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Two Novel Fully Functional Isoforms of CX3CR1 Are Potent HIV Coreceptors¹

Alexandre Garin, Nadine Tarantino, Sophie Faure, Mehdi Daoudi, Cédric Lécureuil, Anne Bourdais, Patrice Debré, Philippe Deterre, and Christophe Combadière²

We identified two novel isoforms of the human chemokine receptor CX3CR1, produced by alternative splicing and with N-terminal regions extended by 7 and 32 aa. Expression of the messengers coding these isoforms, compared with that of previously described V28 messengers, is lower in monocytes and NK cells, but higher in CD4⁺ T lymphocytes. CX3CR1 and its extended isoforms were expressed in HEK-293 cells and compared for expression, ligand binding, and cellular responses. In steady state experiments, all three CX3CR1 isoforms bound CX3CL1 with similar affinity. In kinetic binding studies, however, kₘₐₜ and kₘₐₜ were significantly greater for the extended CX3CR1 isoforms, thereby suggesting that the N-terminal extensions may alter the functions induced by CX3CL1. In signaling studies, all three CX3CR1 isoforms mediated agonist-dependent calcium mobilization, but the EC₅₀ was lower for the extended than for the standard isoforms. In addition, chemotactic responses for these extended isoforms shifted left, also indicating a more sensitive response. Finally, the longer variants appeared to be more potent HIV coreceptors when tested in fusion and infection assays. In conclusion, we identified and characterized functionally two novel isoforms of CX3CR1 that respond more sensitively to CX3CL1 and HIV viral envelopes. These data reveal new complexity in CX3CR1 cell activation and confirm the critical role of the N-terminal domain of the chemokine receptors in ligand recognition and cellular response. The Journal of Immunology, 2003, 171: 5305–5312.

Chemokines are involved in diverse physiological and pathological conditions, mainly through their deployment of leukocytes (1–3). They mediate their effects via specific interactions with extracellular regions of chemokine receptors expressed at the leukocyte cell surface. The N-terminal domain of these receptors has been described as a crucial determinant in ligand binding and signaling (4–7), and thus alterations in this domain may be of critical importance. Furthermore, the N-terminal domain plays a key role in interacting with the HIV envelope gp120 (8–10), at least for CCR5 and CXCR4, the two principal HIV coreceptors (11–13). Abs raised against the N terminals of CXCR4 and CCR5 inhibit cell fusion and infection with HIV-1 (8, 14), and the first 20 N-terminal residues of CCR5 function as a coreceptor when grafted onto a CCR2 backbone (15, 16). A current model of chemokine binding suggests a two-step mechanism involving initial recognition of the chemokine receptor N-terminal domain, followed by more specific interactions with the other extracellular domains. Recent findings of post-transcriptional modifications in the N-terminal domain of chemokine receptors indicate that regulation of receptor function is still more complex.

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Materials and Methods
Cells and culture conditions
Resting PBMCs of heparinized venous blood taken from healthy volunteers were isolated by a one-step centrifugation on a Ficol separating solution (Biochrom, Berlin, Germany). CD8+ T lymphocytes, CD14+ T lymphocytes, and NK cells were then isolated by positive selection through the use, respectively, of anti-CD4 mAbs, anti-CD8 mAbs, and a mixture of anti-CD16 and anti-CD56 mAbs coupled with magnetic beads (Miltenyi Biotec, Paris, France). Negative selection, using a mixture of anti-CD2, -CD7, -CD16, -CD19, and -CD56 mAbs coupled with magnetic beads (Dynal, Compiègne, France), was used to enrich monocytes. Flow cytometric analysis confirmed that the enriched cell populations were 95% pure. U373MG-C4, HeLa, and HEK-293 cell lines were maintained in DMEM (Life Technologies SARL, Cergy Pontoise, France), supplemented with 10% FCS, 1 mM l-glutamine, and penicillin-streptomycin (100 U/ml). Cells used for HIV fusion and infection were provided by Dr. M. Alizon (Institut National de la Santé et de la Recherche Médicale, Unité 567, Université Paris V-Rene Descartes, Paris, France). The U373MG-C4 cells express the CD4 receptor and the lacZ gene driven by the long terminal repeat element. HeLa-Env_LAI cells express HIV-1 LAI, HeLa-Env_ADA cells express HIV-1 ADA envelope glycoproteins, and both express HIV-1 trans-activator Tat.

