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A RAPID AND EFFICIENT WAY TO OBTAIN MODIFIED CHEMOKINES FOR FUNCTIONAL AND BIOPHYSICAL STUDIES

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Abstract

Chemokines and their receptors control cell migration associated with routine immune surveillance, inflammation and development. They are also implicated in a large number of inflammatory diseases, cancer and HIV. Here we describe a rapid and efficient way to express and purify milligram quantities of multiple chemokine ligands (CCL7/MCP-3, CCL14/HCC-1, CCL3/MIP-1 α and CXCL8/IL-8) containing C-terminal modifications to enable coupling to fluorescent dyes or small molecules such as biotin, *in vitro*. These labeled chemokines display wild-type behavior in both receptor binding and calcium mobilization assays.

The ability to rapidly and inexpensively produce labeled chemokines opens the way for their use in many applications, including non-traditional chemokine-receptor interaction studies, both on intact cells and with purified receptor reconstituted in artificial membranes *in vitro*. Furthermore, the ability to immobilize chemokines to obtain ligand affinity columns aids in efforts to purify chemokine receptors for structural and biophysical studies, by facilitating the separation of functional proteins from their non-functional counterparts.

Keywords

Fluorescent chemokine; chemokine biotinylation; chemokine affinity columns; chemokine binding assays; chemokine expression

1. Introduction

Chemokines (*chemotactic cytokines*) represent a large family of signaling molecules that are produced by a variety of cells in response to signals associated with host defense and wound repair. They play a pivotal role in the immune system, controlling migration and activation of leukocytes, by binding to and signaling through seven transmembrane G-protein coupled receptors (GPCRs) on the surface of leukocytes [1, 2]. However, chemokines and their receptors are often likened to double-edged swords because they are also associated with a

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number of pathologies and are essential cofactors during viral entry of HIV into host cells [3-5]. For these reasons, many laboratories are interested in the development of tools to facilitate chemokine:receptor studies.

Studies of chemokine:receptor binding in intact cells or membrane fragments have been traditionally conducted using ^{125}I -radiolabeled chemokines. However, this method has a number of drawbacks, including changes in receptor binding affinity caused by iodination of the chemokine, and the necessity of custom labeling if a particular chemokine of interest is not commercially available. Also, the assays are time-consuming and require multiple wash steps to remove unbound chemokine. More recently, homogeneous assays such as the Scintillation Proximity Assay (SPA) (PerkinElmer) have become popular, particularly in the pharmaceutical industry, as they can be easily miniaturized and require no wash steps. However, they still use radiolabeled chemokines that may need to be custom-labeled, and require safe handling procedures and hazardous waste disposal.

Recently, it has become possible to express, purify and solubilize GPCRs, including chemokine receptors at sufficient levels for structural and biophysical studies [6-11]. Progress in this area recently culminated in the first high resolution crystal structures of a chemokine receptor; those of CXCR4 in complex with small molecule and peptide antagonists [7]. Nevertheless, there are few reports describing chemokine binding to their receptors in solution and due to the lack of alternative approaches, radiolabeled ligands (e.g. ^{125}I -chemokine and ^{35}S -GTP- γS) have been used for this purpose too. Expression and purification of non-radioactively labeled chemokines has been described previously [12-14], though sporadically, and for only one or two ligands. Chemical synthesis of fluorescently labeled chemokines is also possible [15], but is expensive, making the use of these ligands impractical for large-scale studies.

Here we describe methods to systematically express and purify milligram quantities of four different functional, derivatized chemokines for use in a range of assays. To date, we have successfully obtained fluorescent and/or biotinylated versions of CCL7, CCL14, CCL3 and CXCL8, but we believe that this method is also valid for many other chemokines. The applications tested in this study include non-traditional radioligand assays, in which biotinylated chemokines are used in conjunction with ^{125}I -labeled streptavidin, circumventing the need for radiolabeled chemokines and problems associated with reduced binding affinity of the radiolabeled probe. In addition, we describe the use of biotinylated chemokines for use as ligand affinity columns, and the production of fluorescently labeled chemokines for solution-based fluorescence anisotropy-based binding assays. The ability to readily produce these chemokine reagents should also aid in many other types of studies including fluorescence resonance energy transfer-based studies, drug discovery efforts, and array based screening of interacting partners such as chemokine:glycosaminoglycan interactions [16].

