CX3CR1 Tyrosine Sulfation Enhances Fractalkine-induced Cell Adhesion*

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Fractalkine is a unique CX3C chemokine/mucin hybrid molecule that functions like selectins in inducing the capture of receptor-expressing cells. Because of the importance of tyrosine sulfation for ligand binding of the selectin ligand PSGL1, we tested the role of tyrosine sulfation for CX3CR1 function in cell adhesion. Tyrosine residues 14 and 22 in the N terminus of CX3CR1 were mutated to phenylalanine and stably expressed on K562 cells. Cells expressing CX3CR1-Y14F were competent in signal transduction but defective in capture by and firm adhesion to immobilized fractalkine under physiologic flow conditions. In static binding assays, CX3CR1-Y14F mutants had a 2–4-fold decreased affinity to fractalkine compared with wild type CX3CR1. By surface plasmon resonance measurements of fractalkine binding to biosensor chip-immobilized cell membranes, CX3CR1-Y14F mutants had a 100-fold decreased affinity to fractalkine. CX3CR1-expressing cell membranes treated with arylsulfatase to desulfate tyrosine residues also showed a 100-fold decreased affinity for fractalkine. Finally, synthesized, sulfated N-terminal CX3CR1 peptides immobilized on biosensor chips showed a higher affinity for fractalkine than non-sulfated peptides. Thus, we conclude that sulfation of tyrosine 14 enhances the function of CX3CR1 in cell capture and firm adhesion. Further, tyrosine sulfation may represent a general mechanism utilized by molecules that function in the rapid capture of circulating leukocytes.

Fractalkine (FKN, neurotactin, CX3CL1) is a structurally unique CX3C chemokine/mucin hybrid molecule on activated endothelium, epithelium, dendritic cells, and neurons that exists both in transmembrane and soluble shed forms (1, 2). In its cell surface form, FKN has remarkable cell adhesion properties and can induce the capture and firm adhesion of leukocytes under physiologic shear stresses (3, 4). This adhesion capacity of FKN with its receptor, CX3CR1, has been postulated to promote leukocyte migration of CX3CR1-expressing cells and to enhance the cytotoxicity of natural killer (NK) and cytotoxic T lymphocytes by promoting effector cell-target conjugate formation (5–7). Indeed, animal studies in which CX3CR1, the receptor for FKN, has been blocked by antibodies or deleted have shown an important role for CX3CR1 in cardiac allograft rejection (8, 9).

Selectins and their ligands are examples of other molecules that have the capacity to mediate the rapid capture of circulating leukocytes. Partly validating the hypothesis that FKN and CX3CR1 utilize many of the same molecular mechanisms as endothelial cell selectins (E- and P-selectin) and their ligands to capture circulating leukocytes, studies have shown that the FKN mucin domain is structurally and functionally similar to the short consensus repeats of E- and P-selectin (10). The FKN mucin domain functions as a stalk to extend and present the CX3C chemokine domain away from the endothelial cell surface, and it can be functionally replaced by the short consensus repeats of E-selectin (10). Thus, CX3CR1 may utilize the same mechanisms as selectin ligands to function in leukocyte capture. The most well studied selectin ligand is P-selectin glycoprotein ligand-1 (PSGL-1), a ligand for both E- and P-selectin (11–14). Comparing CX3CR1, a heptahelical G-protein-coupled receptor (GPCR), with PSGL-1, a heavily glycosylated dimeric mucin, reveals little structural homology. However, PSGL-1 tyrosine sulfation plays a critical role in high affinity binding to E- and P-selectin (14–17). Recently, it has been found that tyrosine sulfation of the N-terminal region of another chemokine receptor, CCR5, greatly enhanced binding to its ligands macrophage inflammatory protein-1α (CCL3) and macrophage inflammatory protein-1β (CCL4) as well as to HIV gp120 (18). Based on these studies and the presence of two tyrosine residues in the N-terminal region of CX3CR1 at positions 14 and 22 (Tyr-14 and Tyr-22), we hypothesized that these two residues may be contact points for FKN. Furthermore, the sulfation of these residues may be important for high affinity binding and adhesion to FKN.

EXPERIMENTAL PROCEDURES

Cell Culture—K562 erythroleukemic cells were grown at 37 °C in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum and supplemented with penicillin/streptomycin. 293/EBNA cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum. Fractalkine-secreted alkaline phosphatase (FKN-SEAP) fusion proteins were produced and purified from these cells as previously described (3). EA.hy 926 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum. Resting human peripheral blood mononuclear cells were isolated from whole blood using lymphocyte separation medium (Organon Teknika, Durham, NC).