RNA isolation and cDNA synthesis
Total RNA was extracted with the RNeasy Kit (Qiagen, Hilden, Germany) from 10^7 CD4+ T lymphocytes, monocytes, and NK cells from two healthy blood donors. Total RNA of 10^7 CD8+ T lymphocytes was extracted from two other healthy blood donors. Treatment with DNase I (30 U) prevented any genomic DNA contamination. Samples were then heated to denature DNase I. For each sample 1 µg of total RNA was reverse transcribed for 1 h at 37°C with an RT-PCR kit (Stratagene, La Jolla, CA).

SYBR-Green quantitative PCR
The SYBR-Green PCR kit (Applied Biosystems, Foster City, CA) was used according to the manufacturer’s instructions in a model 7700 sequence detector (Applied Biosystems). For each PCR, 150 nM forward (clone 10–3P1), AGAGCTCCTGGCTGTTCCGGAGAA; 28–3P1, TGCGCTAGTGGCAGATCCAGAGGT) and reverse (RACE-3P1, GGCCCTGCAAGAAGATGAGGGATA) primers were used to amplify 1 µM cDNA. Fluorescence was detected at the end of each elongation phase.

CX3CR1 isoform constructs
The open reading frame (ORF) corresponding to the CX3CR1 protein isoform Met+1, Met−7, and Met−32 were produced from cDNA specific from clone 10 mRNA. Forward primers, designed to be specific to the 5’ end of each isoform, were HindIII-tailed in the 5’ end (+32 clone), GGCGATATA AGCTGCACCATGAGAGAACTGAGGCGG; +7 clone, GCG CATATAAGCTTGCCACCATGGATCAGTTCCCTGAATCAGTG). The reverse primer, common to all isoforms, was chosen to be specific to the 3’ end of the CX3CR1 ORF and was XhoI-tailed in its 5’ end (LT3-CX3CR1, GCG CATATAAGCTTGCCACCATGAGAGAACTGAGGCGG). The PCR conditions were as follows: denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The PCR products were then digested with HindIII/XhoI, subcloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen, Leek, The Netherlands), and then sequenced on both strands with the BigDye Terminator kit (Applied Biosystems, Warrington, U.K.).

Stable expression of CX3CR1 isoforms by HEK-293 cells
Two micrograms of each expression vector were transfected by 6 µg of Transfast (Promega, Charbonnieres, France) into 10^6 HEK-293 cells grown in DMEM. Two days later, transfected cells were selected with 1.5 µg/ml G418 (Life Technologies, Gaithersburg, MD), and cell clones were isolated and tested for CX3CR1 expression by FACS analysis with FITC-anti-CX3CR1 Ab (MBL, Nagoya, Japan). Clones expressing each isoform protein were maintained in DMEM containing 0.5 µg/ml G418. Three stable clones of each CX3CR1 isoform were independently assayed in further functional assays.

Generation of specific Met−32 isoform polyclonal Abs
Rabbit polyclonal Abs were raised against the human Met−7 and Met−32 CX3CR1 isoforms (AGRO-BIO, La Ferrière Saint Aubin, France) and further purified.

Western blot analysis of CX3CR1 protein
HEK-293 cells were suspended at 10^6 cells/ml in 20 mM Tris (pH 7.5), 1 mM EDTA, and 1 mM DTT supplemented with complete protease inhibitor cocktail (Roche, Meylan, France). The cells were then homogenized at 4°C in a Dounce homogenizer (Kontes, Vineland, NJ). Nuclear and cellular debris was removed by centrifugation for 10 min at 10,000 × g. The supernatant was then centrifuged at 100,000 × g at 4°C for 30 min. The pellet was suspended in lysis buffer (20 mM Tris, 140 mM NaCl, 1 mM EDTA, and 0.1% Nonidet P-40, pH 7.4) supplemented with complete protease inhibitor cocktail. The samples were then assayed for protein content and resuspended in sample buffer (50 mM Tris (pH 7), 3% SDS, 1% sodium dodecyl sulfate, 5% 2-ME, and bromophenol blue). Proteins were separated by SDS-PAGE with Porzio and Pearson’s method (40). Acrylamide gels (10%) were electrotransferred to Hybond-P nitrocellulose membrane (Amersham Pharmacia Biotech, Orsay, France), and the blots were probed with either polyclonal Abs raised against a peptide corresponding to aa 2–21 of the human CX3CR1 (Chemicon International, Temecula, CA), in accordance with the manufacturer’s instructions or with our polyclonal Abs raised against the human Met−7 or Met−32 isoform. For detection, we used HRP-conjugated goat anti-mouse IgG (Bio-Rad, Marnes-la-Coquette, France) and an ECL detection system (Amersham Pharmacia Biotech, Issy-les-Moulineaux, France).

