Mutational Analysis of the Fractalkine Chemokine Domain

BASIC AMINO ACID RESIDUES DIFFERENTIALLY CONTRIBUTE TO CX3CR1 BINDING, SIGNALING, AND CELL ADHESION*

Received for publication, November 10, 2000, and in revised form, March 5, 2001
Published, JBC Papers in Press, March 8, 2001, DOI, 10.1074/jbc.M010261200

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Fractalkine (FKN/CX3CL1) is a unique member of the chemokine gene family and contains a chemokine domain (CD), a mucin-like stalk, a single transmembrane region, and a short intracellular C terminus. This structural distinction affords FKN the property of mediating capture and firm adhesion of FKN receptor (CX3CR1)-expressing cells under physiological flow conditions. Shed forms of FKN also exist, and these promote chemotaxis of CX3CR1-expressing leukocytes. The goal of the present study was to identify specific residues within the FKN-CD critical for FKN-CX3CR1 interactions. Two residues were identified in the FKN-CD, namely Lys-7 and Arg-47, that are important determinants in mediating an FKN-CX3CR1 interaction. FKN-K7A and FKN-R47A mutants exhibited 30–60-fold decreases in affinity for CX3CR1 and failed to arrest efficiently CX3CR1-expressing cells under physiological flow conditions. However, these mutants had differential effects on chemotaxis of CX3CR1-expressing cells. The FKN-K7A mutant acted as an equipotent partial agonist, whereas the FKN-R47A mutant had marked decreased potency and efficacy in measures of chemotactic activity. These data identify specific structural features of the FKN-CD that are important in interactions with CX3CR1 including steady state binding, signaling, and firm adhesion of CX3CR1-expressing cells.

Leukocyte trafficking is a complex physiological process that is dependent upon a number of cellular events that include mechanisms of cell chemotaxis and adhesion (1, 2). In this regard, chemokines are a growing family of small molecular mass proteins whose role(s) are primarily involved in leukocyte migration (3). Fractalkine (FKN)† is a newly described member of the chemokine gene family and the only known member of the CX3C subfamily (4, 5). The extended structure of FKN is distinct among the chemokine gene family in that it affords the molecule unique membrane localization where it can mediate FKN receptor, CX3CR1, dependent cell adhesion (6–8). FKN is expressed primarily by neurons (9–11) and endothelial cells (6, 12). Neuronally expressed FKN is presumably involved in the central nervous system-based communication with CX3CR1-expressing microglia, whereas FKN expressed on endothelial cells likely mediates extravasation of CX3CR1-expressing cells, which include monocytes, T cells, and NK cells, out of the lumen of the blood vessel into the tissue parenchyma. FKN is also expressed by dendritic cells and intestinal epithelial cells, and CX3CR1 has recently been demonstrated on mast cells (13–16).

The dissection of molecular processes involving chemokines and chemokine receptors is an active area of investigation as development of therapeutic agents targeting these systems are likely to yield novel beneficial approaches in the treatment of inflammation, cancer, as well as inhibition of viral (human immunodeficiency virus) pathogenesis. Given the localization of FKN to endothelial, dendritic, and neuronal cells, this molecule also constitutes a potential therapeutic target for modulating several physiological processes including leukocyte trafficking and/or mechanisms involved neuroinflammation. The goal of the present study was aimed at identifying amino acid residues in the FKN molecule that are important determinants for binding to, and activating its receptor, CX3CR1. We employed deletional and site-directed mutagenesis in order to evaluate the effect of different lengths of the mucin stalk and determine the role of specific amino acid residues present in the FKN chemokine domain, in mediating FKN binding, signaling, and CX3CR1-dependent cell adhesion.

