Identification of CX₃CR1
A CHEMOTACTIC RECEPTOR FOR THE HUMAN CX₃C CHEMOKINE FRACTALKINE AND A FUSION CORECEPTOR FOR HIV-1*

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Fractalkine is a multimodular human leukocyte chemoattractant protein and a member of the chemokine superfamily. Unlike other human chemokines, the chemokine domain of fractalkine has three amino acids between two conserved cysteines, referred to as the CX₂C motif. Both plasma membrane-associated and shed forms of fractalkine have been identified. Here, we show that the recombinant 76-amino acid chemokine domain of fractalkine is a potent and highly specific chemotactic agonist at a human orphan receptor previously named V28 or alternatively CMKBRL1 (chemokine β receptor-like 1), which was shown previously to be expressed in neutrophils, monocytes, T lymphocytes, and several solid organs, including brain. CMKBRL1/V28 also functioned with CD4 as a coreceptor for the envelope protein from a primary isolate of HIV-1 in a cell-cell fusion assay, and fusion was potently and specifically inhibited by fractalkine. Thus CMKBRL1/V28 is a specific receptor for fractalkine, and we propose to rename it CX₃CR1 (CX₃C chemokine receptor 1), according to an accepted nomenclature system.

The chemokine superfamily consists of specific leukocyte chemoattractant proteins that can be classified by structure into four groups, designated CC, CXC, C, and CX₃C, depending on the number and spacing of conserved cysteines (1–5). The only known CX₃C chemokines are human fractalkine (4) and its apparent mouse counterpart, named neurotactin (5). The C subgroup also only contains one known member in mouse and human, whereas both CC and CXC subgroups each have many members. Unlike other chemokines, fractalkine and neurotactin are both multimodular proteins consisting of four distinct domains: a chemokine domain N-terminal to an extended mucin-like stalk; a transmembrane domain, which tethers the molecule to the plasma membrane; and a cytoplasmic domain. After transfection of HEK 293 cells, human fractalkine can be detected on the cell surface, and the cells adhere to human monocytes, T cells, and neutrophils. In addition, a truncated 95-kDa “shed” form, which can be found in the supernatant, induces chemotaxis of monocytes and T cells (4). Consistent with this, the recombinant chemokine domain of mouse neurotactin (amino acids 22–105) induces in vitro chemotaxis of human neutrophils and T cells and in vivo accumulation of mouse neutrophils, monocytes, and T cells (5).

Based on sequence similarity between CC chemokines and the chemokine domain of fractalkine (4, 5), we reasoned that the putative receptor that mediates its action might be most closely related to known CC chemokine receptors (CCR1; 6–12), which are all members of the seven-transmembrane domain G protein-coupled receptor superfamily. In this regard, CMKBRL1 (chemokine β receptor-like 1) (13), a previously identified “orphan” receptor also known as V28 (14), stood out as a particularly good candidate because it has ~40% amino acid sequence identity to most CCRs; its gene is located on chromosome 3p21–3pter near a cluster of CCR genes (13–15); it is expressed in human neutrophils, monocytes, and T cells, which are targets for fractalkine and/or neurotactin (13, 14, 16), and its RNA, as well as RNA for fractalkine, are both present constitutively at high levels in human brain. CMKBRL1/V28 appears to be the human orthologue of RBS11, a rat orphan receptor expressed in leukocytes and brain (17).

Since identification of the chemokine receptor CXCR4 as the first HIV-1 coreceptor, several other chemokine receptors and chemokine receptor-like proteins have been shown to have similar activity for HIV-1 and in some cases for HIV-2 and simian immunodeficiency virus (18). Because of its sequence relationship to chemokine receptors, CMKBRL1/V28 has previously been tested and shown to be a coreceptor for certain isolates of HIV-2 (19). Here we identify functional interactions of CMKBRL1/V28 with HIV-1 and demonstrate that it is a receptor highly specific for fractalkine.