Gene Mutation and Transfection—cDNA constructs encoding for CX3CR1 containing tyrosine to phenylalanine mutations were gener-
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**Expression of wild type and mutant CX₃CR1 fusion proteins.** A, the amino acid sequence of the N terminus of CX₃CR1 and the mutations induced. The positions of the tyrosine residues changed to phenylalanine are indicated in bold. B, shown are flow cytometric analyses of untransfected K562 cells and stable K562 cell transfectants expressing wild type CX₃CR1-GFP (YY) and CX₃CR1-GFP mutants (FF, YF, FY). The dot plots depict GFP expression (x axis) compared with the level of surface CX₃CR1 (y axis) as measured by indirect immunofluorescence with antibody 2A9. Also shown are negative (IgG1) controls. The percentage of cells within the quadrants is indicated. Wild type and mutant CX₃CR1 molecules are expressed at equal levels on the surface of stable K562 cell transfectants. Data are representative of three independent experiments.
surfactant P20) was used as the flow buffer. After each immobilization, sufficient time was allowed to generate a stable ligand immobilized surface with no base-line drift. Membrane vesicle preparations from K562 cells expressing CX3CR1 or the tyrosine mutants were captured on a L1 sensor chip (BIAcore, Inc.), which allows capture of bi-layer membrane vesicles. Membrane preparations were made by freeze thawing 1 × 10^7 cells in hypotonic lysis buffer (10 mM Tris, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride, and 0.01 mg/ml aprotinin) followed by ultracentrifugation at 200,000 × g for 90 min. The L1 surface was first primed with a short injection of octyl glucoside (40 mM, 20 μl at 40 μl/min), and then membrane preparations were injected at 3 μl/min for 10 min. Finally, a short injection (10 μl at 100 μl/min) of 10 mM Na2SO4 was allowed to remove nonspecifically adsorbed membranes and to provide a stable surface of membrane vesicles. A similar membrane preparation from K562 cells served as a control. Equivalent response units of membranes (200–300 resonance units) were captured for each cell type. Prior to each cycle of capture, the membrane preparation was briefly sonicated to provide particles of uniform size. In some cases, membrane preparations were treated with 1 unit of arylsulfatase overnight at 37°C (Sigma) as described previously (14). FKN-SEAP was then injected at a flow rate of 5–20 μl/min. Rate constant measurements and curve fitting analyses were performed using the BIAevaluation 3.0 (BIAcore, Inc.) software.

RESULTS

Cell Surface Expression of CX3CR1 Mutants—CX3CR1 contains four tyrosine residues on its extracellular face at positions 14 and 22 in the N terminus, 179 in the second extracellular loop, and 259 in the third extracellular loop. To function in leukocyte capture, it was hypothesized that the interacting residues would need to be extended away from the cell surface (10). The largest possibility for extension was in the N-terminal region of the receptor; thus Tyr-14 and Tyr-22 were targeted for mutation. Vectors encoding wild type CX3CR1 (YY) and both single (Y14F, YY; and Y22F, YY) and double (Y14F/Y22F, YY, or YF) tyrosine to phenylalanine mutations fused with GFP were generated (Fig. 1A). The mutation to phenylalanine is conservative, and phenylalanine is not subject to sulfation. Stable K562 cell transfectants expressing equivalent levels of GFP were sorted by flow cytometry. All four GFP fusion proteins were expressed at equivalent levels on the cell surface as determined by confocal microscopy (data not shown) and flow cytometry using a CX3CR1-specific monoclonal antibody (Fig. 1B).

Kinetics of FKN Binding to CX3CR1—The kinetics of CX3CR1 and CX3CR1 mutant binding to fractalkine was tested by two different techniques. First, static binding experiments were performed with FKN-SEAP. Using the static binding assay, the measured $K_d$ of YY to FKN-SEAP was 49 pM as compared with 56 pM for YY, 106 pM for YY, and 191 pM for YY cells. The kinetics of FKN binding to YY was also determined by surface plasmon resonance. Membranes from K562 cells expressing YY or the various mutants were prepared and immobilized onto an L1 biosensor chip. FKN-SEAP fusion proteins bound well to K562 cell membranes. Membranes from untransfected K562 cells or K562 cells expressing YY were purified and immobilized on L1 biosensor chips. The kinetics of binding to soluble 0.5 mg/ml FKN-SEAP fusion proteins was determined by surface plasmon resonance using a Biacore 3000. B, kinetics of fractalkine binding to wild type and mutant CX3CR1 proteins. Shown are representative tracings from three independent experiments.