Radioligand competition CX3CR1 binding assay
Binding assays (22) were performed using 5 × 10^4 CX3CR1-expressing HEK cells as previously described (37). Briefly, cells were incubated in a total volume of 200 µl of PBS containing 1 mg/ml BSA and 0.01% azide (pH 7.4) with 50 pM [125I]CX3CL1 and with or without increasing concentrations of unlabeled human CX3CL1 (PeproTech, Rocky Hill, NJ) or 50 nM unlabeled chemokines (CCL2, CCL5, CCL20, CCL21, CXCL1, CXCL8, CXCL12, and v-macrophage inflammatory protein 1B (PeproTech, Newark, NJ)). Azide was added to prevent ligand-mediated internalization of CX3CR1. After 2 h at 37°C, unbound chemokines were separated from cells by centrifugation in 1 ml of PBS complemented with 10% sucrose. Gamma emissions were then counted in the cell pellet. For association studies, cells were incubated with [125I]CX3CL1 for increasing periods of time and washed. For dissociation studies, cells were incubated for 2 h and washed to remove excess [125I]CX3CL1. Cells were then incubated in PBS/BSA/azide and harvested after various periods of incubation. After washing, cells were processed for radioactivity measurements.

Intracellular calcium measurements
Intracytoplasmic free calcium was measured with Fura-2/AM (Molecular Probes, Leiden, The Netherlands). HEK-293 cells (3 × 10^5) were washed once and loaded for 45 min at 37°C in the dark with 2 µM Fura-2/AM and 2 µM pluronic acid in 1 ml of HBSS buffer supplemented with 10 nM HEPES, 0.5 mM MgCl₂, and 1 mM CaCl₂. Cells were centrifuged, and the pellet was resuspended in 2 ml of the same buffer and transferred to a quartz cuvette for reading. CX3CL1 was added to the 2-ml cell volume at various concentrations. Fluorescence was monitored with a spectrofluorometer (SafiS, Monaco) in cuvettes thermostatically controlled at 37°C and stirred continuously. The cell suspension was excited alternately at 340 and 380 nm, and fluorescence was measured at 510 nm. Ten-nanometer slit widths were used for both excitation and emission. Graphic representations of intracellular calcium concentrations were computed with the equation: Ca²⁺ concentration = 225 × (R min − R)/R max × SBF/300, previously determined by Grynkiewicz et al. (41). R was the ratio of the fluorescence measured at the 340 and 380 nm excitations. R min was assessed by lysing the cells with 0.5% Triton X-100 for R max and R min was determined by adding excess EGTA. SBF and SBF were the fluorescence levels with 380 nm excitation, both determined under the same conditions.

Chemotactic activity of CX3CL1 on CX3CR1-transfected HEK-293 cells
Chemotaxis was assayed in a 96-well chemotaxis chamber with a filter porosity of 10 µm (NeuroProbe, Cabin John, MD). HEK-293 cells were washed twice with PBS, resuspended in serum-free RPMI 1640 containing
0.1% BSA, then labeled for 30 min at 37°C with CFSE (Molecular Probes) in PBS. Cells were then washed in PBS and resuspended in RPMI 1640 medium (106 cells/ml); 60 μl of cell suspension was then loaded onto the filter. A final volume of 25 μl of medium with various concentrations of CX3CL1 was placed in the lower chamber. The 96-well plate was incubated for 2-3 h at 37°C in 100% humidity and 5% CO2. The filter-top surface was rinsed with PBS, and the plate was centrifuged for 2 min at 1500 rpm. Fluorescence was measured with a Packard Fusion microplate analyzer (PerkinElmer, Boston, MA).

