Ultrastructure and Function of the Fractalkine Mucin Domain in CX₃C Chemokine Domain Presentation

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Fractalkine (FKN), a CX₃C chemokine/mucin hybrid molecule on endothelium, functions as an adhesion molecule to capture and induce firm adhesion of a subset of leukocytes in a selectin- and integrin-independent manner. We hypothesized that the FKN mucin domain may be important for its function in adhesion, and tested the ability of secreted alkaline phosphatase (SEAP) fusion proteins containing the entire extracellular region (FKN-SEAP), the chemokine domain (CX₃C-SEAP), or the mucin domain (mucin-SEAP) to support firm adhesion under flow. CX₃C-SEAP induced suboptimal firm adhesion of resting peripheral blood mononuclear cells, compared with FKN-SEAP, and mucin-SEAP induced no firm adhesion. CX₃C-SEAP and FKN-SEAP bound to CX₃CR1 with similar affinities. By electron microscopy, fractalkine was 29 nm in length with a long stalk (mucin domain), and a globular head (CX₃C). To test the function of the mucin domain, a chimeric protein replacing the mucin domain with a rod-like segment of E-selectin was constructed. This chimeric protein gave the same adhesion of peripheral blood mononuclear cells as intact FKN, both when immobilized on glass and when expressed on the cell surface. This implies that the function of the mucin domain is to provide a stalk, extending the chemokine domain away from the endothelial cell surface to present it to flowing leukocytes.

Leukocyte trafficking of cells out of the bloodstream and into sites of inflammation requires multiple steps (1, 2). In the classic pathway of leukocyte migration, the first step involves transient, selectin-mediated interactions between the rolling leukocytes and the endothelium (3, 4). In the next step, integrins are activated by locally produced chemokines to induce firm adhesion of the leukocyte to endothelial cells (5–7). Leukocytes then extravasate through the vascular wall and into the tissue.

Recently, a new pathway by which leukocytes can be induced to firmly adhere to endothelium and traffic into inflamed tissues has been described. This process is mediated by the chemokine fractalkine (FKN), expressed on endothelial cells, and its G-protein coupled receptor, CX₃CR1, expressed on PBMC. Fractalkine (neurotactin in the mouse) is the first, and thus far only, member of the CX₃C chemokine family, so named because the first two conserved cysteine residues are separated by three amino acids (8, 9). Another unique characteristic of fractalkine is that it is expressed on the cell surface. By nucleotide sequence analysis, fractalkine consists of a chemokine head tethered to the cell surface by a mucin stalk, followed by a single transmembrane spanning domain and a short cytoplasmic tail (8, 9). In addition, fractalkine expression on endothelium is increased by proinflammatory cytokines interleukin-1 and tumor necrosis factor-α. The receptor for fractalkine, CX₃CR1 (V28), is expressed on T cells, monocytes, macrophages, and natural killer cells (10, 11). Fractalkine and CX₃CR1 function as cell adhesion molecules under both static and dynamic conditions (10, 12). Unlike other chemokine/G-protein coupled receptor interactions that require signal transduction and integrin activation for cell adhesion to occur, the adhesive interaction between fractalkine and CX₃CR1 is independent of signal transduction or integrin function (12, 13). Therefore, fractalkine and CX₃CR1 provide an integrin-independent mechanism for leukocyte migration.

Compared with other chemokines, fractalkine has at least three unique features that may mediate its function as a cell adhesion molecule: 1) it is a transmembrane molecule with a cytoplasmic tail that may participate in signal transduction; 2) it has a mucin domain; 3) it is the only CX₃C chemokine and has a three-dimensional structure that is slightly different from other chemokines (14). In this report, we sought to determine the role of the various fractalkine domains, but in particular the mucin domain, in cell adhesion.