Binding Site for FKN. These data suggest that CX3CR1 tyrosine 14 is an important binding site for FKN. Phenylalanine is similar to tyrosine except for the fact that it cannot be post-translationally modified by sulfation or phosphorylation. To test the hypothesis that sulfation of Tyr-14 is important, numerous immunoprecipitation studies using our and other commercially available polyclonal and monoclonal antibodies directed against CX3CR1 or the GFP fusion partner were attempted but were unsuccessful. CX3CR1 may be tightly complexed with other proteins in the membrane, cytosol, or lipid rafts such that its isolation by immunoprecipitation is difficult. To ascertain the importance of sulfation for binding, two approaches were taken. First, YY membranes were treated with arylsulfatase to cleave off the sulfate residues and tested for binding to FKN by surface plasmon resonance. Active arylsulfatase also caused an approximate 150-fold decrease in $K_d$ (1.9 versus 290 nM) similar to the difference between YY and YY using the same assay (Fig. 3A). Second, peptides encoding the N-terminal 20 amino acids of CX3CR1 that contained either a non-sulfated Tyr-14 (CX3CR1 1–20 Y14-OH) or a sulfated Tyr-14 (CX3CR1 1–20 Y14-SO4) were immobilized on a CM5 biosensor chip and tested for their ability to bind to FKN-SEAP by surface plasmon resonance. FKN bound to the sulfated peptide with an
approximate 10-fold greater affinity as compared with the non-sulfated peptide (K_d 0.79 versus 6.6 nM) (Fig. 3B). These data suggest that CX3CR1 is sulfated and that the sulfation enhances the ability of CX3CR1 to bind FKN under flow conditions.

**Functional Analysis of CX3CR1 Mutants**—The above data suggest that Tyr-14 is a critical residue for CX3CR1-FKN interactions under flow conditions, but it may not be as critical for interactions under static conditions. To address this possibility, we tested the function of cells expressing wild type and mutant CX3CR1 molecules in cell capture and adhesion under flow and in calcium mobilization assays.

To examine cell capture and adhesion under flow conditions, the ability of YY, YF, FY, FF, and K562 control cells to bind and firmly adhere to immobilized FKN-SEAP fusion proteins under shear stress were tested in the parallel plate flow chamber assay. Cells were allowed to capture on FKN-SEAP or SEAP control protein-coated glass coverslips at 0.25 dynes/cm^2 for 5 min. The cells that were able to transiently interact with the fractalkine substrate for at least 5 s were categorized as captured cells. We observed that FY and FF cells containing a tyrosine to phenylalanine mutation at position 14 were ineffective at being captured by fractalkine (data not shown). After the initial loading period, the cells were subjected to increasing shear stresses, and the number of cells remaining adherent after 10 dynes/cm^2 were quantified. Fig. 4 shows a representative experiment that wild type YY cells bound to FKN at significantly higher levels than K562 control cells (p < 0.05). In contrast, the FF clone in which both tyrosines 14 and 22 were changed to phenylalanine bound to FKN at the same level as K562 cells (p = not significant, n = 3) and was only 10% of the level of YY cells. Analysis of the single mutants revealed that

![Surface plasmon resonance measurements of sulfated versus non-sulfated CX3CR1.](http://www.jbc.org)
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TABLE I

| Binding affinity (K_ds) | On rate (ka, 1/ms) | Off rate (kd, 1/ms) | Ca²⁺ mobilization (EC₅₀) | Cell adhesion (% max) |
|------------------------|-------------------|-------------------|------------------------|---------------------|
| YY                     | 45                | 5.8 × 10³         | 2.6 × 10⁻⁴             | 0.4                 | 100               |
| YF                     | 64                | 5.6 × 10³         | 3.6 × 10⁻⁴             | 0.6                 | 95                |
| FY                     | 6600              | 0.28 × 10³        | 0.18 × 10⁻⁴            | 1.0                 | 10                |
| FF                     | 3500              | 0.37 × 10³        | 0.13 × 10⁻⁴            | 2.2                 | 10                |

* Reported are the binding affinities of FKN-SEAP to membrane-bound CX₃CR1 proteins as measured by surface plasmon resonance.

Tyr-14 was a critical residue for CX₃CR1-expressing cell adhesion to FKN as FY bound at only 10% of the level of YY whereas YF bound 95% as well as YY (p = not significant and <0.05, respectively, compared with K562 cells, n = 3). Similar results were observed in flow chamber experiments using FKN-transfected EA.hy 926 cells as the substrate (data not shown).