Fusion and infection assays

Fusion and infection assays were performed as previously described by Sol et al. (42). Briefly, for both assays, 2 × 104 U373MG-CD4 cells were transiently transfected in DMEM with 1 μg of each CX3CR1 isoform and 3 μg of Transfast (Promega, Charbonnieres, France). After 48 h, 5 × 104 of these cells were cocultured with HeLa-P4.2-EnvLA1 and HeLa-P4.2-EnvVADA at a 1:1 ratio for the fusion assay. At the same time, 5 × 103 of these cells were infected in DMEM (DEAE-dextran, 60 μg/ml) for 3 h with 10 ng of LAL-VIA, or V7 virus (respectively, R5-tropic, X4-tropic, and triple-tropic) and then extensively washed in DMEM to remove adsorbed virus.

Twenty-four hours later, cells were lysed with 100 μl of lysis buffer (5 mM MgCl2 and 0.1% Nonidet P-40), and chlorophenolred-β-D-galactopyranoside (Calbiochem-Novabiochem, Bad Boden, Germany) was added at a final concentration of 600 mM to assess β-galactosidase activity. This reading was made at an excitation length of 570 nm and an emission length of 690 nm, with an Exmax precision microplate reader (Molecular Devices, Wokingham, U.K.). For all experiments, the low background signal obtained with target cells transfected by pCDNA3 was subtracted from the signal reported. Results were also standardized according to CX3CR1 expression, measured as previously described, to estimate the total number of CX3CL1 binding sites.

Statistical analysis

Data handling, analysis, and graphic representation were performed using PRISM 2.01 (GraphPad Software, San Diego, CA). Curve regression and analysis used nonlinear regression analysis. Statistical analyses assessed the means by paired two-sample t tests.

Results

Expression of novel human CX3CR1 transcripts

We previously identified three CX3CR1 transcripts that differ only in their 5'-untranslated regions and demonstrated that they are controlled by three independent promoters (43). Interestingly, one of the three messengers, clone 10, contains two other in-frame AUG codons that can extend the protein by 7 or 32 aa (Fig. 1A). The first AUG codon is flanked by an A in −3, essential for the 40S ribosomal subunit trapping and for fixation of the 60S ribosomal subunit. Downstream, the AUG is flanked by an A in +4, which is less optimal for initiating protein translation than a G (44). This context is reported to be favorable for leaky scanning, so that initiation of translation may also occur at the downstream AUG codon. The second AUG conforms to a fully functional Kozak consensus sequence (A in −3 and G in +4), which should preclude leaky scanning. Finally, the third AUG, which corresponds to the initiating AUG previously described for the classic CX3CR1 isoform, should not be used by clone 10. Thus, clone 10 is probably translated in two isoforms of CX3CR1, which we call Met −32 and Met−7, both longer than Met +1, the classic isoform.

A key factor affecting chemokine receptor functions is the presence of charged amino acids in the extracellular N terminus. The first 35 aa are thus known to be critical for ligand binding and signaling. Interestingly, the global charge of the additional amino acids in Met −32 is neutral: five are basic, and four are acidic (Fig. 1A). Likewise, all the additional amino acids in the CX3CR1 Met−7 isoform are neutral. Hence, the Met−7 and Met−32 isoforms do not change the global charge of CX3CR1 and thus prevent repulsion. Nevertheless, the presence of four serines in Met−32 and one in Met−7 might provide additional O-glycosylated potential sites that could enhance ligand binding, as reported for CCR5 (45).

To determine the relative expression of these isoforms in PBMCs, we performed SYBR-Green quantitative PCR with primers designed specifically to amplify clone 10, which contains the three AUG and the previously described V28 transcripts that translate only CX3CR1 Met +1. Both transcripts were detected in each of the leukocyte subpopulations tested: CD4+ T lymphocytes, CD8+ T lymphocytes, NK cells, and monocytes. As expected, V28 messengers were expressed mainly by NK cells and monocytes, while clone 10 messenger expression was more homogeneous throughout the subpopulations (Fig. 1B). Nonetheless, the relative expression of V28 and clone 10 messenger expression by SYBR-Green quantitative PCR analysis. Total RNA extracted from various cell populations was reverse-transcribed, and cDNA products were subjected to specific PCR amplification. Data are expressed as the level of V28 and clone 10 messenger expression after normalization based upon V28 messenger expression within NK cells, according to the following calculations: 2ΔΔCt(NK-V28-CX32a) × 100, where C represents the cycle at which a significant increase in SYBR-Green signal intensity is first detected, and X is the messenger within a subpopulation tested. Data are the mean ± SEM of three independent experiments with cDNA from four healthy blood donors (N1 to N4).