2. Materials and methods

2.1 Materials

Fluorescein-5-maleimide and Alexa Fluor 647 C2-maleimide were obtained from Invitrogen (Carlsbad, CA). *E. coli* codon optimized variants of full-length CCL3, CCL7, and CXCL8, along with the functional form of CCL14 containing residues 9-74 [17] were purchased from Genscript (Piscataway, NJ). The pHUE vector containing an N-terminal His tag and ubiquitin (ub), along with pET15b containing the catalytic core of the deubiquitylating enzyme Usp2 (Usp2-cc) were kind gifts from Rohan Baker (JCSMR, The Australian National University, ACT0200, Canberra, Australia). A variant of ubiquitin in which three destabilizing mutations were introduced (I3L, V17L, I23V) [18] was cloned into pET27

(EMD Chemicals, Gibbstown, NJ) in-house to obtain pHUE3D3 (Douglas *et al*, manuscript in preparation). ^{125}I -labeled CCL3 and streptavidin were purchased from Perkin Elmer (Waltham, MA), and anti-CCR1 antibody was obtained from Thermo Fisher Scientific (Rockford, IL).

2.2 Cloning and expression of chemokines as pHUE- and pHUE3D3-chemokine fusions

Chemokines were cloned onto the 3' end of ubiquitin in pHUE [19] (CCL7, CCL14) or ubiquitin-3D3 in pHUE3D3 (Douglas *et al*, in preparation) (CCL3, CXCL8) using SacII and HindIII restriction sites at the chemokine N and C-termini respectively. For derivatization, DNA coding for a cysteine residue or an Avitag sequence (KLGSGGLNDIFEAKIEWHE) was also added to the C-terminus of the chemokine. After verification by sequencing, pHUE or pHUE3D3-based constructs were transformed into BL21(DE3)pLysS cells (Novagen, Madison, WI). For pHUE constructs, cells were grown in Luria Broth in the presence of carbenicillin (200 $\mu\text{g}/\text{mL}$) and chloramphenicol (34 $\mu\text{g}/\text{mL}$) at 30°C. For pHUE3D3 constructs, cells were grown in Luria Broth in the presence of kanamycin (40 $\mu\text{g}/\text{mL}$) and chloramphenicol (34 $\mu\text{g}/\text{mL}$) at 37°C. In all cases, expression of the ubiquitin-chemokine fusions was induced using 0.5mM IPTG at $\text{OD}_{600\text{nm}} \sim 0.6$. After induction, cells were grown for 4 hours before harvesting.

2.3 Chemokine purification and derivatization

Cell pellets containing ubiquitin-chemokine fusions were resuspended in buffer A (50mM HEPES, pH7.4, 0.5M NaCl, 40mM imidazole, protease inhibitor tablets (no EDTA) (Roche, Indianapolis, IN)). 5mM MgCl_2 , DNase I (Roche) and 0.1% Tween-20 (Sigma, St. Louis, MO) were added and cells were disrupted using an Emulsiflex (Avestin, Ottawa, Canada) (2 runs, 12,000 psi). For soluble chemokines expressed using pHUE, insoluble material was removed by centrifugation (48,000g, 45 minutes, 4°C) and the supernatant was loaded onto a Ni-sepharose column (GE Life Sciences, Piscataway, NJ). Ub-chemokine fusion proteins were eluted using a linear gradient from 40mM to 500mM imidazole. CCL3 expressed predominantly insolubly as a pHUE3D3 fusion, so was purified and refolded from inclusion bodies before being loading onto a Ni-affinity column as described above.

