Synthetic chemokines directly labeled with a fluorescent dye as tools for studying chemokine and chemokine receptor interactions

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ABSTRACT. Chemokines constitute a protein family that exhibit a variety of biological activities involved in normal and pathological physiological processes. CCL11 (eotaxin), CCL19 (MIP-3β), CCL22 (MDC), CXCL11 (I-TAC) and CXCL12 (SDF-1α) chemokines, modified with the Alexa Fluor® 647 fluorescent dye at specific positions along their sequence, were produced by a chemical route and their biological activities were characterized. In a migration assay, fluorescent chemokines were as biologically active as the unmodified forms. All labeled chemokines specifically stained cell lines transfected with the appropriate human chemokine receptors. The specificity of binding was further established by showing that the unlabeled ligands efficiently competed with the labeled chemokines for binding to their respective receptor. A low molecular weight antagonist of CXCR4 prevented binding of labeled CXCL12 to CXCR4 comparably to a neutralizing anti-CXCR4 antibody. Finally, labeled CCL19 was used for the staining of primary cells, illustrating that this reagent can be used for studying CCR7 expression on different cell types. Together, these results demonstrate that fluorescent synthetic chemokines constitute promising ligands for the development of chemokine receptor-binding assays on intact cells, for applications such as cell-based, high throughput screening, and studies of chemokine receptor expression by primary cells.

Keywords: Alexa Fluor® 647, antagonist, flow cytometry, fluorescence, high throughput screening

Chemokines constitute a family of close to forty small, basic proteins that are involved in numerous physiological processes, as varied as embryogenesis and homeostasis of the immune system [1-3]. Most chemokines are secreted proteins of 60 to 130 amino acid residues, with the exception of two chemokines, which are transmembrane proteins. The chemokine family is made up of two major types, the CC and the CXC chemokines. As regards the first type, represented by the CCL1 to CCL28 chemokines, the two N-terminal cysteine residues are adjacent, while in the second type, represented by the CXCL1 to CXCL8 chemokines, the two N-terminal cysteine residues are separated by a single residue. The conserved N-terminal region of the chemokines is critical for receptor binding, and the C-terminal residues represent a binding site for glycosaminoglycans of the extracellular matrix and are expressed on cell surfaces. The chemokine receptors constitute a protein family of about seventeen G protein-coupled receptor members. The CCR1 to CCR9 receptors bind the CC chemokines, while the CXCR1 to CXCR6 receptors bind the CXC chemokines.

Chemokine receptor engagement has been shown to result in complex intracellular signaling events, such as activation of phospholipase β, phosphoinositide 3-kinases and Src family tyrosine kinases, ultimately leading to reorganization of the cytoskeleton and chemotaxis [1, 3]. Not surprisingly, many disease states are also much influenced by dysregulated chemokine and chemokine receptor expression [1, 2]. In this respect, it is now well established that chemokines and their corresponding receptors play a pivotal role in the development of various inflammatory states such as asthma, rheumatoid arthritis and atherosclerosis [4-7], of autoimmune disorders such as type I diabetes [8], and in infectious diseases such as AIDS [9]. Development of graft-versus-host disease [10] and spread of tumor cells in some types of cancer [11] are also controlled by chemokines. The broad spectrum of diseases, for which interference with chemokine functions might have a beneficial role, indicated that chemokines and their receptors constitute a new array of targets for the pharmaceutical industry. Over recent years, a great deal of effort has been directed towards the isolation of small molecular weight
antagonists of chemokine receptors [12-15]. As of today, many compounds have been evaluated in preclinical studies and a handful of them have now reached clinical testing in humans [16, 17]. Although chemical entities interfering with chemokine functions have been successfully developed and are being evaluated in humans, there is still a pressing demand from many investigators for the development of new chemokine tools needed for the study of chemokine physiology. In particular, the imaging of chemokine binding to their receptors is still problematic, due to the lack of appropriate reagents. Simple and reliable binding assays for chemokines are available for only a subset of them and, in addition, most of them are based on radiolabeled chemokines [18, 19]. This situation prompted us to take advantage of our ability to produce chemokines by total chemical synthesis for the development of chemokines labeled with fluorescent tags at site-specific positions along the sequence. Five chemokine species, namely CCL11, CCL19, CCL22, CXCL11 and CXCL12, were manufactured and singly labeled with Alexa Fluor® 647 dye on a specific residue in their C-terminal sequence. One of these reagents, Alexa Fluor® 647-labeled CXCL12, was previously characterized by others [20]. In this report, these reagents were used in migration assays and were shown to be as efficient as their non-labeled counterparts. Furthermore, using the flow cytometry application, their specificity, their use for screening of low-molecular weight antagonists or neutralizing antibodies and, finally, their capacity to stain different cell types, alone or in combination with antibodies, were documented. Collectively, the results reported in the present work indicate that synthetic chemokines labeled at site-specific positions with fluorescent dyes constitute promising tools for the study of many aspects of chemokine physiology.