![FIGURE 1](https://www.jimmunol.org/content/5307/2/312/F1.large.jpg)
clone 10 messengers, but this ratio was reversed for blood donor 4 (28 fold less), a finding that suggests a subtle regulation of CX3CR1 transcription within CD8+ T cells. These results indicate that clone 10 messengers are present in each leukocyte subpopulation we tested and may thus cause the production of CX3CR1 Met −7 and CX3CR1 Met −32 isoforms.

**CX3CR1 isoforms identification**

To test whether the CX3CR1 isoforms were functional, we generated stable HEK-293 cell lines that expressed each isoform and tested them for the presence of cell surface CX3CR1 by flow cytometric analysis. Despite the N-terminal extension, both Met −7 and Met −32 isoforms could be detected with polyclonal CX3CR1-specific Abs. Three clones of each isoform, chosen for their high expression on FACS analysis, underwent further functional testing to ensure that any hypothetical function differences were not clone specific.

The presence of specific CX3CR1 isoforms in the transfected HEK-293 cells was tested by Western blotting (Fig. 2, A and B). The commercial Ab revealed two specific bands, at ∼27 and 30 kDa, in the CX3CR1 Met +1 cell extract compared with the control CCR5 transfected HEK-293 cells. The weaker intensity of the 30-kDa band may reflect post-translational modifications occurring with Met +1 or a CX3CR1 protein denaturation defect. Similarly, two specific bands with slightly higher molecular masses appeared in the Met −7 cell extract, and two more with apparent molecular weights of ∼31 and 34 kDa in the Met-32 cell extract. With our polyclonal Abs specific to the Met −32 isoform, no bands were detected in either the CX3CR1 Met +1 or CCR5 cell extracts, and a single specific band was observed for the CX3CR1 Met −32 cell extract (Fig. 2B). Polyclonal Abs raised against the Met −7 isoform had no activity. Moreover, the polyclonal Abs raised against the Met −32 isoform did not reveal any identifiable specific bands within the primary cell extract, probably because CX3CR1 expression in these cells was so low compared with that in the HEK-transfected cells (data not shown).

Together, these data indicate that the extended isoforms of CX3CR1 are effectively produced in HEK-transfected cells and suggest that the Met −32 isoform may be produced within primary cells.

**CX3CR1 isoforms bind CX3CL1**

To compare the capacity of each protein isoform to bind CX3CL1, we performed binding assays on CX3CR1-transfected HEK-293 cells. In steady state experiments, the three isoforms bound the [125I]CX3CL1 in the presence of 0.05 nM [125I]CX3CL1. Cells were then washed, and the bound labeled CX3CL1 was quantitated in a gamma counter. These data were analyzed with PRISM software (GraphPad) and are the mean ± SEM of four or six independent experiments performed in duplicate. The IC_{50} calculated for each isoform is indicated at the right of the curves. B, CX3CR1-transfected HEK-293 cells were incubated with 0.05 nM [125I]CX3CL1 for the times indicated, and cell-associated radioactivity was measured. Data are the mean ± SEM of three independent experiments performed in duplicate. C, CX3CR1-transfected HEK-293 cells were incubated with 0.05 nM [125I]CX3CL1 for 2 h, washed, and resuspended in chemokine-free binding medium. Cells were then harvested at the times indicated, and cell-associated radioactivity was measured. Data are the mean ± SEM of three independent experiments performed in duplicate.