Inclusion body preparation and refolding of CCL3 was undertaken according to a modified version of Zabel *et al*. [20]. Briefly, cell pellets were detergent-solubilized by three rounds of homogenization and centrifugation (40,000g, 45 mins) in the presence of 0.25% (w/v) sodium deoxycholate. The insoluble inclusion body pellet was solubilized in a denaturing buffer (6M guanidine HCl, 0.1M sodium phosphate, 10mM Tris, pH8.0) and loaded onto a nickel-nitrilotriacetic acid (Ni-NTA) column (Qiagen, Valencia, CA). CCL3 was eluted by decreasing the pH to 4, and refolded by rapid dilution into refolding buffer (55mM MES, pH6.5, 264mM NaCl, 11mM KCl, 0.055% (w/v) PEG3350, 1.1mM EDTA, 550mM L-arginine, 0.3mM n-dodecyl maltoside, 1mM reduced L-glutathione, 0.1mM oxidized L-glutathione), at a final CCL3 concentration of 0.1mg/mL. After stirring overnight at 4°C, refolded CCL3 was dialyzed into buffer A before loading onto a Ni-affinity column. After Ni-affinity chromatography, ubiquitin fusion proteins were either directly cleaved using the Usp2-cc (see below), or further purified by loading onto a C18 reversed-phase liquid chromatography column (Grace Vydac, Deerfield, IL) in 0.1% trifluoroacetic acid (TFA)/25% acetonitrile and eluted using an increasing gradient of acetonitrile. In the latter case, chemokines were lyophilized and stored as fusion proteins.

For deubiquitylation by Usp2-cc, samples were resuspended to approximately 1mg/mL in a buffer containing 20mM Tris, pH8.0, 0.2M NaCl. Usp2-cc, prepared as described in Catanzariti *et al*. [19], was added at a molar ratio of 50:1 chemokine:Usp2-cc, and samples were placed at room temperature for 2 hours before loading onto a Ni-NTA column

(Qiagen) in 50mM Tris, pH7.5, 40mM imidazole, 500mM NaCl. Ni-NTA was used at this stage instead of Ni-sepharose, due to the tendency of untagged chemokines to bind to Ni-sepharose resin (data not shown). Column flow-through samples containing chemokine were quickly pooled and derivatized by addition of fluorophore or BirA enzyme as described below. For fluorescent labeling, chemokines containing a C-terminal cysteine were mixed with a 1:5 molar ratio of chemokine to fluorescein-5-maleimide or Alexa Fluor 647, and incubated in the dark at 4°C overnight. Biotinylation of Avitagged chemokines was undertaken using the BirA enzyme, prepared as previously described [21]. After derivatization, labeled chemokines were further purified using a C18 reversed-phase liquid chromatography column as described above. This step efficiently removed unbound small molecules such as fluorophore and biotin, along with BirA and any remaining underivatized chemokine. After purification, samples were flash-frozen and lyophilized.

The number of moles of biotin incorporated per mole of protein (i.e. % incorporation) was determined using the Pierce Biotin Quantitation Kit (Thermo Fisher Scientific), following manufacturer's instructions. For all labeled samples, identity, purity and % incorporation values were verified using electrospray and MALDI mass spectrometry. Due to dye interference at 280nm, concentrations of fluorescently labeled chemokines were determined by monitoring the absorbance of the dye conjugate at 492nm (fluorescein) or 651nm (Alexa-647). Extinction coefficients of the dyes in the sample buffer were experimentally determined using known concentrations of unconjugated dyes diluted into the same buffer.

2.4 Cell culture and calcium mobilization assays

HEK293 cells expressing CCR1 were cultured as previously described [8]. Calcium flux activity assays were performed using a FLIPR Calcium 4 assay kit (Molecular Devices), using 1.3×10^5 cells per well in a 96-well assay format. Chemokine-dependent increases in cytosolic Ca^{2+} were measured using a FlexStation 3 microplate reader (Molecular Devices).