MATERIALS AND METHODS

Peptide synthesis, purification and folding

The sequences of human CCL11, CCL19, CCL22, CXCL11 and CXCL12 were assembled using solid phase Fmoc chemistry. The most C-terminal Lys residue (Lys73 in CCL11, Lys73 in CCL19, Lys66 in CCL22, Lys71 in CXCL11 and Lys67, followed by an extra Lys, in CXCL12) was replaced by a (Nα-serine) diaminopropionic acid residue [Dpr(Ser)] (Novabiochem, Laufelfingen, Switzerland). Following synthesis, the peptides were cleaved from the resin, reverse phase (RP-HPLC)-purified on a C18 column (Vydac, Hesperia, CA, USA) run on an analytical reverse phase high pressure liquid chromatography (RP-HPLC) on a Waters Alliance system. Samples were run with a linear gradient of 0.1% aqueous trifluoroacetic acid (TFA) in 0.09% TFA-acetonitrile at 1 mL/min on a C18 Vydac column (238TP54). Elution was monitored at 214 nm.

Cell culture

The cell culture medium used for all the cells was RPMI 1640 medium supplemented with 2 mM glutamine, 1-fold concentrated mixture of nonessential amino acids, 1 mM sodium pyruvate, 100 μg/mL kanamycine, 5x10^{-5} M 2-mercaptoethanol (all from Invitrogen, Basel, Switzerland) and 10% fetal calf serum (FCS). Human CCR3-, CCR4-, CCR7-, CXCR3- and CXCR4-transfected B300.19 mouse B lymphoma cells were obtained as described [24] and cultured in medium containing 1.5 μg/mL puromycin for the CCR3-, CCR4-, CCR7- and CXCR4-transfected cell lines, or 1 mg/mL geneticin for the CXCR3-transfected cell line. Under these culture conditions, expression of the transfected receptors remained stable over a period of > 6 months. T cell blasts were obtained after activation of peripheral blood mononuclear cells (PBMC) for 5 days in the presence of 1 μg/mL phytohemagglutinin (PHA), purified phytohaemagglutinin, Murex Biotech, Dartford, UK) and 1% human interleukin-2.

Migration assay

Migration induced by the chemokines was determined using 5 μm polycarbonate Transwell inserts (Costar, Corning, NY, USA). Briefly, 3x10^5 cells in 100 μL RPMI 1640 and 0.5% bovine serum albumin (BSA) were dispensed into the upper chambers of the migration wells. Chemokine in the same medium was added to the lower chambers in a final volume of 600 μL. After 3 hours of incubation at 37 °C, cell migration was determined using a lactate dehydrogenase assay-based colorimetric assay (CytoTox 96 non-radioactive cytotoxicity assay, Promega, Madison, WI, USA) on the cells recovered in the lower chambers. Results are given as means of duplicate determinations of the optical density (O.D.) at 490 nm.

Flow cytometry

Cells (3x10^7/well) in phosphate buffered saline (PBS) and 3% FCS were incubated with Alexa Fluor® 647-labeled chemokine at the indicated concentrations for 1 hour at 4°C. After 2 washings with PBS, cells were fixed in PBS with 1% FCS and 1% paraformaldehyde, before flow cytometry analysis (FACSCalibur cytofluorometer, BD Bio-
Production and chemical characterisation of synthetic chemokines labeled at defined positions with fluorescent dyes

Chemokines were prepared by solid phase peptide synthesis, using a modification of the method for the preparation of site-specifically biotinylated proteins [23]. The labeled polypeptides were analyzed by analytical RP-HPLC and MALDI-TOF (figure 1). Integration of peak areas of the analytical RP-HPLC revealed that the Alexa Fluor® 647-labeled CXCL12 preparation contained a main peptide species representing 84% of the material and a minor peak of a more hydrophobic species (figure 1A). Mass spectrometry analysis established that this material had an m/z ratio corresponding to 8846 Da, which is consistent with the expected mass of 8844 Da (figure 1B). Similar results were obtained with Alexa Fluor® 647-labeled CCL11, CCL19, CCL22 and CXCL11.