To assess the roles of CX₃CR1 Tyr-14 and Tyr-22 in signal transduction, the ability of soluble FKN-SEAP to induce calcium mobilizations in YY, YF, FY, FF, and K562 cells was tested. All four CX₃CR1 types mobilized calcium in response to soluble FKN-SEAP (Fig. 5). There was a slight rightward shift in the dose-response curve for FF > FY > YF > YY (EC₅₀ = 0.4 ± 0.2, 0.6 ± 0.3, 1.0 ± 0.2, and 2.2 ± 0.8 nM for YY, YF, FY, and FF, respectively) with no significant differences (p > 0.05) among Tyr-14-containing proteins (YY and YF) and Phe-14-containing proteins (FY and FF). Although the 2.5-fold difference in EC₅₀ between YY and FY was statistically significant (p < 0.05), the physiologic relevance is unclear. It could be that Tyr-14 is necessary for optimal interactions of membrane-tethered FKN under conditions of flow but not under static conditions (similar to L-selectin).

DISCUSSION

CX₃CR1 is a unique chemokine receptor in that it has a dual function in signal transduction and cell adhesion. Because no other GPCR has been identified as a cell adhesion molecule, little is known about the mechanisms by which this or other GPCRs may function in this capacity. Based on our original hypothesis that the FKN-CX₃CR1 interaction may share the same mechanisms as selectin-selectin ligand interactions (10), we tested whether N-terminal tyrosine residues in CX₃CR1 were involved in CX₃CR1-FKN interactions and whether sulfation of these residues affected adhesion.

Our study showed that a conservative mutation in CX₃CR1-Y14 to phenylalanine had numerous functional effects (Table I). First, it did not abolish soluble FKN binding to CX₃CR1, but it did decrease the affinity as measured by two different assays. Second, signaling through CX₃CR1-FY was intact, but ~2.5-fold higher concentrations of FKN were needed to produce similar responses. Third, Y14F nearly abolished all cell adhesion activity to immobilized FKN. In most assays, mutating Tyr-22 had little effect on FKN binding or CX₃CR1 function. However, the slight but statistically insignificant decrease in cell adhesion and G-dependent signaling in YF cells may suggest a small role for Tyr-22 in FKN-CX₃CR1 interactions. An important consideration for any mutation is whether it induces structural changes that render the protein inactive. Two lines of evidence argue against major structural changes being induced by mutating Tyr-14 and/or Tyr-22 to F; soluble FKN was able to bind and transduce signals in YY, FY, YF, and FF cells, and monoclonal antibody 2A9 directed to the N terminus of CX₃CR1 was able to bind well to both wild type and mutated CX₃CR1 proteins.

Tyrosine sulfation is critically important for selectin-mediated cell capture under physiologic flow (15–17) and is also important in other protein-protein interactions, including those of chemokines with their receptors (18, 20–23). Recently, CCR2 and CCR5 have been shown to undergo tyrosine sulfation, and this tyrosine sulfation is functionally relevant (18, 21). Unlike the present study, mutations in N-terminal tyrosine residues caused a loss in binding to their natural ligands. CCR2B mutants failed to form lamellipodium and migrate whereas CCR5 mutants were unable to bind to HIV gp120 (18, 21). Although these other chemokine receptors have been shown to contain sulfated tyrosine residues, CX₃CR1 could not be directly evaluated in this study by immunoprecipitation because of technical reasons related to the receptor and reagents available. However, CX₃CR1 does contain an important feature for tyrosine sulfation with an acidic residue (glutamic acid) located directly before Tyr-14. The parameters that determine tyrosine sulfation are not entirely understood, but features that enhances tyrosine sulfation are the presence of acidic residues within 3 bases upstream of the tyrosine residue, a neutral or acidic residue at −1, and the lack of a basic residue at −1 (24). Further, an indirect method wherein arylsulfatase (used to desulfate tyrosine residues) reduced the affinity of FKN binding to the level of the mutated TF protein suggests that the endogenous CX₃CR1 is sulfated. The importance of Tyr-14 sulfation to FKN binding was demonstrated by the increased affinity of sulfated compared with non-sulfated CX₃CR1 N-terminal peptide binding to FKN. Thus, Tyr-14 sulfation is important for adhesion to fractalkine under physiologic flow conditions.