[125I]CX3CL1, which in competition assays was displaced by increasing concentrations of nonradioactive CX3CL1. As the competition curves show (Fig. 3A), the affinity was similar for all three isoforms, with a slight tendency toward better affinity for the elongated forms (IC_{50} = 0.4 ± 0.1 nM for Met −7 and IC_{50} = 0.4 ± 0.1 nM for Met −32) compared with the classic form (IC_{50} = 1.3 ± 0.6 nM for Met +1). These modest increases in affinity did not, however, reach statistical significance. In contrast, the similarity of the total number of CX3CL1 binding sites within each isoform clone suggests that N-terminal additions do not affect membrane expression. In addition, because the N-terminal regions of chemokine receptors are critically involved in ligand recognition, we tested whether N-terminal extension altered CX3CR1 specificity. None of the other human chemokines (CCL2, CCL5,
CCL20, CCL21, CXCL1, CXCL8, and CXCL12) or the HHV8-produced viral ligand v-macrophage inflammatory protein-II (tested up to 50 nM) competed significantly against [125I]CX3CL1 (data not shown).

Because the IC_{50} for the extended isoforms showed slight differences in the steady state experiments, we performed kinetic experiments to characterize in more detail CX3CL1 affinity to each CX3CR1 isoform. Surprisingly, the association rate constants (k_{on}) for Met−7 and Met−32 were higher (k_{on} = 5.13 ± 0.57 and 6.20 ± 0.53 nM/min, respectively) than that for Met +1 (k_{on} = 3.17 ± 0.63 nM/min; Fig. 3B), thereby indicating that Met−7 and Met−32 associate more slowly with CX3CL1 than does Met +1. Reciprocally, when the excess ligand was washed, and dissociation was monitored, Met−7 and Met−32 had higher dissociation rate constants (k_{off} = 199 ± 51 and 398 ± 84 min⁻¹, respectively) than Met +1 (k_{off} = 66 ± 18 min⁻¹; Fig. 3C); this indicates that the Met−7 and Met−32 isoforms dissociated more slowly. Together, these data show that the CX3CR1 N-terminal addition modestly affected the binding and release of CX3CL1. It is nonetheless possible that such modest changes in binding may affect isoform signaling and downstream functions.

CX3CL1 is a better agonist for extended isoforms of CX3CR1

To determine whether changes in binding affected functional moieties, we monitored calcium fluxes in CX3CR1 isoform-transfected HEK-293 clones. Stimulation of each CX3CR1 isoform with 10 nM CX3CL1 elicited a transient increase in intracellular calcium concentration, of similar intensity in each case (Fig. 4A). Interestingly, as Fig. 4B shows, the dose-response curves revealed that the threshold, half-maximal, and saturating concentrations of CX3CL1 were lower for the extended CX3CR1 isoforms (EC_{50} for Met−7 = 0.13 ± 0.05 nM; EC_{50} for Met−32 = 0.08 ± 0.04 nM) than for Met +1 (EC_{50} = 0.57 ± 0.19 nM; p < 0.05). We also performed desensitization analyses with all three isoforms. A concentration of 10 nM CX3CL1 fully abrogated the response to a subsequent stimulation with 100 nM CX3CL1, whereas 1 nM inhibited 50% of the calcium mobilization in response to a second optimal challenge (data not shown). Comparison of the desensitization curves of each isoform showed no differences between them.

Chemotactic activity of CX3CL1 on CX3CR1 isoforms

All CX3CR1 isoforms responded to CX3CL1 chemotactically in a dose-dependent fashion, producing the expected bell-shaped curve with similar maximal chemotaxis indexes (~1.70; Fig. 5). The Met−32 HEK clones responded to lower CX3CL1 concentrations than Met +1 or Met−7 HEK clones. The half-maximal chemotactic response for Met−32-transfected HEK occurred at ~0.1 nM CX3CL1 while at that concentration, Met +1 and Met−7 cell responses represented only 15% of the maximal migration (p < 0.05). Moreover, desensitization of the migration required lower concentrations of CX3CL1 for Met−7 and Met−32 HEK clones than for Met +1 HEK clones (mid-desensitization at ~10 nM compared with ~100 nM; p < 0.05). Maximum migration of the isoforms was observed at different CX3CL1 concentrations (~0.5 nM for extended CX3CR1 and ~1 nM for CX3CR1 Met +1). Together, these data suggest that cells that express extended isoforms of CX3CR1 are more prone to migration in response to CX3CL1.