RESULTS

Unaltered biological activity of fluorescent chemokines as compared to unlabeled chemokines

We investigated whether modification with Alexa Fluor® 647 dye had an impact on the biological activity of the chemokines. The fluorescent chemokines were thus evaluated for their capacity to induce migration of cells transfected with their corresponding receptor. Migration of CXCR4-transfected cells induced by unmodified CXCL12 and Alexa Fluor® 647-labeled CXCL12 is shown as a representative example (figure 2A). Similarly to the bell-shaped dose response curve observed with unmodified CXCL12, Alexa Fluor® 647-labeled CXCL12 promoted maximal migration at a dose of 100 ng/mL and migration was still clearly detected at 10 ng/mL. At higher concentrations (1000 ng/mL) of both CXCL12 and Alexa Fluor® 647-labeled CXCL12, the extent of migration decreased. This low chemotactic response at high chemokine concentrations might have been due to sub-optimal chemokine receptor triggering. As a result, a bell-shaped migration response curve was obtained over the range of chemokine concentrations tested. By contrast, untransfected cells did not migrate at all over the whole range of concentrations of CXCL12 tested, indicating that CXCL12-induced migration was strictly dependent on CXCR4 expression (data not shown). These results, showing that Alexa Fluor® 647-labeled CXCL12 fully retained its ability to trigger migration of the appropriate chemokines receptor-expressing cell, indicated that the characteristics of binding between CXCL12 and CXCR4 were, overall, not altered by the introduction of the Alexa Fluor® 647 molecule. Similar observations were made when comparing unlabeled and Alexa Fluor® 647-labeled CCL11, CCL19, CCL22 and CXCL11 for their capacity to induce migration of CCR3-, CCR7-, CCR4- and CXCR3-transfected cells, respectively (data not shown).
down to 12 ng/mL and maximal CXCR4-specific staining was obtained at 300 ng/mL (figure 2B). At these concentrations, untransfected cells were negative or weakly positive in the case of the 300 ng/mL concentration. These results indicated that the staining was due to the binding of Alexa Fluor® 647-labeled CXCL12 to CXCR4. However, at concentrations higher than 300 ng/mL, untransfected cells became clearly stained and these concentrations were thus not used in further studies.

In order to compare the efficacy of staining by the two labeled CXCR4-binding reagents, untransfected (figure 3A and B) and CXCR4-expressing cells (figure 3C and D) were double-stained using Alexa Fluor® 647-labeled CXCL12 and the PE-labeled anti-CXCR4 MoAb. Alexa Fluor® 647-labeled CCL22 and isotype PE-labeled MoAb were used as negative controls. As shown, both types of cells remained negative when stained simultaneously with the negative controls (figure 3A and C). In addition, untransfected cells were stained neither by Alexa Fluor® 647-labeled CXCL12, nor by PE-labeled anti-CXCR4 MoAb (figure 3B). By contrast, the CXCR4-transfected cells were double-stained by these two reagents (figure 3D), indicating that labeled CXCL12 and anti-CXCR4 MoAb (12G5) can simultaneously bind to the CXCR4 receptor.

Inhibition of Alexa Fluor® 647-labeled CCL22 and CXCL11 cell staining by the respective unlabeled synthetic chemokines

Additional synthetic chemokines labeled with Alexa Fluor® 647 dye were manufactured to assess whether the observations made with CXCL12 could be extended to other chemokines. The next chemokine tested was CCL22, which also constituted a first example of a directly labeled chemokine belonging to the CC-type of chemokines. It was evaluated whether Alexa Fluor® 647-labeled CCL22 and unmodified CCL22 had similar binding characteristics.
for CCR4. Preliminary experiments indicated that the intensity of staining of CCR4-transfected cells progressively increased with the concentration of Alexa Fluor® 647-labeled CCL22, up to 300 ng/mL, while staining of untransfected cells remained negative. Then, samples of increasing concentrations of unlabeled CCL22 were added to a fixed concentration of Alexa Fluor® 647-labeled CCL22 (50 ng/mL) giving sub-optimal FACS staining intensity. These chemokine solutions were then used to stain CCR4-transfected and untransfected cells. Flow cytometry analyses revealed that cell staining of CCR4-transfected cells was reduced by 50% with an equimolar addition of unlabeled CCL22 (50 ng/mL). Weak inhibition was detected with competition by 17 ng/mL of unlabeled CCL22 and complete inhibition was reached with 450 ng/mL of unlabeled CCL22 (figure 4A). By contrast, untransfected cells were not stained under the different conditions tested (figure 4A). These results indicated that the binding characteristics, in particular the on- and off-rates, of Alexa Fluor® 647-labeled CCL22 were not greatly

**Figure 2**

Migration and staining of CXCR4-transfected cells treated with Alexa Fluor® 647-labeled CXCL12.