EXPERIMENTAL PROCEDURES

Production and Use of Anti-CX₃CR1 Antiserum—A synthetic peptide corresponding to amino acids 1–27 of the CMKBRL1/V28 sequence was produced by FMOC-[(9-fluorenylmethoxycarbonyl)amidomethyl]cysteine, conjugated to KLH, and injected into rabbits. Preimmune sera and sera harvested after serial boosts were tested for reactivity with various cell lines by flow cytometry using a previously published method (20) with two modifications. A donkey anti-rabbit phycocerythrin-conjugated F(ab')₂ (Jackson ImmunoResearch Laboratory, West Grove, PA) was used as the secondary antibody, and seven-aminomethylcoumarin D (Sigma) was used to gate dead cells. Sera harvested after the third immunization was used for all experiments at a dilution of 1:50.

Creation of Cell Lines Expressing Chemokine Receptors—The cloning of a 1.6-kilobase pair cDNA named clone 74.2 coding for CMKBRL1, construction of recombinant pBluescript and pREP9 plasmids containing this cDNA, and creation of HEK 293 cell lines stably transfected with pREP9-CMKBRL1 have been described previously (13). In addi-
tion, the cDNA was excised from Bluescript using BanHI (5') and XhoI (3') sites in the polylinker and subcloned between the corresponding sites of the mammalian expression vector pCDNA3 (Invitrogen, Carlsbad, CA), and additional HEK 293 cell lines were derived using previously described methods (13). The production of HEK 293 cell lines stably expressing CCR1, CCR5, and CCR6 have been previously described (9, 11, 21, 22). A CCR6 cell line was a generous gift of F. Liao and J. Farber (NIAID, Bethesda, MD). HEK 293 cell lines were maintained in DMEM with 10% fetal bovine serum containing streptomycin 100 μg/ml and penicillin 100 units/ml, supplemented with 1 mg/ml G418.

Intracellular [Ca2+] Measurements—Changes in [Ca2+]i were measured using ratio fluorescence of FURA-2-loaded cells in suspension exactly as described previously (21). The following recombinant human chemokines were tested: SDF-1, HCC-1, lymphoactin, and I-309 (R&D, Minneapolis, MN); neutrophil-activating peptide 2, Bachem (Philadelphia, PA); macrophage inflammatory protein (MIP)-1α, MIP-1β, MCP-4, etoxin, interleukin-8, growth-related oncogene α (GROα), GROβ, GROγ, and ENA-78 (Peprotech, Rocky Hill, NJ). cjk4 (also known as MIP-3α, exodus, and liver activation-related chemokine) was kindly provided by G. Garrotta (Human Genome Sciences, Rockville, MD). The 76-amino acid chemokine domain of fractalkine (amino acids 25–100) produced in Escherichia coli was purchased from R&D (product name = recombinant human fractalkine). In this paper, we will refer to this material as "fractalkine." Ligation Binding Analysis—Binding experiments were carried out using 125I-fractalkine obtained from Amersham Pharma Biotech (specific activity = 2200 Ci/mmol). After washing in phosphate-buffered saline, 106 cells were incubated in triplicate with 125I-labeled fractalkine and calcium flux response was concentration-dependent. We carried out a detailed study for the 3.2 cell line and the fractalkine-induced calcium flux response was concentration-dependent. We carried out a detailed study for the 3.2 cell line and the fractalkine-induced calcium flux response was concentration-dependent.

RESULTS

Surface Expression of CXC.CR1—We previously reported that HEK 293 cell lines stably transfected with a pREP9 plasmid encoding CMKBR1 expressed CMKBR1 RNA in large amounts but did not exhibit calcium flux responses to any agonists tested at the time, including the CC chemokines MIP-1α, RANTES, MIP-1β, MCP-1, MCP-2, MCP-3 and I-309; the CXC chemokines interleukin-8, IL-10, neutrophil-activating peptide-2, GROα, GROβ, platelet factor 4, and Mig; and the nonchemokine leukocyte chemoattractants formylmethionyl-leucylphenylalanine, C3a and C5a (13). Because of the functional specificity that we now describe, we have renamed CMKBR1/V28 as CXC.CR1 (CXC chemokine receptor 1), which is consistent with nomenclature guidelines established at the 2nd Gordon Conference on Chemotactic Cytokines (Plymouth, NH, 1996).