EXPERIMENTAL PROCEDURES

Mutagenesis—DNA sequences encoding human fractalkine were cloned to the EcoRI and/or site of pcDNA3 (huFKN/pcDNA3). Five truncation mutants of FKN were prepared by site-directed mutagenesis using huFKN/pcDNA3 as the template and five paired sets of complementary oligonucleotides encoding a different region of the mucin stalk (see Fig. 1). The oligonucleotides used are as follows A, 5′-CGA TGT GGT CCT CGA GCC CGA AGG-3′ and 5′-CGC CAA AGG-3′; B, 5′-CGC CAA AGG CTC AGG CTC GAG CTC GAG CTC-3′ and 5′-CGC CAA AGG CTC AGG CTC GAG CTC GAG CTC-3′; C, 5′-GGA GGA

The abbreviations used are: FKN, fractalkine; CD, chemokine domain; CX3CR1, receptor for fractalkine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; HEK, human embryonic kidney; Ni-NTA, nickel-nitriceltrate acid; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; WT, wild type; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody.
TGC TCC GTG TCG AGG CCA GCG TGG GTG G-3' and 5'-CCA CAC ACG CTT GCG TCC AGC AGG ATC TTC C-3'; D, 5'-CCA TGC CAC GAT GCA CTT GAC GGC GGT GGT C-3' and 5'-GGC CGC CTC GCA GGT CAG TGC GAT C-3'; and E, 5'-CGC CGC CTC GGC TGG GAT TTC GCT G-3'. The entire protein coding sequence of each plasmid was subject to DNA sequence analysis in order to confirm the mutation and the fidelity of the DNA polymerase (Fyu) used in the PCR. An EcoRI/XhoI fragment of each truncation mutant was then subcloned to the same site(s) of pDNA3.1/myc/His6 (Invitrogen); the specific version used depended on the frame in which the XhoI site was engineered. DNA sequence analysis of the final plasmid constructs was also performed in order to verify that the nucleotides encoding the myc/His6 in frame with the nucleotides encoding the FKN.

Site-directed mutagenesis of the chemokine domain of FKN (FKN-CD) was also carried out using oligonucleotide-directed PCR and subsequent DpnI treatment. Mutants were generated in the huFKN/pDNA3 (full length) and the truncation mutant, form E (described above). The specific oligonucleotide pairs used for the mutagenesis are as follows (see Fig. 1 in reference to the specific residues mutated): K7A, 5'-GCA CCA CGG TGC GAC GGC ATG CAA CAT CAC GTG C-3' and 5'-GCA CCT GTT GAT GTC GCA GTC CAC ACC GTG C-3'; K1A, 5'-GCA ACA TCA CGT GCG CGC TGA CAT CAA AGA TAC C-5' and 5'-GAT ATC TTT GAT ATC TTC TCG TTC TCG C-3'; K16A, 5'-GCC GAC GCC CCC CGG CAG CCG CAA AAC AAT CAT GGC CGG-3' and 5'-AGA TGC CAA GAT GAT TGC-5'.

Expression of FKNs in HEK293T Cells—Approximately 10 million HEK293T cells (seeded in two 100-mm dishes) were transfected using 10 μg of plasmid DNA/plate and LipofectAMINE according to the manufacturer's instructions. Cells were incubated in DMEM containing 10% FBS for the first 20 h post-transfection and subsequently incubated with MEM containing 10% FBS. The cells were harvested and subjected to Ni-NTA chromatography according to the manufacturer's (Qiagen, Inc.) recommended procedures. Briefly, conditioned media were mixed with an equal volume of 2x buffer (100 mM NaH2PO4, pH 8.0, 600 mM NaCl, 20 mM imidazole, pH 8.0) and 1 ml of the Ni-NTA slurry. The mixture was rocked for 1 h at 4 °C. After rocking, the mixture was loaded into a 0.5 ml Ni-NTA column elution buffer had no inhibitory or enhancing effects on the cells. After washing three times in wash buffer (0.5% Tween 20 in PBS), the cells were blocked with 1% BSA, 5% sucrose, 0.05% NaN3 for 1 h. Following three more washes, 100 μl of each diluted protein sample was added and incubated for 1 h at 37 °C. After washes, 100 μl of 10 ng/ml biotinylated anti-FKN (R & D Systems, Minneapolis, MN) was incubated overnight at room temperature in a 96-well plate. After washing three times in wash buffer (0.05% Tween 20 in PBS), the wells were treated with 1% BSA, 5% sucrose, 0.05% NaN3 for 1 h. Following three more washes, 100 μl of 1:4000 dilution streptavidin horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, CA) was added and incubated for 20 min at room temperature. After washes, horseradish peroxidase activity was detected colorimetrically using the TMB microwell peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, MD) and the results read on a Dynex microplate reader. Protein concentrations were determined by comparing the spectral readings with the standard curve of the independent ELISA.