A) Graded concentrations of Alexa Fluor® 647-labeled and unmodified CXCL12 were tested for their capacity to induce migration of CXCR4-transfected cells. The extent of cell migration was assessed in duplicate in a colorimetric lactate dehydrogenase assay on the cells recovered in the lower chamber of the migration wells after a 3 hour incubation period. Similar results were obtained in two other, independent experiments.

B) CXCR4-transfected (left panel) and untransfected (right panel) cells were stained with graded concentrations of the CXCL12 Alexa Fluor® 647-labeled chemokine and fluorescence intensity was determined by flow cytometry. The continuous bold, dotted, dashed, dashed/dotted and continuous fine lines in panels B correspond to staining using 333, 111, 37, 12 and 0 ng/mL Alexa Fluor® 647-labeled CXCL12, respectively.
modified as compared with unlabeled CCL22. This feature is certainly of chief importance for the use of Alexa Fluor® 647-labeled CCL22 in pharmacological applications, such as the identification of small molecular weight CCR4 antagonists. Identical results were obtained when competition was performed in a two-step protocol in which incubation of cells with unlabeled chemokine was followed by the addition of labeled CCL22 (data not shown).

A similar experiment was then conducted using the CXCL11 and CXCR3 pair. In this case, labeling of CXCR3-transfected cells was detected down to 0.4 ng/mL and a staining plateau was already obtained with 10 ng/mL of Alexa Fluor® 647-labeled CXCL11, while incubation of control untransfected cells with this reagent resulted in no detectable staining over the entire range of concentrations tested (data not shown). Staining was competed with unlabeled chemokine in the two-step protocol and, similar to what was reported for CCL22, cell staining by labeled CXCL11 (10 ng/mL) was gradually blocked with increasing concentrations of the corresponding unmodified chemokine. In this case, reduction of staining by 50% was attained with 37 ng/mL of unlabeled chemokine (figure 4B).

Similar results were also obtained with CXCL12 binding to CXCR4. By contrast, and for reasons that remain unclear, competition of Alexa Fluor® 647-labeled CCL11 staining on CCR3-expressing cells was found to require a larger than equimolar excess of unmodified CCL11 (data not shown).

**Inhibition of binding of Alexa Fluor® 647-labeled CXCL12 and PE-labeled anti-CXCR4 MoAb by a low molecular weight CXCR4 antagonist**

Screening of small molecular weight compound libraries for the identification of chemokine receptor antagonists is certainly an area of application for fluorescent chemokines. This idea prompted us to validate the use of fluorescent chemokines for the identification of molecules interfering with the binding of chemokines to their receptors. The example of CXCL12 and CXCR4 was chosen, since for this chemokine and chemokine receptor pair, a commercially available, low molecular weight CXCR4 antagonist has previously been described as effective in a cell-based, high throughput screening assay [25]. Pre-incubation of CXCR4-transfected cells with the AMD3100 antagonist prevented binding of PE-labeled anti-CXCR4 MoAb in a dose-dependent fashion. Staining of CXCR4-transfected cells was reduced by 50% with 3 ng/mL AMD3100, and was completely extinguished with 200 ng/mL (figure 5A). AMD3100 was then evaluated for its ability to prevent staining of CXCR4-transfected cells by Alexa Fluor® 647-labeled CXCL12. The MFI was diminished by 50% following incubation of
CXCR4-transfected cells with 6 ng/mL AMD3100 and a maximal inhibition of 92% was obtained with 1000 ng/mL AMD3100 (figure 5B). The mean IC50 determined in three independent experiments on CXCR4-transfected cells were 32 ng/mL and 28 ng/mL for Alexa Fluor® 647-labeled CXCL12 and PE-labeled anti-CXCR4 reagents, respectively.

It should also be mentioned that, in a similar manner, the interaction of Alexa Fluor® 647-labeled CCL11 with its CCR3 receptor was inhibited by the low molecular weight CCR3 antagonist SB 328437 compound [26], as well as by a neutralizing anti-CCR3 MoAb (data not shown). All these results illustrate that fluorescent chemokines are promising reagents for the study of the interactions between chemokines and their receptors.