2.5 Construction of chemokine affinity columns

For each column, 200 μL slurry of high capacity streptavidin agarose resin (Thermo Scientific) was equilibrated in 50mM Tris, pH 8.5, 150mM NaCl. Resin was mixed with 1 mg biotinylated CCL14 or buffer alone in a total volume of 1mL. After 20 minute incubation at 20°C, columns were washed with 40 column volumes of equilibration buffer followed by binding buffer (50mM Tris pH8.5, 150mM NaCl, 10% glycerol, 0.1% (w/v) dodecyl maltoside and 0.01% (w/v) cholesterol hemisuccinate). Detergent-solubilized CCR1, prepared as previously described [8], was loaded onto the CCL14 and control columns by gravity flow, and the column was washed three times with 1mL binding buffer. CCR1 was eluted by washing twice with 1mL binding buffer supplemented with 1M NaCl.

2.6 Radioligand binding assays

Scintillation proximity assay (SPA) binding assays (GE Healthcare, Piscataway, NJ) were conducted essentially as previously described [8, 22, 23]. HEK293 cells stably expressing CCR1 were used for these assays, and untransfected HEK293 cells were used as a control. Assays were undertaken in a 96-well plate format and each data point was assayed in triplicate on a Microbeta plate counter (Perkin Elmer) for 1 min per well. Each well contained 20,000 cells, 0.2mg WGA-PVT-SPA beads, increasing concentrations of unlabeled competitor chemokine, and either 50pM ^{125}I -CCL3 or 100pM ^{125}I -Streptavidin + 400pM CCL3-Biotin, in a total volume of 100 μL . IC_{50} values were obtained by nonlinear regression curve fitting using Prism software (GraphPad, La Jolla, CA).

3. Results and discussion

3.1 Purification of chemokines

E. coli codon-optimized variants of four chemokines (CCL3, CCL7, CCL14, and CXCL8) were cloned into pHUE and pHUE3D3 vectors to produce a fusion protein containing an N-terminal 6-His tag, ubiquitin and the chemokine of interest. Depending upon the application, the chemokine was either native, or modified at the C-terminus by addition of an extra cysteine residue or an Avitag sequence, to permit labeling (Figure 1A). The N-terminal ubiquitin fusion system was developed, as cleavage with Usp2-cc results in the native sequence. This is important because the exact nature of the N-terminal residues of chemokines affects their activity. In contrast, chemokines are generally tolerant to modifications at their C-termini, so this region was chosen for the addition of non-native residues for labeling. Two different versions of ubiquitin were used in this study: wild-type ubiquitin (pHUE vector) was used for expression of CCL7, CCL14 and CXCL8, as these express solubly at high levels and ubiquitin promotes soluble expression. However, a core-repacked mutant of ubiquitin (Ub3D3 in the pHUE3D3 vector) was used for the expression of CCL3, in order to increase the overall yield of this protein by enhancing its expression in inclusion bodies (Douglas *et al.*, in preparation).

An overview of the purification scheme is described in Figure 1B, and representative gels and mass spectrometry data for CCL14 are shown in Figure 2A/B as an example. For the Avitagged chemokine constructs, 6-His-ubiquitin-chemokine-Avitag fusions were purified by Ni-affinity chromatography, the chemokines were cleaved from 6-His-ubiquitin using Usp2-cc, derivatized as required and loaded onto Ni-NTA resin prior to a final reverse phase chromatography and lyophilization. As ubiquitin elutes from a C18 reverse phase chromatography column at a similar acetonitrile concentration as many chemokines, the addition of a Ni-NTA column served to remove His-tagged ubiquitin, and increase the purity of the final product. This procedure was modified slightly for chemokines containing C-terminal cysteine residues such that an additional Reversed Phase HPLC column was inserted into the method prior to cleavage and derivatization. This optional additional step allowed chemokines to be stored in an underivatized state with minimal aggregation, as described in section 3.2. As shown in Figure 2 for CCL14, cleavage of ubiquitin-chemokine fusion is >90% efficient (Figure 2A, lane 1), and the column efficiently separates chemokine that flows through the column (Figure 2A, lanes 2 and 3) from His-tagged ubiquitin that efficiently binds to the resin (Figure 2A, lane 4). After purification, mass spectrometry confirmed the correct mass of the purified, derivatized proteins, and confirmed that in each case, little or no underivatized protein remained in the final sample (Figure 2B, Table 1). Representative chromatography traces are shown in the supplementary material (Figure S1).