Extended forms of CX3CR1 are potent HIV fusion and infection cofactors

CX3CR1 has been described as an HIV-1 coreceptor for a limited number of viral envelopes. Because N-terminal regions of CCR5...
and CXCR4 have been involved in interactions with HIV envelopes, we compared the CX3CR1 isoforms in both fusion and infection assays to investigate their HIV-1 coreceptor potencies. The results were normalized to the relative binding capacities to iodinated CX3CR1 of U373MG-CD4 cells transfected with each isoform. Surprisingly, U373MG-CD4 cells transfected with Met−7 and Met−32 isoforms fused with both HeLa-EnvLAI and HeLa-EnvADA cells 5 times more than did Met+1 (p < 0.05), with no difference in terms of viral envelope specificity (Fig. 6A). Fusions with extended isoforms of CX3CR1 occurred at one-third and one-fifth the rates of those obtained with CCR5- and CXCR4-transfected cells, respectively (data not shown). Similarly, both extended CX3CR1 isoforms responded more vigorously to infection (4.5- to 15-fold increased) from three types of viruses, LAI, VIA, and V7 (Fig. 6B). These data showed that both types of viruses of extended CX3CR1 may serve as potent HIV-1 coreceptors for R5 and X4-tropic strains.

**Discussion**

We previously showed the existence of three distinct CX3CR1 mRNAs among leukocyte subpopulations and demonstrated that they were controlled by three different promoters (43). Here, we have identified a novel complexity in CX3CR1 regulation; we characterized two novel CX3CR1 isoforms that differ from the form previously described by N-terminal additions of 7 and 32 aa. These extended isoforms of CX3CR1 are fully functional and may be more sensitive to CX3CL1, as binding, calcium, and chemotaxis assays show. These novel isoforms are potent in vitro HIV coreceptors.

Both isoforms may be translated from a single mRNA (clone 10) that also contains, in-frame, the AUG used for the translation of the classic CX3CR1 form. Accordingly, we measured the relative expression of clone 10 and V28 transcripts in several leukocyte subpopulations. Clone 10 messengers were present within every leukocyte subpopulation tested and were expressed predominantly by CD4+ T lymphocytes. If leaky scanning occurs from the far upstream AUG, the Met−7 isoform may be translated as well as the Met−32 isoform. The environment of the second AUG, however, precludes any leaky scanning and thus prevents translation of the Met+1 form. Therefore, the latter may be synthesized only by the other two alternative messengers described (V28 and clone 6). The presence of multiple consecutive start codons within messengers is a well-known mechanism in leukocyte translation regulation, as Kozak noted for lymphocytes (46). Surprisingly, Abs raised against the N terminus of the Met+1 form can also detect the extended CX3CR1 isoforms on HEK clones. Consistent with the presence of both V28 and clone 10 messengers within the leukocyte subpopulation, CX3CR1 Abs may bind only to the Met+1 form without detecting the longer isoforms. Nevertheless, the increased molecular mass in Fig. 2 and the functional differences in Figs. 3–6 reveal the presence of the extended isoforms. The functional differences between the isoforms may be underestimated, because all three isoforms may be present simultaneously.

The CX3CR1 isoforms described here differ from previously reported chemokine receptor isoforms that are generated by alternative splicing. Two isoforms of CXCR4 have been described (18): one results from a splicing event between a 5’ exon coding for the first five N-terminal amino acids and a 3’ exon coding for the remaining ORF, and the second transcript, called CXCR4-lo, arises from an intron-coding sequence that exchanges the first nine N-terminal amino acids. Assays of intracellular calcium release and of chemotaxis in transfected cells have shown that both isoforms are responsive to CXCL12. Another example of splicing variants that code for chemokine receptor isoforms is CCR9A and CCR9B, both of which are functional (17). Nevertheless, CCR9B, which is 12 aa shorter than CCR9A, is more sensitive to its ligand CCL25. CCR2 splice variants, known as CCR2A and CCR2B, also result from alternative splicing and differ in the carboxyl-terminal region (19). While both forms are functional, CCR2A is far less abundant than CCR2B in monocytes and macrophages. CX3CR1 Met+1 and the extended isoforms, on the other hand, are driven by two independent promoters. This fact may allow different levels of expression during cell differentiation or maturation and may explain the differential expression we found in leukocyte subpopulations. The two promoters may be differentially regulated upon stimulation as well; this hypothesis remains to be elucidated.