Chemotaxis Assays—Chemotaxis toward soluble FKN and FKN-CBD mutants was addressed by migration through transwells. Various dilutions of Ni-NTA-eluted fractions containing wild type and mutant proteins were made in 600 μl of medium (RPMI 1640 containing 10% FBS) and placed in the bottom of a transwell (6.5 mm diameter, 3-μm pore size, Costar, Corning, NY). One million L1.2 cells expressing CX3CR1-GFP were resuspended in 100 μl of medium and placed in the bottom of a transwell (6.5 mm diameter, 3-μm pore size, Costar, Corning, NY). The transwell was incubated for 2 h at 37 °C. After 3 washes, 100 μl of 1:4000 dilution streptavidin horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, CA) was added and incubated for 20 min at room temperature. After washes, horseradish peroxidase activity was detected colorimetrically using the TMB microwell peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, MD) and the results read on a Dynex microplate reader. Protein concentrations were determined by comparing the spectral readings with the standard curve of the independent ELISA.

Expression of FKNs in EA.hy 926 Cells—EA.hy 926 cells were grown in DMEM containing 10% FBS. Cells (grown to 80% confluence) were transfected with 10 μg of DNA using a calcium phosphate precipitation-based method. Forty-eight hours post-transfection, cells were seeded into media containing 0.5 mg/ml G418. G418-resistant cells were analyzed by counting a 20-μl aliquot using flow cytometry ( Coulter Epics XL-MCL). Ni-NTA column elution buffer had no inhibitory or enhancing effects on L1.2 cell chemotaxis. For each experiment, values were normalized to wild type FKN with maximal chemotaxis to wild type FKN set at 100%. Concentration-response curves were generated by curve fit interpolation analysis using Cricket Graph III (Computer Associates International, Islandia, NY). Statistical analysis was carried out using Statistics 3.0b (SAS Inc.).
FIG. 1. Alignment of human fractalkine (huFKN) with rat and murine fractalkine chemokine domains (rat-FKN-CD and mur-FKN-CD). Basic residues conserved within the three FKN orthologs (Lys-7, Lys-14, Lys-36, Arg-37, Arg-47, Lys-54, and Arg-74) were changed to alanine and are indicated in boldface. Boldface methionine (Met-15) was mutated to a lysine. The unique N-linked glycosylation consensus sequence (NIT) present in human and rat FKN-CDs is underlined. The starred residues denote the four conserved cysteine residues; the boldface and underlined sequences correspond to the transmembrane spanning domain.

RESULTS

Although FKN is expressed as a membrane-attached protein, extracellular forms of this protein are readily detected. The specific cleavage site(s) that generate these shed forms of FKN are not known. FKN released from the cell surface has an observed molecular mass greater than predicted and thus is likely to contain all or a portion of the glycosylated mucin-like stalk. Our first goal was directed toward determining the effect of various lengths of this mucin-like domain on the affinity and efficacy of the FKN-CD for binding and activating CX3CR1. Fig. 1 depicts the entire protein sequence of human FKN, in alignment with the chemokine domains of the two known rodent orthologs (rat and murine FKN-CD). In order to evaluate the effect of various lengths of the mucin stalk on FKN-CD function, five truncation mutants of human FKN (designated A–E) were prepared in which DNA sequences encoding the signal peptide, the chemokine domain, and differential lengths of the mucin stalk were cloned in frame with C-terminal six histidine residues (His$_6$). The C-terminal addition of the His$_6$ residues facilitated purification of the various secreted FKN truncation mutants from the conditioned media of the HEK293T cell transfectants (using Ni-NTA chromatography). In addition, the figure highlights conserved lysine and arginine residues, present in the chemokine domain of the various fractalkine orthologs, that were targeted for site-directed mutagenesis (to alanine). Since chemokine receptors typically contain a number of aspartate and glutamate residues within their extracellular regions, these negatively charged side chains present in the receptor are likely candidate amino acids for interaction with positively charged amino acids that are prevalent not only in FKN but also in other chemokine sequences. Thus, we hypothesized that specific basic residues present within the FKN molecule are critical for interactions with its receptor CX3CR1. In addition, a single methionine residue (at position 15) and a unique N-linked glycosylation sequence (NIT) present in human and rat FKN are highlighted. Met-15 was previously suggested to interact with a peptide containing the sequences of the N terminus of human CX3CR1 (17); this residue was mutated to a lysine. From structural studies, the single N-linked glycosylation in the human FKN sequence, which is also present in the cognate region of rat FKN, appears to be a readily available substrate for this post-translational modification. Thus, in order to assess the role of this putative glycosylation site in the interaction of FKN with CX3CR1, we mutated the N-linked glycosylation sequence to “EIM”; this tri-amino acid motif is EIM in the murine ortholog.