3.2 Fluorescent labeling of CCL7 and CCL14

Fluorescently labeled chemokines are attractive as they can be used to easily determine dissociation constants for the binding of chemokines to solubilized chemokine receptors [8]. In order to obtain fluorescently labeled chemokines, the proteins were expressed with a non-native C-terminal cysteine, and maleimide chemistry was used to couple fluorescent labels at this position after purification.

During preliminary purifications of CCL7-cys, we attempted to cleave ubiquitin early in the purification and store the protein as lyophilized underivatized protein, as was customary for wild-type CCL7. However, after cleavage and storage the efficiency of derivatization was very low indicating that the reactive cysteine was inaccessible. Electrospray mass spectrometry confirmed that chemokine dimers had formed, as the major mass observed was 18108.5 (expected mass of dimer = 18109.0). This dimer was presumably facilitated by the

formation of an additional time-dependent disulfide bond between the non-native C-terminal cysteine residues.

Chemokines contain multiple disulfide bonds (CCL3, CCL7, CCL14 and CXCL8 each contain two) and attempts to selectively reduce the non-native disulfide bond with reasonable efficiency failed. However, in small-scale purification studies the CCL7 fusion protein was monomeric until the ubiquitin was cleaved, based on SDS-PAGE gels run under non-reducing conditions. Therefore, we decided to purify the chemokine as a fusion, and remove ubiquitin as a last step, just prior to derivatization. This method was successful for both chemokines tested, and the yields of purified CCL7 and CCL14 prior to derivatization were approximately 5mg/L and 1mg/L cells respectively. How ubiquitin is able to prevent dimerization of chemokines is unclear without the existence of a high-resolution structure, but is likely due to steric hindrance. The recovery of pure, labeled CCL7 and CCL14 was 2.5mg/L and 0.5mg/L respectively (Table 1).

In a previous publication, we showed that these fluorescently tagged chemokines displayed activity indistinguishable from native chemokines on cell membranes [8]. Figure 2C shows representative calcium flux data generated by exposing HEK293s cells stably expressing CCR1 to a dilution series of CCL14-fluorescein. The EC₅₀ for the dose response curve was 2nM, similar to that (2.8nM) previously published for unlabeled CCL14 [17]. We also previously demonstrated the use the fluorescently labeled chemokines in fluorescence polarization studies to obtain dissociation constants for chemokine binding to solubilized chemokine receptors [8].

3.3 Biotinylation of CCL3, CCL14 and CXCL8

The addition of an Avitag, and biotinylation with the BirA enzyme is a well characterized method for producing protein that is biotinylated at a single, specific lysine residue [21]. Biotin has been used as a molecular handle in numerous published studies, but the ability to biotinylate chemokines is especially attractive because chemokine ligand affinity columns can be used to separate functional chemokine receptors from non-functional chemokine receptors during receptor purification. Three chemokines (CCL3, CCL14 and CXCL8) were tested in this study, and all three could be purified and biotinylated with an efficiency of >95%. In all three cases, the final yield of biotinylated chemokine was at least 0.5mg/L, and masses were as expected according to electrospray mass spectrometry (Table 1, columns 4 and 5). To demonstrate the utility of this approach, CCR1 solubilized in detergent micelles [8] was loaded onto a column containing biotinylated CCL14 immobilized on streptavidin-agarose resin. Figure 3 shows a representative anti-CCR1 western blot of column samples from this experiment. The CCR1 sample likely contains a mixture of functional and non-functional CCR1, as illustrated by the presence of CCR1 in both the flow-through and the elution fractions of the chemokine column. A control column without immobilized CCL14 did not bind CCR1 (data not shown).