On the other hand, signaling through CX₃CR1 is not greatly affected. These results are different from the ones published for CCR2. In this case, mutation of the tyrosine residue at the N terminus which is sulfated (position 26) to phenylalanine caused a total loss in signaling as measured by calcium mobilization (21). We and others have previously demonstrated that signaling through the G protein is not necessary for cell adhesion (3, 4). CX₃CR1 couples exclusively to Go₁ subunits, and poisoning this G protein by pertussis toxin had no effect on cell adhesion (3). In addition, mutation of the G protein docking site in CX₃CR1 abolished G-dependent signaling but had no effect on cell adhesion (4). One possible explanation for the lack of a large effect on signal transduction while the adhesion defect is dramatic could be that the calcium fluxes are measured using soluble fractalkine added under static conditions. In contrast, our adhesion experiments used immobilized surface-bound fractalkine under dynamic conditions. Indeed we see a rightward shift in the calcium flux dose-response curves, indicating that the signaling through the mutant receptors is suboptimal. The results of the binding experiments, both static and Biacore analysis, suggest that the mutant CX₃CR1 containing the phenylalanine at position 14 can bind to fractalkine, though not as well as those containing the native tyrosine residue. Therefore, tyrosine 14 and its sulfation may be most important for the initial capture of CX₃CR1-expressing cells to fractalkine under physiologic flow conditions and less important for other potential downstream functions.
It is interesting to note that the difference in $K_d$ values between wild type and tyrosine 14 mutants of CX$_3$CR1 obtained for the Biacore analysis is greater (150-fold) than those calculated for the static binding experiments. Biacore measurements are taken using membranes lacking any cytosolic components whereas the static binding experiments are performed on whole cells. It is possible that some of these components may alter or affect the $K_d$. It has previously been shown that measurements of T cell receptor affinity using live cellular assays were substantially higher than those measured in cell-free systems (25). Furthermore, Biacore $K_d$ values are calculated using on and off rates (apparent $K_d$) and thus may not reflect the true physiological equilibrium. Consistent with other heptahelical receptors, CX$_3$CR1 may bind using the two-site model of ligand association in which the tyrosine-sulfated N terminus mediates the initial docking interaction with fractalkine (20).

Fractalkine-CX$_3$CR1 interactions are important in a number of systems and may play a role in various disease states. In the brain, fractalkine expressed on neurons can mediate the activation and migration of CX$_3$CR1-expressing microglial cells (26, 27). Fractalkine-CX$_3$CR1 interactions are believed to be involved in NK cell-mediated cytosis (6). Kidneys undergoing allograft rejection have elevated levels of FKN, and antibodies against CX$_3$CR1 can protect a cardiac allograft from rejection (8). Furthermore, a mouse deficient in CX$_3$CR1 has been shown to have prolonged cardiac allograft survival (9). The mechanism of this finding appears to be NK cell-mediated, and fractalkine-CX$_3$CR1 interactions may be important for the recruitment of NK cells to the transplanted organ. Finally, CX$_3$CR1 may be a weak co-receptor for HIV (28, 29).

In summary, we have identified that tyrosine residue 14 at the N terminus of CX$_3$CR1 is necessary for FKN-dependent rapid capture and firm adhesion. This negatively charged sulfated residue may form an important contact point for binding to fractalkine. There are many conserved positively charged residues among species, and the crystal structure of the chemokine domain of FKN was recently solved (30). We have previously published that the CX$_3$C chemokine head contains most of the conserved basic residues and have identified Lys-7 and Arg-47 as potentially important contact points for CX$_3$CR1 function in cell adhesion (31). It is possible that these amino acids may interact with sulfated tyrosine 14. Because tyrosine sulfation is essential for cell capture under dynamic conditions by two families of molecules, chemokines and selectins, this post-translational modification may be considered a global mechanism to increase the types of adhesive interactions that lead to the capture of circulating inflammatory cells.

REFERENCES

1. Banaz, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A., and Schall, T. J. (1997) Nature 385, 640–644
2. Pan, Y., Lloyd, C., Zhou, H., Delich, S., Deeds, J., Gonzalez, J. A., Vath, J., Gusselin, M., Ma, J., Huttner, W. B., Adhikari, S., Thompson, D. A., Botti, P., McEver, R. P., and Patel, D. D. (1998) J. Exp. Med. 188, 1413–1419
3. Haskell, C. A., Cleary, M. D., and Charo, I. F. (1999) J. Biol. Chem. 274, 10553–10558
4. Imai, T., Hieshima, K., Haskell, C., Babu, M., Negira, M., Nishimura, M., Kakizaki, M., Takagi, S., Noniyama, H., Schall, T. J., and Yoshi, O. (1997) J. Clin. Invest. 100, 670–678
5. Haskell, C. A., Hancock, W. W., Salant, D. J., Gao, W., Cimazuddin, V., Peters, W., Faia, K., Fitouri, O., Rottman, J. B., and Charo, I. F. (2001) J. Clin. Invest. 108, 10901–10907
