needed to determine whether CCL2-CCL8 heterodimers actually do occur in vivo as has been observed for CCL3-CCL4 in cultured supernatants and lysates (37). Further, experiments using more biologically relevant GAGs such as heparan and chondroitin sulfate should be conducted to better understand what effects they have on heterodimerization and whether different GAG sequences have unique activities. If heterodimerization is functionally relevant, another question is that of mechanism. For other reports of heterodimer formation, there is a correlation between heterodimer formation and functional synergy between pairs of chemokines (12, 14, 38). However, these observations do not prove that the functional consequences are directly due to receptor activation by the heterodimer. In fact, the current model of the signaling complex of chemokines with receptors is that a ligand monomer binds to a receptor, at least with respect to migration. This has been demonstrated with monomeric variants of chemokines that bind and activate receptors equally well as their oligomerizing counterparts in migration experiments in vitro (24, 54, 55). Furthermore, at the interface of these CC dimers are amino acids known to be important for receptor binding and signaling (55, 56); these are exposed in the monomer but masked in the context of a dimer and unavailable for receptor engagement. These data argue against a direct interaction of a C.C-type heterodimer with the receptor and raise the question of how the heterodimer might function. In this regard, it is possible that the heterodimer state regulates the pre-binding concentration and availability of the chemokine, which may also involve changes in the binding affinity and/or selectivity of the chemokines to GAGs. Another is that the two chemokines act independently at the cell surface, while their signals interact downstream through integration and/or cross-talk to produce novel effects inside the target cells. On the other hand, in contrast to the monomer–binds-receptor paradigm, it remains entirely possible that the heterodimer binds directly to the receptor via a different binding surface than the monomers, thereby producing alternative signals.

In summary, although there is a fair amount of information regarding the response of a single receptor to a given chemokine in a particular cell type, much less is known about the ramifications of stimulation by more than one. Nevertheless, it is becoming very clear that cells migrate through environments that have complex profiles of not only chemokines but also cytokines and GAGs. Thus, experiments that aim to investigate the interplay of molecules or molecular complexes that act at the cell surface, and the subsequent downstream signals that they produce, will contribute to a better understanding of how complex immunological processes are triggered outside, and integrated inside, the cell. Our phenomenological observations of CCR2 ligand heterodimers and the influence of GAGs on their stability represent a first step toward these goals.

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