Another useful application of biotinylated chemokines is in non-conventional radioligand binding assays. Radioligand assays are accepted as the gold-standard for determining binding affinities between chemokines and their receptors, and as such are desired for chemokine receptor studies. The drawback of this approach is that although many common chemokines can be purchased as ¹²⁵I versions, some chemokines need to be custom labeled, and it is generally accepted that iodination can affect receptor binding affinities. However, it is possible to purchase ¹²⁵I-labeled streptavidin, and to use it in conjunction with biotinylated chemokine as a surrogate for labeled chemokine in binding assays.

In order to test the use of ¹²⁵I-streptavidin as a surrogate radiolabel in a chemokine SPA assay, we undertook competition assays using membranes containing the chemokine

receptor CCR1. CCL3 was used for these assays because ^{125}I -CCL3 is commercially available, allowing for a direct comparison. After immobilization of membranes to SPA beads, either radiolabeled chemokine, or a combination of biotinylated chemokine and ^{125}I -labeled streptavidin was added.

Figure 4 shows a representative SPA assay for CCR1, and demonstrates that unlabeled CCL14 competes with ^{125}I -CCL3 or a combination of CCL3-biotin/ ^{125}I -streptavidin for binding to HEK293 membranes containing CCR1 with a similar affinity (IC_{50} = 2.6nM versus 0.9nM for displacement of ^{125}I -CCL3 or CCL3-biotin/ ^{125}I - streptavidin respectively). In contrast, no binding of CCL3 or competition with CCL14 was observed when CCR1-expressing membranes were replaced with untransfected HEK293 membranes. In addition, the ^{125}I -Streptavidin signal was CCL3-biotin dependent and while the highest signal was observed with a 4:1 ratio of chemokine to streptavidin, avidity effects due to the tetrameric state of streptavidin were not observed. These data indicate that the assay is a valid surrogate for traditional radioligand assays, and opens the door to radioligand assays using any chemokine, while only purchasing a single radiolabeled species (^{125}I -streptavidin). These assays may be further improved with appropriately engineered versions of monomeric streptavidin [24].

In conclusion, we show that it is possible to efficiently and inexpensively produce milligram quantities of labeled chemokines that can be used in many cell-based, biochemical and biophysical assays. These assays include fluorescence-based binding assays, radioligand binding assays, and the generation of chemokine affinity columns. In addition to traditional assays, our laboratory is particularly interested in structure-function studies of chemokine receptors, and the use of labeled chemokines to guide these studies has many advantages. Specifically, easily separating functional chemokine receptors from non-functional counterparts, and conducting binding studies in solution enables us to identify detergents/lipids that retain receptors in a chemokine-binding competent state [8]. Given the breakthroughs in expression and purification of GPCRs at high level (Rev. in [25]), and the ability to determine GPCR structures [26] including that of the chemokine receptor CXCR4 [7], it is anticipated that the flood gates of biophysical studies of chemokine receptors will open, where such reagents will prove extremely useful.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Moser B, Willmann K. Chemokines: role in inflammation and immune surveillance. *Ann Rheum Dis.* 2004; 63(2):ii84–ii89. [PubMed: 15479880]
2. Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol.* 2000; 18:217–42. [PubMed: 10837058]
3. Gerard C, Rollins BJ. Chemokines and disease. *Nat Immunol.* 2001; 2:108–15. [PubMed: 11175802]