EXPERIMENTAL PROCEDURES

Fusion Proteins—FKN-SEAP, CX₃C-SEAP, and mucin-SEAP fusion proteins were produced as described previously (10). A plasmid encoding CX₃C-ESCR hybrid molecules was generated by polymerase chain reaction and subsequent ligation of the DNA fragments into pDREF-Hyg-SEAP. DNA encoding the CX₃C domain was synthesized using primers 5'-CGCGTGCAGCTCACTGATGGCTCCGATA-3' and 5'-CGCA- GATCTTTAGGGCCAGACGCTGGCGCC-3', digested with SacI and BglII. DNA encoding the E selectin consensus repeat (ESCR) domain was generated by polymerase chain reaction amplification of E-selectin cDNA in pM2 (15) using the primers 5'-CGCGATCTCTATTTGCTGAGC- TGTTACGCG-3' and 5'-GGGGTCTAGATTAACAGTGGTAGTACCAG-3'. These fragments were digested with BglII and XbaI and then ligated together into the pDREF-Hyg SEAP expression vector digested with SacI and XbaI. The nucleotide sequence of both strands of the new construct (pCX₃C-ESCR-SEAP) was determined to verify its identity. A plasmid encoding a CX₃C-ESCR-GFP fusion protein in which the mucin domain of full-length fractalkine was replaced by ESCR was constructed by amplifying the transmembrane and cytoplasmic tail of fractalkine by polymerase chain reaction using primers 5'-CGCCTCTA- GACAGCGTGTTGGGGCTGCTG-3' and 5'-CTAGAGATCCCGACGG- CACAGGAC-3'. This fragment and the CX₃C-ESCR fragment described above (prepared by digesting pCX₃C-ESCR-SEAP with SacI and BglII) were ligated together into the pDREF-Hyg SEAP expression vector digested with SacI and XbaI.

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and XbaI) were cloned into the pEGFP-N2 expression vector (CLONTECH, Palo Alto, CA) digested with XbaI and BamHI. The nucleotide sequence of the resulting plasmid (pCX3C-ESCR-GFP) was determined for verification. SEAP fusion proteins were produced in mammalian 293/EBNA-1 cells and soluble fractalkine was generated in a baculovirus system as described previously (10).

Cell Culture and Transfection—K562 erythroleukemic cells were grown at 37°C in RPMI 1640 medium (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum and supplemented with penicillin/streptomycin. 293/EBNA-1 cells were grown in Medium 199 (Life Technologies, Inc.) containing 10% fetal bovine serum, ECV-304 cells were grown in Medium 199 (Life Technologies, Inc.) containing 10% fetal bovine serum and supplemented with penicillin/streptomycin. 293/EBNA-1 cells were grown in Medium 199 (Life Technologies, Inc.) containing 10% fetal bovine serum and supplemented with penicillin/streptomycin. 293/EBNA-1 cells and soluble fractalkine were generated in a baculovirus system as described previously (10).

Gradient Sedimentation and Rotary Shadowing—Samples of fractalkine-SEAP or of fractalkine alone were layered on 15–40% glycerol gradients in 0.2M ammonium bicarbonate and centrifuged in a SW50Ti rotor (Beckman) at 30,000 rpm for 16 h at 20°C. Sedimentation standards (catalase, 11.3 S; bovine serum albumin, 4.6 S; and ovalbumin, 3.5 S) were centrifuged in a separate gradient. Gradients were fractionated into 12 fractions, and the protein peaks were identified by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. The molecular weights (in Kd) of selected standards are indicated. Data are representative of three different experiments.

Gradient Sedimentation of FKN-SEAP (A) and FKN (B). Shown are proteins in the 12 fractions of glycerol gradients after SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. The lines indicate the positions of standard proteins run in separate gradients. The second lane from the right contains the starting material (L) located on the gradient, and the last lane contains standards. The molecular weights (in Kd) of selected standards are indicated. Data are representative of three different experiments.

Receptor Binding Assay—The kinetics of receptor binding were determined as described previously (10). Briefly, 2 x 105 L1.2 cells expressing CX3CR1 (V28) were incubated with various concentrations of FKN-SEAP, FKN-ESCR-SEAP, and CX3C-SEAP for 1 h at 16°C in 200 µl of RPMI containing 20 mM HEPES (pH 7.4), 1% bovine serum albumin, and 0.02% sodium azide. Cells were then washed and lysed in 50 µl of 10 mM Tris (pH 8), 1% Triton X-100 and heated at 65°C for 10 min. Following centrifugation, lysates were collected and alkaline phosphatase activity was determined by chemiluminescence. Binding data were analyzed by the LIGAND program.