The effect of the length of the mucin stalk on the binding of the FKN-CD to CX3CR1 and the stimulation of intracellular calcium mobilization in CX3CR1-expressing cells were assessed. Western blot analysis (Fig. 2A) of five 293T cell-expressed truncation mutants of FKN, purified by Ni-NTA chromatography, indicated that all five forms were expressed and secreted into the HEK293T cell-conditioned media. All of the secreted proteins migrated as broad, diffuse bands through SDS-PAGE at molecular masses greater than their calculated molecular masses based upon the predicted protein backbones (Table I). This is likely due to the glycosylated characteristics of the mucin stalk. Nevertheless, the relative sizes of the various truncation mutants were consistent with the protein containing the CD and progressively increasing lengths of the mucin stalk. Competition binding analysis ($^{125}$I-FKN-CD binding to huCX3CR1-CHO cells) of each of these truncation mutants indicated that each form displayed a comparable affinity for human CX3CR1 (Fig. 2B). Furthermore, each form of the protein stimulated similar increases in intracellular calcium levels in the fura-2-loaded huCX3CR1-CHO cells (Fig. 2C). Collectively the data indicate that the mucin stalk does not contribute to FKN binding affinity and efficacy for stimulating calcium mobilization in CX3CR1-expressing cells.

The effect of mutating the species conserved basic residues, the single N-linked glycosylation consensus sequence, and Met-15, in the FKN-CD, on the binding of FKN to CX3CR1 was evaluated. Site-directed mutants of these residues were prepared in the context of the longest secreted truncation mutant (CD with all of the mucin stalk, i.e. form E) and purified by
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Ni-NTA chromatography. Western blot analysis (Fig. 3A) indicated that all of the Ni-NTA-purified mutants were present at concentrations equivalent to the levels of the purified "wild type" form. Ni-NTA-purified proteins were then evaluated for their relative potency for inhibiting the binding of 125I-huFKN-CD to the huCX3CR1-CHO cells. The K7A and R47A mutants displayed significantly lower binding potencies than the wild type and all other mutant forms (Fig. 3B). Estimates of the relative shift in potencies of these two forms were ~30–60-fold. FKN-CD mutants showing comparable binding affinities for CX3CR1 were evaluated for their ability to stimulate

in intracellular calcium in fura-2-loaded huCX3CR1-CHO cells. At concentrations that achieve near full receptor occupancy (~90%), each of these FKN mutants stimulated increases in intracellular calcium that were comparable to the "wild type" FKN (Fig. 3C). At concentrations equivalent to ~75% receptor occupancy by the wild type FKN, FKN-K7A, and -R47A mutants stimulated lower increases in intracellular calcium that correlated with the decreased binding affinity of these mutants.

The concentration-dependent effect of FKN to promote chemotaxis of CX3CR1-expressing cells was evaluated for each of the mutants (Fig. 4). FKN-K7A and -R47A mutants displayed reduced chemotactic activity relative to that observed with wild type FKN. The FKN-R47A mutant was nearly ineffective at stimulating a chemotactic response, with maximal activity less than 10% of the wild type activity, whereas the FKN-K7A mutant retained ~60% of the wild type maximal activity. The FKN-K36A mutant had slightly enhanced maximal chemotactic activity. Mutation of Met-15 (to Lys) yielded an FKN that was 10-fold less potent at promoting chemotaxis; these data are consistent with the slight reduction in binding affinity of FKN-M15K (see Fig. 3B). Table II summarizes the effect of the predicted and observed molecular masses of FKN truncation mutants

| Form | Predicted (protein backbone) | Observed (approximate range) |
|------|-----------------------------|------------------------------|
| A    | 14.7                        | 21–30                        |
| B    | 19.6                        | 35–50                        |
| C    | 26.2                        | 43–80                        |
| D    | 34.7                        | 60–100                       |
| E    | 36.6                        | 66–100                       |