4. Lusso P. HIV and the chemokine system: 10 years later. *EMBO J.* 2006; 25:447–56. [PubMed: 16437164]
5. O'Hayre M, Salanga CL, Handel TM, Allen SJ. Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment. *Biochem J.* 2008; 409:635–49. [PubMed: 18177271]
6. Park SH, Prytulla S, De Angelis AA, Brown JM, Kiefer H, Opella SJ. High-resolution NMR spectroscopy of a GPCR in aligned bicelles. *J Am Chem Soc.* 2006; 128:7402–3. [PubMed: 16756269]
7. Wu B, Chien EY, Mol CD, Fenalti G, Liu W, Katritch V, Abagyan R, Brooun A, Wells P, Bi FC, Hamel DJ, Kuhn P, Handel TM, Cherezov V, Stevens RC. Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science.* 2010; 330:1066–71. [PubMed: 20929726]
8. Allen SJ, Ribeiro S, Horuk R, Handel TM. Expression, purification and in vitro functional reconstitution of the chemokine receptor CCR1. *Protein Expr Purif.* 2009; 66:73–81. [PubMed: 19275940]
9. Blackburn PE, Simpson CV, Nibbs RJ, O'Hara M, Booth R, Poulos J, Isaacs NW, Graham GJ. Purification and biochemical characterization of the D6 chemokine receptor. *Biochem J.* 2004; 379:263–72. [PubMed: 14723600]
10. Mirzabekov T, Bannert N, Farzan M, Hofmann W, Kolchinsky P, Wu L, Wyatt R, Sodroski J. Enhanced expression, native purification, and characterization of CCR5, a principal HIV-1 coreceptor. *J Biol Chem.* 1999; 274:28745–50. [PubMed: 10497246]
11. Nisius L, Rogowski M, Vangelista L, Grzesiek S. Large-scale expression and purification of the major HIV-1 coreceptor CCR5 and characterization of its interaction with RANTES. *Protein Expr Purif.* 2008; 61:155–62. [PubMed: 18588983]
12. Inglese J, Samama P, Patel S, Burbaum J, Stroke IL, Appell KC. Chemokine receptor-ligand interactions measured using time-resolved fluorescence. *Biochemistry.* 1998; 37:2372–7. [PubMed: 9485384]
13. Datta-Mannan A, Stone MJ. Chemokine-binding specificity of soluble chemokine-receptor analogues: identification of interacting elements by chimera complementation. *Biochemistry.* 2004; 43:14602–11. [PubMed: 15544331]
14. Zoffmann S, Turcatti G, Galzi J, Dahl M, Chollet A. Synthesis and characterization of fluorescent and photoactivatable MIP-1 α ligands and interactions with chemokine receptors CCR1 and CCR5. *J Med Chem.* 2001; 44:215–22. [PubMed: 11170631]
15. Strong AE, Thierry AC, Cousin P, Moulon C, Demotz S. Synthetic chemokines directly labeled with a fluorescent dye as tools for studying chemokine and chemokine receptor interactions. *Eur Cytokine Netw.* 2006; 17:49–59. [PubMed: 16613763]
16. de Paz JL, Seeberger PH. Deciphering the glycosaminoglycan code with the help of microarrays. *Mol Biosyst.* 2008; 4:707–11. [PubMed: 18563243]
17. Detheux M, Standker L, Vakili J, Munch J, Forssmann U, Adermann K, Pohlmann S, Vassart G, Kirchhoff F, Parmentier M, Forssmann WG. Natural proteolytic processing of hemofiltrate CC chemokine 1 generates a potent CC chemokine receptor (CCR)1 and CCR5 agonist with anti-HIV properties. *J Exp Med.* 2000; 192:1501–8. [PubMed: 11085751]
18. Lazar GA, Desjarlais JR, Handel TM. De novo design of the hydrophobic core of ubiquitin. *Protein Sci.* 1997; 6:1167–78. [PubMed: 9194177]
19. Catanzariti AM, Soboleva TA, Jans DA, Board PG, Baker RT. An efficient system for high-level expression and easy purification of authentic recombinant proteins. *Protein Sci.* 2004; 13:1331–9. [PubMed: 15096636]
20. Zabel BA, Allen SJ, Kulig P, Allen JA, Cichy J, Handel TM, Butcher EC. Chemerin activation by serine proteases of the coagulation, fibrinolytic, and inflammatory cascades. *J Biol Chem.* 2005; 280:34661–6. [PubMed: 16096270]
21. O'Callaghan CA, Byford MF, Wyer JR, Willcox BE, Jakobsen BK, McMichael AJ, Bell JI. BirA enzyme: production and application in the study of membrane receptor-ligand interactions by site-specific biotinylation. *Anal Biochem.* 1999; 266:9–15. [PubMed: 9887208]