**Alexa Fluor® 647-labeled chemokines as reagents for primary cell staining**

The staining reported above was conducted on chemokine receptor-transfected cells in order to assess the binding specificity of the Alexa Fluor® 647-labeled reagents. However, the labeled chemokines could also represent a useful tool to study the expression of chemokine receptors on primary cells. To illustrate this application, binding of CCL19/MIP-3β/ELC was analyzed on ex vivo PBMC. CCL19 was shown to be the only ligand of CCR7 that is expressed by activated lymphocytes. For this reason, CCL19 binding could constitute an alternative method for the detection of recently activated lymphocytes. It was shown that following incubation with Alexa Fluor® 647-labeled CCL19, CCR7-transfected cells were stained, while untransfected cells remained negative. Staining of CCR7-transfected cells was detected down to 0.05 ng/mL, and maximal CCR7-specific staining was obtained at 250 ng/mL. Then, single staining of PHA-activated PBMC were performed using PE-labeled anti-CD4 and anti-CD8 MoAbs, or Alexa Fluor® 647-labeled CCL19 (figure 6A and B). Negative controls consisted of PE-labeled isotype MoAb and Alexa Fluor® 647-labeled CCL11 (figure 6A and C). CCL11, which is bound by CCR3 predominantly...
double staining analyses. CD4+ cells were strongly stained by CCL19 (panel H), while CD4– cells were not, or only weakly stained by this labeled chemokine. Taken together, these results further extended the application of Alexa Fluor® 647-labeled chemokines to the detection of chemokine receptors expressed by primary cells (figure 6D to 6I).

**DISCUSSION**

Chemokines are polypeptides of 60 to 130 residues, with 1 to 3 disulfide bonds, of which only a few are post-translationally modified by glycosylation [13, 27-29]. The relatively small size and simple structural features of the members of this protein family make them amenable to total chemical synthesis. Indeed, over recent years, our laboratory has accumulated a good deal of experience in the manufacturing of synthetic chemokines [23]. After the production and use of biotinylated chemokines in whole cell binding assays, it came as a logical next step to attempt the development of synthetic chemokines directly labeled with tags, such as fluorescent dyes, at defined positions along the sequence rather than simply at the N-terminus [30]. The initial characterization of one of these directly labeled chemokines, Alexa Fluor® 647-labeled CXCL12, was recently reported in the literature [20]. To our knowledge, chemokines modified with fluorescent dyes in this manner were not previously commercially available. This procedure was expected to minimize the impact on the biological activity of the chemokines. It would be more difficult to achieve such a favorable situation for chemokines of recombinant origin. For polypeptides of this nature, labeling would be expected to affect the native molecule and would typically result in a mixture of proteins modified at different positions with consequently reduced biological activity. For example, labeling with the NHS ester modifies the N-terminal, which is particularly important for chemokine activity, as well as the side-chain of lysine residues.

After having successfully produced these reagents, different lines of evidence support the notion that the Alexa Fluor® 647-labeled chemokines are indeed as biologically active as their unmodified counterparts. Firstly, we showed that the labeled and unlabeled ligands had similar potency in migration assays. Then, under various experimental conditions, these fluorescent chemokines were able to stain cells transfected with the corresponding receptors, while untransfected cells remained unstained. This interaction was specific, as it could be inhibited with the corresponding unlabeled ligand. Indeed, partial displacement of the labeled chemokine from its receptor was achieved by an equimolar concentration of corresponding unlabeled ligand. After this was recently reported in the literature [20]. To our knowledge, chemokines modified with fluorescent dyes in this manner were not previously commercially available. This procedure was expected to minimize the impact on the biological activity of the chemokines. It would be more difficult to achieve such a favorable situation for chemokines of recombinant origin. For polypeptides of this nature, labeling would be expected to affect the native molecule and would typically result in a mixture of proteins modified at different positions with consequently reduced biological activity. For example, labeling with the NHS ester modifies the N-terminal, which is particularly important for chemokine activity, as well as the side-chain of lysine residues.