% Bound as a function of volume of the eluate added. Results are plotted as % Bound as a function of volume of the eluate added. Results are expressed as mean ± S.E. from three independently conducted experiments performed in triplicate. Inset depicts competition by huFKN-CD. C, stimulation of intracellular calcium mobilization by huFKN truncation mutants. Representative traces from a single experiment are depicted. Results are representative of three independently conducted experiments.

Ni-NTA chromatography, and an aliquot (1 µl) of one of the elution fractions was subject to SDS-PAGE and Western blot analysis using the anti-hufractalkine antibody according to procedures indicated under "Experimental Procedures." The 1st lane contains 2.5 pmol of human FKN chemokine domain (CD) purchased from R & D Systems. Numbers to the left denote relative molecular mass of protein standards. B, competition binding analysis of huFKN truncation mutants. Radioligand binding analysis of 125I-huFKN-CD binding to huCX3CR1-CHO cells. Whole cell binding analysis of 125I-huFKN-CD in the absence and presence of increasing amounts of the Ni-NTA-eluted protein was performed according to procedures outlined under "Experimental Procedures." Data are plotted as % Bound as a function of volume of the eluate added. Results are expressed as mean ± S.E. from three independently conducted experiments performed in triplicate. Inset depicts competition by huFKN-CD. C, stimulation of intracellular calcium mobilization by huFKN truncation mutants. Representative traces from a single experiment are depicted. Results are representative of three independently conducted experiments.

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mutations on the relative concentration dependence and maximal chemotactic activities of the mutant FKNs relative to the respective wild type FKN parameters.

As stated, the specific basic residues targeted for mutagenesis are conserved in human, rat, and mouse FKNs. Analysis of the relative potencies of the mutants in binding to rat CX3CR1 revealed similar decreased affinities of the K7A and R47A mutants. This was evident regardless if the source of rat CX3CR1 was recombinant (Fig. 5A, rat CX3CR1-CHO cells) or native cell expressed (Fig. 5B, rat microglia). These data indicate similar interactions of the FKN with CX3CR1 in humans and rodents and point to conserved amino acids in these receptor orthologs as the counter amino acid residues interacting with amino acids Lys-7 and Arg-47 in FKN.

Parallel mutations were generated in the full-length FKN, in order to assess the role of these residues on FKN-dependent cell adhesion. The endothelial cell line, EA.hy 926, was transfected with each of these mutant constructs, and G418-resistant clonal cells were sorted. EA.hy 926 cells are derived from human umbilical vein endothelial cells and display a number of endothelial cell-like properties. Cell surface expression levels of these mutant FKNs in the stably transfected cells were roughly equivalent in all cell lines generated, as evidenced by flow cytometry (Fig. 6). The ability of CX3CR1-expressing cells to bind to the EA.hy 926 cells expressing the mutant FKNs was determined by a parallel plate flow chamber assay. Fig. 7 shows that K562-CX3CR1 cells efficiently bind to all of the mutant FKN EA.hy 926 cells to the same extent as the wild type FKN with 100% representing the peak chemotaxis to wild type FKN in each experiment.

**TABLE II**

| Form   | Relative EC<sub>50</sub> | Maximal activity |
|--------|-------------------------|------------------|
| WT     | 1                       | 100              |
| K7A    | 0.81 ± 0.22             | 58 ± 18<sup>a</sup> |
| K14A   | 1.8 ± 0.16              | 78 ± 13          |
| M15K   | 9.8 ± 4.95<sup>a</sup>  | 113 ± 34         |
| K36A   | 2.54 ± 0.7              | 116 ± 8<sup>a</sup> |
| R37A   | 2.28 ± 0.63             | 105 ± 33         |
| R47A   | ND                      | 8 ± 4<sup>a</sup> |
| K54A   | 1.36 ± 0.06             | 115 ± 21         |
| R74A   | 1.71 ± 0.51             | 122 ± 24         |
| NIT/EIM| 1.68 ± 0.34             | 118 ± 20         |

<sup>a</sup> p < 0.04, relative to WT.
type FKN-expressing EA.hy 926 cells with the exception of the K7A and R47A mutant FKN-expressing cells. A 60% reduction in both the capture and firm adhesion of K562-CX3CR1 cells to these two mutant FKN-expressing EA.hy 926 cells, as compared with wild type FKN-EA.hy 926 cells, was evident.