22. Hesselgesser J, Ng HP, Liang M, Zheng W, May K, Bauman JG, Monahan S, Islam I, Wei GP, Ghannam A, Taub DD, Rosser M, Snider RM, Morrissey MM, Perez HD, Horuk R. Identification and characterization of small molecule functional antagonists of the CCR1 chemokine receptor. *J Biol Chem.* 1998; 273:15687–92. [PubMed: 9624164]
23. Vaidehi N, Schlyer S, Trabanino RJ, Floriano WB, Abrol R, Sharma S, Kochanny M, Koovakat S, Dunning L, Liang M, Fox JM, de Mendonca FL, Pease JE, Goddard WA 3rd, Horuk R. Predictions of CCR1 chemokine receptor structure and BX 471 antagonist binding followed by experimental validation. *J Biol Chem.* 2006; 281:27613–20. [PubMed: 16837468]
24. Wu SC, Wong SL. Engineering soluble monomeric streptavidin with reversible biotin binding capability. *J Biol Chem.* 2005; 280:23225–31. [PubMed: 15840576]
25. Chiu ML, Tsang C, Grihalde N, MacWilliams MP. Over-expression, solubilization, and purification of G protein-coupled receptors for structural biology. *Comb Chem High Throughput Screen.* 2008; 11:439–62. [PubMed: 18673272]
26. Hanson MA, Stevens RC. Discovery of new GPCR biology: one receptor structure at a time. *Structure.* 2009; 17:8–14. [PubMed: 19141277]

HIGHLIGHTS

- Method to obtain milligram quantities of chemokines with C-terminal modifications.
- We couple fluorescent dyes or biotin to chemokines *in vitro*.
- Labeled chemokines show wild-type function.
- Chemokines can be used in a variety of biochemical and biophysical studies.

Figure 1. Schematic and purification scheme for chemokines

A) Schematic of chemokine constructs used in this study. B) The purification scheme for obtaining pure, labeled chemokines.

Figure 2. Purification and properties of fluorescently labeled CCL14

A) Coomassie-stained SDS-PAGE gel, B) representative electrospray mass spectrometry trace, and C) calcium-mobilization of CCR1-expressing HEK293 cells, to illustrate the purification, cleavage, derivatization and function of fluorescently labeled CCL14. A) Lane 1: ubiquitin and CCL14-cys after Usp2-cc treatment (sample loaded onto Ni-NTA column), Lanes 2 and 3: Ni-NTA flow-through, Lane 4: Ni-NTA imidazole elution.

Figure 3. CCR1 binds to a chemokine-affinity column

Detergent-solubilized CCR1 was loaded onto a streptavidin column preloaded with biotinylated CCL14. Samples of the column load, flow-through, final wash and column elutions (eluate 1 and eluate 2) were subjected to SDS-PAGE and western blotting analysis using an anti-CCR1 antibody.

	Membranes	Ligand(s)	IC ₅₀ (nM)	SEM (nM)
◆	Con	¹²⁵ I CCL3	-	-
■	CCR1	¹²⁵ I CCL3	2.61	1.12
●	CCR1	CCL3-biotin ¹²⁵ I-streptavidin	0.90	1.38

Figure 4. SPA competition assays

HEK293 membranes, either untransfected (Con) or expressing CCR1 (CCR1) were incubated with increasing concentrations of CCL14, in the presence of either ¹²⁵I-labeled CCL3 (shown as squares or diamonds), or a combination of CCL3-biotin and ¹²⁵I-labeled streptavidin (shown as circles).

Table 1

Summary of masses of labeled chemokines, and final yields after derivatization

Chemokine	Mass of derivatized protein by electrospray mass spectrometry (Da)			Yield of derivatized chemokine (mg/L)
	+ Fluorescein	+ Alexa Fluor 647	+ Biotin	
CCL7/MCP-3	9482.2 (Expected: 9482.3)	10038.3	nd	2.5
CCL14/HCC-1	8328 (Expected: 8326.9)	8881	10220 (Expected: 10223.3)	0.5
CCL3/MIP-1 α	nd	nd	10138 (Expected: 10135.3)	4.0
CXCL8/IL-8	nd	nd	10805 (Expected: 10804.5)	5.0