Flow Cytometry—The level of cell surface expression was determined by labeling the transfected cells with antibodies against either the chemokine domain of fractalkine (4F2) or the mucin domain (1D6) as described previously (12). The secondary reagent used was phycoerythrin-conjugated goat anti-mouse IgG (Sigma). Analysis was performed on a Coulter Epics XL flow cytometer (Beckman-Coulter, Fullerton, CA) and data analyzed using CellQuest software (Becton-Dickinson, Mountain View, CA).
RESULTS

To determine which domains of fractalkine may be necessary for leukocyte capture and firm adhesion, we constructed fusion proteins of the chemokine (CX3C) and mucin domains separately linked to SEAP. Based on our previous studies in which immobilized fusion proteins containing the entire extracellular region of fractalkine (FKN-SEAP) functioned as well in leukocyte capture and firm adhesion as cells expressing fractalkine, we reasoned that the extracellular domains (CX3C and mucin) contained all of the structures necessary for fractalkine-mediated cell adhesion (12). Both PBMC and K562 cells expressing CX3CR1 (K562-CX3CR1) on the cell surface were able to firmly adhere to immobilized fractalkine-SEAP but not to control SEAP proteins alone (Fig. 1A). CX3C-SEAP induced capture and firm adhesion of resting PBMC, but at a reduced number compared with FKN-SEAP (17 × 10^5 versus 10^6 cells/mm², p = 0.005). In contrast, no interactions or firmly adherent cells were observed in association with immobilized mucin-SEAP. These results suggested that the chemokine domain provided the specificity of interaction with CX3CR1 and that the mucin domain acted to increase the affinity or avidity of CX3CR1 interaction.

The mucin domain could have acted either as a co-receptor with the CX3C domain or as an extender to present the CX3C domain to CX3CR1-expressing cells. To determine if the mucin domain may itself act as a cell adhesion molecule, mucin-SEAP fusion proteins were tested for their ability to bind to K562-CX3CR1-expressing cells. To determine if the mucin domain could act as a stalk or presentation molecule, we determined the ultrastructure of the fractalkine extracellular domain. Information on the structures of FKN-SEAP and soluble FKN was obtained by glycerol gradient sedimentation. Fig. 2 shows SDS-polyacrylamide gel electrophoresis of the two proteins after fractionating the gradients. Each ran as a discrete peak, indicating a homogeneous sample. FKN-SEAP showed a broad band at ~150 kDa on SDS-polyacrylamide gel electrophoresis. The predicted mass of the protein backbone of FKN-SEAP is 100.6 kDa, indicating that FKN-SEAP has approximately 50 kDa of O-linked carbohydrates. FKN-SEAP sedimented between catalase and bovine serum albumin, at an estimated 6.3 S. For a hydrated sphere that could contain the protein mass, would be 1.1 if the protein were a monomer, and 1.7 if it were a dimer. If the protein were a dimer, the frictional coefficient for an unhydrated sphere that could contain the protein mass, would be 1.1 if the protein were a monomer, and 1.7 if it were a dimer. Each run as a discrete peak, indicating a homogeneous sample. FKN-SEAP showed a broad band at ~150 kDa on SDS-polyacrylamide gel electrophoresis. The predicted mass of the protein backbone of FKN-SEAP is 100.6 kDa, indicating that FKN-SEAP has approximately 50 kDa of O-linked carbohydrates. FKN-SEAP sedimented between catalase and bovine serum albumin, at an estimated 6.3 S. For a hydrated sphere that could contain the protein mass, would be 1.1 if the protein were a monomer, and 1.7 if it were a dimer.
Baculovirus-expressed FKN sedimented much more slowly than FKN-SEAP, giving a peak near the top of the gradient. Based on the calculated mass of the protein backbone of soluble FKN (36.6 kDa), the baculovirus-derived FKN which migrated as a diffuse band between 46 and 56 kDa contains 10 to 20 kDa of O-linked carbohydrates. Since FKN ran well behind our slowest standard (ovalbumin at 3.5 S) we could only estimate the S by extrapolation, which was not very accurate. Values from 0.6 to 2.5 S were estimated in three different experiments. These values are consistent with an elongated 50-kDa protein. See Ref. 18 for more extensive examples of \( f/f_{\text{min}} \) for elongated proteins.