A chief concern in the analysis of functional properties of site-directed mutants is whether or not the specific mutation alters global protein conformation, as opposed to disrupting a specific protein-protein interaction. Thus, the effect of the various site-specific mutations on global FKN-CD conformation was assessed using a panel of monoclonal antibodies directed against the CD of human FKN. Cell surface reactivity of each of the mAbs was assessed using the EA.hy 926-expressed proteins. Reactivity of each of the mAbs toward the wild type FKN and the K7A, K14A, K36A, R74A, and NIT-EIM FKN mutants were equivalent (Table III). In contrast, the reactivities of mAbs 2A5 and 8C5 were reduced in the M15K, K36A, and R37A mutants. The monoclonal antibody, mAb 1B7, was less reactive toward the M15K and R37A mutants, whereas mAb 4F2 did not react with the R47A mutant. The spatial proximity of Met-15, Lys-36, and Arg-37 (Fig. 8) is consistent with similar reductions in reactivity by mAbs 2A5, 8C5, and 4F2.

DISCUSSION

The major goal of the current study was aimed at identifying specific basic residues in the chemokine domain of FKN that are critical for receptor interactions. Two amino acid residues, namely Lys-7 and Arg-47, were identified as important amino acid determinants in the binding of FKN to CX3CR1. The significance of these residues in binding interactions was confirmed in two independent methods used to assess binding of FKN to CX3CR1, namely in steady state binding analysis and in artificial flow chamber assays in which the FKN-CX3CR1 interaction is measured under conditions mimicking physiological blood flow. Whereas the K7A and R47A mutants behaved similarly in assays quantifying binding activities, these mutants had differential effects in measures of signaling via CX3CR1. Although FKN-R47A was essentially ineffective at stimulating intracellular calcium mobilization and chemotaxis of CX3CR1-expressing cells, the FKN-K7A mutant retained signaling activity. Three additional findings were noted in the present study. First, the single N-linked glycosylation site present in human and rat FKN is not glycosylated to any appreciable extent in heterologous expression systems and also does not appear to have any functional significance in the binding or activation of CX3CR1 by FKN. Second, a methionine residue (Met-15), previously suggested to interact with the N terminus of CX3CR1, does not appear to be critical at interacting with CX3CR1, as expressed in mammalian cells. Third, the affinity and efficacy of the FKN-CD are independent of the length of the mucin stalk.

FKN is a dual function chemokine capable of promoting CX3CR1-expressing cell chemotaxis and cell adhesion. Shed or secreted forms of FKN stimulate leukocyte chemotaxis, whereas the membrane-attached form of FKN mediates cell adhesion (4, 6, 7). A limited level of structure-function analysis of FKN and CX3CR1 has been reported. Although the chemokine domain of FKN is critical for interaction of FKN with CX3CR1, less information is available on the role of the mucin stalk. Under conditions mimicking physiological blood flow, cells expressing “full-length” FKN with the entire mucin domain are more efficient at capturing leukocytes as compared with FKN-CD proteins, i.e. without the mucin stalk (18). Although the arrest of CX3CR1-expressing cells under flow conditions is dependent on the chemokine domain of FKN, the function of the mucin stalk in this setting appears to be as a molecular extender of the FKN-CD to circulating leukocytes. The ability of FKN to arrest cells appears to be a unique property of the FKN chemokine domain as chimeric molecules in which the mucin domain was replaced with a stalk from E-selectin was fully functional in leukocyte arrest (18). Furthermore, chimeric molecules in which other chemokines were “fused” to the FKN mucin stalk failed to arrest cells expressing the cognate chemokine receptor (19).