We created an additional set of HEK 293 cell transfectants using a pCDNA3 expression plasmid and screened them plus the first generation of transfectants for cell surface expression of CXC.CR1 based on reactivity with a polyclonal anti-CXC.CR1 rabbit antiserum. We selected two cell lines transfected with the pCDNA3 construct named 3.2 and 3.3 for further study because they exhibited the highest level of specific staining, which was consistently ~10-fold greater than either untransfected HEK 293 cells or HEK 293 cells expressing CCR5 and CXC.CR1-transfected cells stained with preimmune serum (Fig. 1 and data not shown).

Identification of Fractalkine as an Agonist for CXC.CR1—Neither CXC.CR1-expressing cell line exhibited a calcium flux response when stimulated with 100 nM interleukin-8, MCP-1, MCP-2, MCP-3, etoxin, MIP-1α, MIP-1β, RANTES, SDF-1, I-309, MIP-3α, or lymphoactin, whereas both cell lines consistently responded to 100 nM fractalkine (Fig. 1B and data not shown for the ineffective chemokines; n > 7 and = 3 for fractalkine for the 3.2 and 3.3 cell lines, respectively). Consistent with this, we did not detect endogenous RNA by Northern blot analysis for CXCR1, CXCR2, CXCR1–5, or CCR8 in these cells. As previously reported, HEK 293 cells stably transfected with CCR1, CCR5, or CCR6 responded to MIP-1α, MIP-1β, and liver activation-related chemokine, respectively (8, 9, 21, 22), but we observed no responses by any of these cell lines or by untransfected HEK 293 cells to fractalkine (Fig. 1B and data not shown). Thus, we infer from this gain-of-function result that fractalkine is a specific agonist acting at CXC.CR1 in this system.

For both the 3.2 and 3.3 cell lines, the relationship between fractalkine and calcium flux response was concentration-dependent. We carried out a detailed study for the 3.2 cell line (Fig. 1C) and found that the threshold, half-maximal, and saturating concentrations were 0.5, 2, and 10 nM, respectively. This is consistent with the potency range that we and others have reported for other chemokines acting at other chemokine receptors for this response. However, the potency of natural forms of soluble fractalkine and membrane-anchored fractalkine could differ, either in this system or at endogenous CXC.CR1.

When the cells were pretreated with ineffective chemokines at 100 nM, there was no effect on the magnitude or kinetics of the fractalkine-induced calcium flux response, suggesting that other chemokines are not antagonists at CXC.CR1 (Fig. 1B, top tracings). In contrast, when cells were sequentially stimulated with the same concentration of fractalkine, no response to the second application was observed (Fig. 1B, top right tracing), suggesting homologous desensitization of the receptor.

Fractalkine Is a High Affinity Ligand for CXC.CR1—To directly examine the interaction of fractalkine with CXC.CR1,
CX3CR1 is a Chemotactic Receptor—To test the biological function of CX3CR1, we carried out chemotaxis assays using the 3.2 cell line stably transfected with CX3CR1 cDNA. Fractalkine was able to significantly induce concentration-dependent transmigration of CX3CR1-expressing cells across a filter in a modified Boyden chamber assay of chemotaxis, but RANTES had no activity (Fig. 3). The dose-response curve was bell-shaped, which is typical for chemotaxis, and the optimal concentration and \( EC_{50} \) were 100 and 20 nM, respectively. Thus, by gain-of-function criteria we infer that fractalkine is a chemotactic agonist at CX3CR1 in this system.

CX3CR1 Is a Cofactor for HIV-1 Env-dependent Cell Fusion—To determine whether CX3CR1 functions as a coreceptor for HIV-1, we used a vaccinia-based \( \beta \)-galactosidase reporter assay for Env-dependent cell-cell fusion, which models the viral envelope-target cell fusion step in HIV-1 infection (26). This assay was used to identify CXCR4 as the first HIV-1 coreceptor (24), and subsequently several other chemokine receptors and chemokine receptor-like proteins have been shown to have similar activity using the same or related assays (see Ref. 18 for more and cell-type specific references). HIV-1 strains vary in the coreceptors they can use for cell entry, and this determines strain cytropotism. Among other classification schemes, classification of HIV-1 strains according to their ability to infect primary macrophages and use CCR5 (M-tropic or R5 strains) versus transformed T cell lines and CXCR4 (TCL-tropic or X4 strains) has been particularly useful. Some primary isolates
can infect both cell types and use both coreceptors and are classified as dual-tropic strains.