Electron microscopy of FKN-SEAP (Fig. 3) showed a large central globular particle with two thin strands projecting from them. These thin strands are identified as the mucin stalk, and had a length of 26 nm. The mucin stalk was capped by a small globular domain about 3 nm in diameter, which we identify as the cytokine domain. The entire length of the FKN molecule is 29 nm.

The structure of the mucin domain as a long stalk suggested that it functioned to extend the chemokine domain for optimal interaction with cell surface CX3CR1. To confirm that the mucin domain acted as an extender and not as a co-receptor, we replaced the mucin domain in FKN-SEAP with the six short consensus repeat segments of E-selectin (Fig. 4A). ESCR is expected to have the same length (approximately 26 nm) as the mucin domain (19) and does not have any intrinsic binding capacity (15). Soluble CX3C-ESCR bound to L1.2-CX3CR1 cells with the same characteristics as CX3C-SEAP and FKN-SEAP (\( K_d = 130 \mu M \)) (Fig. 4B). Immobilized CX3C-ESCR functioned as well as FKN-SEAP in the capture and firm adhesion of resting PBMC and K562-CX3CR1 under physiologic flow conditions (Figs. 4C). These data indicated that extension was the important feature of the mucin domain.

While the above studies indicate that the mucin domain could be functionally replaced by a stalk of similar length (ESCR), they were performed with purified fusion proteins immobilized on glass coverslips. To determine whether ESCR could functionally replace the fractalkine mucin domain in the context of the cell surface, two chimeric molecules were constructed: a transmembrane fractalkine-green fluorescent protein (FKN-GFP) molecule; and a CX3C-ESCR-GFP fusion protein in which the mucin domain of FKN-GFP was replaced with the ESCR domain (CX3C-ESCR-GFP). Stable ECV-304 transfectants expressing similar surface levels of native fractalkine, FKN-GFP, and CX3C-ESCR-GFP were isolated by fluorescence-activated cell sorting using monoclonal antibodies recognizing the CX3C (4F2) domain of fractalkine. As expected, mCX3C-ESCR-GFP was recognized by monoclonal antibody 4F2, but not 1D6, an antibody against the mucin domain of fractalkine (Fig. 5). Expression of a GFP tag on the cytoplasmic tail of fractalkine did not affect its function in cell adhesion (Fig. 6). ECV304 cells expressing mCX3C-ESCR-GFP induced capture and firm adhesion of CX3CR1-expressing K562 cells as efficiently as ECV304-FKN cells (Fig. 6).

**DISCUSSION**

In this paper we sought to determine the role of the fractalkine mucin domain in the capture and firm adhesion of CX3CR1-expressing leukocytes. From the results presented here, we conclude the following. 1) The FKN mucin domain enhances the ability of CX3C to function in cell adhesion. The CX3C chemokine domain of FKN is sufficient for the rapid capture and firm adhesion of circulating leukocytes, but the capture is more efficient with the mucin domain. 2) The mucin domain does not significantly alter the affinity of soluble CX3C binding to CX3CR1. 3) The mucin domain does not inherently function as a cell adhesion molecule or co-receptor. 4) The FKN...
mucin domain exists as an approximately 26 nm long stalk between the globular CX3C domain and the cell surface. 5) A primary function of the mucin domain in cell adhesion is to extend the CX3C chemokine domain away from the cell surface to present it to flowing leukocytes.

Mucins, including many selectin ligands like PSGL-1, are highly O-glycosylated and exist as long, thin stalk-like structures (20). For most selectin ligands, the O-linked carbohydrates are essential components for binding (21, 22). FKN has 26 potential O-linked glycosylation sites, and is heavily glycosylated (8, 9). Consistent with other mucins, the FKN mucin domain has a long stalk-like ultrastructure, but we could not detect any binding function of the mucin domain. In this respect, the FKN mucin domain shares similarity with the short consensus repeats of L-, E-, and P-selectin that function to extend the ligand-binding lectin stalk that functions to extend the chemokine domain away from the cell surface for optimal interaction of CX3C with its receptor. The FKN mucin domain is functionally similar to the stalk regions of the selectin family of molecules that also function in leukocyte capture and firm adhesion under physiological flow conditions. FKN and CX3CR1 may also share other functional similarities with the selectins and their ligands.

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