Given the importance of the chemokine domain of FKN in binding and functional responses of CX3CR1-expressing cells, our studies primarily addressed the roles of specific amino acids in the FKN-CD in interacting with the receptor. A single N-linked glycosylation site is present in the human and rat orthologs of FKN and, according to structural data, is spatially available for post-translational modification (17). Although the human and rat FKN molecules have the potential N-glycosylation site (CNITC), mouse FKN does not contain an N-glycosylation site in the CX3C bulge (CEIMC), indicating that glycosylation may not be important. However, murine FKN does not support arrest of CX3CR1-expressing cells under flow (20). Furthermore, evidence that N-glycosylation may play a role in the ability of the receptor to mediate CX3CR1-mediated chemotaxis in vivo (21).
the human protein. The relative migration of the NIT to EIM FKN mutant in SDS-PAGE was not significantly different than the mobility of the wild type FKN. Furthermore, these amino acid changes did not affect binding and functional properties, including affinity for the receptor, stimulation of intracellular calcium mobilization, potency, and efficacy in promoting chemotaxis and cell adhesion.

There are six basic residues present within the FKN-CD that are conserved in human, rat, and murine FKN. The similar binding affinities of human and rodent FKN for rat CX3CR1 (9) suggests that conserved residues in FKN are the likely amino acid residues that interact directly with motifs present in the receptor. Mutation of each of these residues to an alanine appeared to have little effect on the global conformation of the

FIG. 6. Expression analysis of “full-length” huFKN-CD mutants in EA.hy 926 cells. EA.hy 926 cells stably transfected with various full-length FKN-CD mutants were tested for surface expression by flow cytometry. Shown is the expression of FKN as determined using mAb 1D6 (dark histograms) that recognizes the mucin domain. Background staining with the control mAb P3 is indicated by the clear histograms.
FKN-CD. This conclusion is based on two observations from the mAb epitope mapping studies. First, in general the mAbs used in this study reacted as efficiently with the mutants as they did with the wild type FKN. Second, those mutants that showed reduced mAb reactivity (e.g. M15K, K36A, and R37A) did not display significant differences in binding affinities, stimulation of calcium mobilization, or cell adhesive properties; a slight reduction in the potency of the M15K mutant at stimulating chemotaxis was seen. Our approach identified two key residues, namely Lys-7 and Arg-47, as important amino acids for interaction with CX3CR1. These mutant FKNs displayed reduced affinity for CX3CR1 in steady state whole cell binding assays and did not support either efficient capture or firm adhesion of CX3CR1-expressing cells under physiological flow. However, despite similar effects of these mutations on FKN-CX3CR1-binding interactions, differential effects of these mutations on CX3CR1 signaling were observed. Mutation of Arg-47 produced a protein largely ineffective at stimulating either intracellular calcium mobilization or chemotaxis, at least at the concentrations tested. However, mutation of Lys-7 yielded a molecule that retained most of the agonist activity, although maximal chemotaxis of the L1.2 cells expressing CX3CR1 toward the K7A mutant was slightly reduced. More notable, the potency of this mutant in this assay was similar to the wild type FKN, which was not expected based on measures of affinity in the whole cell binding assays. This difference may reflect receptor host cell differences (L1.2 versus CHO). Alternatively, K7A may be stimulating chemotaxis via a state of CX3CR1 that is not sensitive to mutation at this amino acid position in FKN. Measurement of the relative binding affinities of the mutant panel in the whole cell binding assay is based upon inhibition of the binding of subsaturating levels of $^{125}$I-FKN-CD and thus represents binding to a high affinity state of the receptor. This state of the receptor may be all or partially distinct from the population of receptors that mediate the chemotaxis response. Nonetheless, the results identify regions of the FKN-CD that differentiate binding and signaling properties of the FKN molecule.