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In a previous work, mono-biotinylated chemokines were produced and used for whole cell binding assays [23]. While this approach was successful for some chemokines, such as CCL2, CCL19 and CCL22, it did not work in our hands for others, in particular for CCL11, and for others, such as CCL1, CCL5 and CXCL13, in spite of an extensive evaluation of binding conditions. The reasons for this
failure are not yet clear, but could be explained by unfavor- 
able on- and off-rates of the chemokine for their recep-
tor [31]. The use of directly labeled chemokines overcame 
this limitation, since Alexa Fluor® 647-labeled CCL11 
specifically stained CCR3-transfected cells. In addition, 
detection of the chemokine did not rely any longer on 
secondary reagents, such as avidin or anti-biotin antibody, 
which can induce chemokine receptor cross-linking and, 
thereby, modify the pharmacological features of the 
chemokine interacting with its receptor. Besides, binding 
assays based on chemokines directly labeled with fluores-
cent dyes were found to be at least 10-fold more sensitive 
than those using biotinylated chemokines [23], and also 
resulted in simplified assays, an importance aspect in the 
context of high throughput screenings. It is anticipated that 
the directly labeled chemokines that we are currently de-
veloping (CCL1/I-309 and CXCL8/interleukin-8) will 
also result in reagents with staining characteristics similar 
to those described for the labeled chemokines reported in 
the present study.

Obviously, fluorescent chemokines represent very useful 
tools in high throughput screening applications. Alexa
Fluor® 647-labeled chemokines (e.g., CCL22 and CXCL11) have been used successfully in FMAT® system-based applications (8200 Cellular Detection System, Applied Biosystems, Foster City, CA, personal communication). Furthermore, Alexa Fluor® 647-labeled chemokines constitute an alternative to radioactive ligands often used in chemokine and chemokine receptor binding assays [20]. In this study, we documented the capacity of a low molecular weight antagonist, AMD3100, to inhibit the Alexa Fluor® 647-labeled CXCL12 and CXCR4 interaction as effectively as it abrogated the binding of an anti-CXCR4 MoAb to the chemokine receptor. Moreover, it was also found that both the small molecular weight CCR3 antagonist SB32837 and a neutralizing anti-CCR3 antibody inhibited the staining of CCR3-transfected cells by fluorescent CCL11 (data not shown). Altogether, these findings validated the use of fluorescent chemokines for the identification of small molecular weight chemokine receptor inhibitors.

If the screening of chemokine receptor antagonists constitutes a straightforward application for fluorescent chemokines, this study also established that these reagents could be used in other applications, for example as surrogates for anti-chemokine receptor antibodies in FACS and confocal microscopy analyses.

Surprisingly, there are still no commercially available antibodies against some mouse and human chemokine receptors. The reasons for this are not clear, but could be due to the intrinsic poor immunogenicity of these receptors and the resulting difficulty of raising antibodies against them. Our findings have shown that labeled chemokines constitute alternative reagents for the study of chemokine receptors. Using the CXCL12 and CXCR4 pair, we showed that the labeled chemokine could be used similarly to, or in conjunction with, an anti-CXCR4 antibody for the staining of CXCR4-transfected cells. The use of labeled chemokine reagents in flow cytometry was then extended to primary cells as Alexa Fluor® 647-labeled CCL19 could specifically stain cells from human peripheral blood expressing the CCR7 receptor. Nevertheless, one should point out that staining of chemokine receptors with fluorescent chemokines on primary cells is difficult to interpret, since comparison with the same cells devoid of only the chemokine receptor under investigation is lacking. Negative controls are constituted by other labeled, expected irrelevant chemokines. In such cases, specificity of binding to the chemokine receptor, rather than interaction with glycosaminoglycans, could be assessed using of neutralizing anti-chemokine receptor antibodies.

The procedure used to covalently graft Alexa Fluor® 647 to one specific, chosen residue in the protein sequence is not restricted to this dye; it can also be applied to others. To this end, we successfully labeled various chemokines with Alexa Fluor 488 (Molecular Probes), EVOBlue 30 (Evotec, Hamburg, Germany), Atto 655 (Fluka, Buchs, Switzerland) and Cy5 (Amersham Biosciences, Cardiff, UK). With the exception of Cy5, the labeled chemokines produced cell staining of lower intensity than that obtained with the corresponding chemokine labeled with Alexa Fluor® 647. Other modifications, for use in additional applications, will certainly be considered in future work in our laboratory, such as polyethylene glycol attachment, and coupling of chemokines to magnetic or fluorescent plastic polymer beads and to surfaces, for the manufacturing of protein arrays.

Site-specific labeling with fluorescent dyes and other tags is not restricted to chemokines, but could be applied to other proteins amenable to total chemical synthesis, i.e. up to about 15 kDa. By combining the methods described in this study, the future will certainly see the manufacture of other proteins and their site-specific labeling in the drug development process.

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