CX3CR1 was able to reconstitute fusion activity in target NIH 3T3 cells expressing CD4 when mixed with effector cells expressing Env from the dual-tropic primary isolate 89.6, inducing approximately half of the level of fusion found for the same Env tested with CXCR4- or CCR5-expressing target cells (Fig. 4). In contrast, no significant level of fusion was detected with Env from three R5-tropic isolates or with Env from a X4-tropic isolate. Thus, by gain-of-function criteria, we infer that CX3CR1 has HIV-1 Env coreceptor activity.

CX3CR1 was analyzed for its sensitivity to inhibition of Env-mediated fusion by various chemokines. Fractalkine, but not any other chemokine tested, was able to inhibit fusion mediated by 89.6 Env-expressing effector cells with CD4- and CX3CR1-expressing target cells (Fig. 5A). Fractalkine inhibited the β-galactosidase signal to background levels. To determine whether the inhibitory effect of fractalkine on fusion was specific for CX3CR1 and not other HIV-1 coreceptors, its effect on fusion mediated by CXCR4 and CCR5 with the 89.6 Env was assayed in a parallel experiment. No significant inhibition of fusion was seen with either CXCR4 or CCR5 (data not shown). Fractalkine inhibited CX3CR1-dependent fusion activity for the 89.6 Env in a concentration-dependent manner (IC50 = 5 ± 1 nM, n = 3 experiments; Fig. 5B). This value correlates well with the IC50 for homologous competition of fractalkine binding to CX3CR1-expressing HEK 293 cells and the EC50 values for fractalkine induction of calcium flux. Complete inhibition was seen at concentrations of 100 nM or greater.

DISCUSSION

In summary, we have shown by four different assays, direct radioligand binding, ligand-induced calcium flux and chemotaxis, and ligand inhibition of HIV-1 coreceptor activity, and by both gain and loss of function criteria that fractalkine is a highly specific ligand for the previously identified orphan receptor CMKBL1/V28, thus justifying renaming it as CX3CR1 according to guidelines of an accepted nomenclature system. While this work was under review, Imai et al. (27) also reported that fractalkine is a functional ligand for the same receptor and also renamed it CX3CR1. In addition to showing as we have here that the receptor mediates chemotactic responses to a soluble form of fractalkine, they also showed that it directly...
mediates cytoadhesion to cells expressing fractalkine on the
surface.

Since RNA for CX₃CR1 is expressed in neutrophils, mono-
cytes/macrophages, and T lymphocytes, it is an excellent can-
didate for the cellular receptor involved in fractalkine-depend-
ent signal transduction in these cell types that have previously
been described; however, specific blocking agents will have to
be developed before this can be specifically tested. Based on its
in vitro properties, future research will be directed toward
examining the role of CX₃CR1 in specific leukocyte trafficking
in vivo. Like other leukocyte chemotactants and chemato-
tractant receptors, fractalkine and its receptor have the poten-
tial to function benefically, for example in host defense and
tissue repair, as well as harmfully, for example in immunolog-
ically mediated inflammatory diseases. In this regard, our
identification of CX₃CR1 may be useful in future research
aimed at evaluating the fractalkine signal transduction path-
way as a target for development of potential anti-inflammatory
therapies.

Also while this paper was in review, Rucker et al. (28) re-
ported that CX₃CR1 functions as a weak fusion cofactor for a small percentage of HIV-1 Envs, including the 89.6 Env and then infected with vP11T7gene1. Target NIH 3T3 cells were transfected with a plasmid expressing CX₃CR1 and then coinfected with vCB-3 and vCB21RLacZ. A, CX₃CR1-mediated fusion inhibition is specific for fractalkine. The indicated chemokines were preincubated with target cells at a concentration of 2 μM for 30–45 min at 37 °C. Upon mixing of the two cell populations in the fusion reaction, the chemokines were dilute to a final concentration of 1 μM. B, dose response of fractalkine inhibition of CX₃CR1-mediated fusion. The indicated concentrations of fractalkine were tested. The dashed line indicates the IC₅₀. Data are representative of three separate experiments.

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