Slight reductions in steady state binding affinity and potency in the chemotaxis assay were observed for the M15K mutant. Met-15 was previously suggested to be a key determinant in the interaction of the FKN-CD with CX3CR1. From solution NMR measurements of bacterially expressed protein, Mizoue et al. (17) determined that this residue displayed a significant chemical shift upon titration with a chemically synthesized peptide corresponding to the N terminus of human CX3CR1. Our approach toward identifying amino acids in FKN important for CX3CR1 binding and signaling was based on investigation of these interactions using longer forms of the FKN molecule, expressed by mammalian cells as either a membrane-attached or secreted form, as well as full-length, mammalian cell-expressed receptor. Thus, the identification of Lys-7 and Arg-47 as critical amino acids suggests that regions other than the N terminus of CX3CR1 contribute to the binding energy. Furthermore, post-translational modifications of the receptor may also confer FKN binding determinants.
Shed forms of FKN are evident in both transfected and native cell systems (3, 9). Transfection of 293 cells with the full-length form of FKN leads to the detection of both cell surface protein as well as protein in the conditioned media (3). Brief exposure of primary rat neurons with glutamate leads to increased levels of shed FKN and corresponding decreases in membrane-attached FKN. This neurotoxicity-induced proteolytic cleavage of FKN may be mediated by specific proteases as the matrix metalloproteinase inhibitor batimastat prevents this cleavage (20). Alterations in the levels of low molecular weight forms of FKN are also evident in the facial motor nucleus after peripheral nerve injury and may play a role in neuronal regeneration (9). The presence of various forms of shed FKN could suggest distinct physiological roles for the cleaved forms of the protein. Previously published data (4, 6, 18) indicated that FKN-CD and FKN-CD with the entire mucin stalk were equally effective at binding and stimulating CX3CR1; these earlier studies did not address the functionality of the FKN-CD with intermediate lengths of the mucin stalk. In order to examine possible biphasic effects of the mucin stalk region (i.e. determine if shorter stalks reduced or enhanced a FKN-CX3CR1 interaction), we generated five soluble recombinant FKN constructs in which the FKN-CD contains various lengths of the mucin stalk. In this domain, the physiological significance of potentially distinct forms of shed FKN remains to be determined. Furthermore, the context within which the receptor is expressed, i.e. the specific cell type, may determine the differential sensitivity toward various shed forms of FKN.

In summary, these data exemplify the importance of amino acid residues within the FKN-CD as the critical determinants in an FKN-CX3CR1 interaction. The structure of the FKN-CD has been reported using both NMR and x-ray crystallography (17, 21). Considering this structural information, the side chains of Lys-7 and Arg-47, while remotely distant from one another, appear to extend out from the same face of the FKN-CD (Fig. 8). Thus, it is conceivable that both of these residues are simultaneously interacting with specific, different amino acid residues found in the extracellular domain of CX3CR1. Mutation of both residues interfered to a similar extent, with the ability of FKN to bind to CX3CR1, as measured in steady state binding and adhesion assays. However, the K7A mutant retained the potency and most of the efficacy of the FKN for stimulating chemotaxis of CX3CR1-expressing cells. The results indicate that residues within the FKN-CD differentially regulate binding to and activation of CX3CR1. Site-directed mutagenesis of other chemokines has revealed similar interactions between these chemokines and their cognate receptors. For instance, mutation of the Arg residue in the ELR motif of interleukin-8 (equivalent to Lys-7 in FKN) yields a molecule that displays reduced affinity for its neutrophil receptor (22). Similarly, mutation of Lys-49 in MCP-1, an amino acid found in the analogous position as Arg-47 of the FKN-CD, yields a mutant with reduced affinity for CCR2 (23). Therefore, common mechanisms of chemokine-chemokine receptor protein interactions seem likely. The identification of the specific counter amino acids residing on the receptor, which interact specifically with the FKN-CD, will require additional experimentation and will have to include further mutational analysis and/or structural determination of the receptor in complex with the ligand. Toward the latter goal, the recent demonstration of the x-ray crystal structure of rhodopsin (24) suggests that more precise structures of other members of the G-protein-coupled receptor superfamily may be forthcoming. Identification of specific FKN-CX3CR1 interactions will aid future development of therapeutic agents that target this receptor system in disease.

Acknowledgments—We thank Aron Boney for performing the FKN ELISA assays and Drs. Art Edison for help with constructing the FKN-CD model, Wolfgang Streit for assistance in culturing rat microglia, and Stephen Baker for critical discussions of the data.

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Mutational Analysis of the Fractalkine Chemokine Domain: BASIC AMINO ACID RESIDUES DIFFERENTIALLY CONTRIBUTE TO CX3CR1 BINDING, SIGNALING, AND CELL ADHESION
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J. Biol. Chem. 2001, 276:21632-21641.
doi: 10.1074/jbc.M010261200 originally published online March 8, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010261200

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