Despite their N-terminal extensions, the two longer isoforms still bind CX3CL1 in steady state experiments with an affinity similar to that of the Met+1 form. The higher association and dissociation rate constants found in the extended isoforms, however, indicate that they not only bind CX3CL1 more slowly, but also retain the ligand much longer. The higher k on and k off that we observed may result from steric environment modifications, since the level of the kinetic constant correlates directly with the size of the isoforms rather than with additional amino acid charges. Our results indicate that amino extension may not drastically affect

**FIGURE 6.** Extended CX3CR1 isoforms are potent HIV coreceptors. Fifty thousand U373MG-CD4-lacZ cells transfected with CX3CR1 isoforms were assessed for fusion or infection. A. Coculture was performed with 50,000 HeLa-Tat cells expressing the prototypical R5 (EnvADA) or X4 (EnvLAI) envelope. Fusion was measured after 24 h of cell coculture by measuring β-galactosidase activities with a microplate reader. B. The infection assay was performed at 2 h with 0.5 ng of virus to infect 50,000 CX3CR1-transfected U373MG-CD4-lacZ cells. Infection was measured after 24 h of incubation by measuring β-galactosidase activities with a microplate reader. Data are expressed as fusion or infection activity and represent the following calculations: OD observed in the presence of CX3CR1 isoforms − OD observed in the presence of pcDNA3/binding observed in presence of CX3CR1 isoforms − binding observed in presence of pcDNA3. Data are the mean ± SEM of eight independent experiments performed in triplicate. *p < 0.05.
specific recognition of CX3CL1, but may instead decrease its accessibility. Nevertheless, the extended tail may anchor the ligand in the binding pocket and limit its release. Moreover, it remains possible that different degrees of post-translational modifications of CX3CR1 isoforms may affect isoform binding differently. Fong et al. (47) showed that tyrosine sulfation of residues 14 and 22 is a key element in CX3CL1 binding. No post-translational modifications of the extended forms of CX3CR1 are currently known, although Western blotting experiments suggest the existence of such phenomena (Fig. 2). The multiple serine residues present in CX3CR1 Met−32 may be susceptible to O-glycosylation and may affect ligand recognition, as proposed for CCR5 (45). However, neither treatment with glycosidase to prevent O-glycosylation nor growing the cells in the absence of sulfate to limit tyrosine sulfation affected the number and size of the bands detected. This suggests either that other post-translational modifications occurred or that CX3CR1 proteins were denatured in various ways.

In addition to their modestly increased affinity, the CX3CR1 extended isoforms appeared slightly more sensitive than the classic form in such functional assays as calcium response and chemotaxis. The threshold, half-maximal, and saturating concentrations required lower levels of CX3CL1. It also remains possible that the signaling pathways mediated by the CX3CR1 isoforms generate different signaling and biological events in response to CX3CL1. We did not, however, observe any significant difference in the phosphorylation of ERK1/2 or in the adherence of CX3CR1 cells to immobilized CX3CL1 (data not shown). Hence, the existence of these CX3CR1 isoforms may expand the range of CX3CL1 concentrations over which a cell can migrate and thus allow more sensitive responses. Although the CX3CR1 isoforms were more sensitive in terms of calcium efflux and chemotaxis, the physiological importance of these slight differences is an unresolved point. We did observe, however, that every difference occurred at physiological CX3CL1 concentrations.

Besides its physiological roles, CX3CR1 is subverted by HIV to allow virus entry. We and others have shown that CX3CR1 is a minor in vitro coreceptor for HIV strains (35–37). Here, we limited our investigations to biochemical studies relevant to HIV infection, namely, the role of CX3CR1 in both fusion and infection assays and therefore its physical involvement as an HIV coreceptor, and we found that both the Met−7 and Met−32 isoforms were more potent coreceptors than the classic form for the R5, X4, and X4R5 strains of HIV-1. To date, CCR5 and CXCR4 are the principal HIV coreceptors used by the virus to enter cells (48). According to our data, CCR5 and CXCR4 were only 3–5 times more potent in the fusion assay than the Met−7 and Met−32 isoforms. These extended isoforms of CX3CR1 may therefore be physiologically relevant for HIV infection in vivo. Although the presence of messengers could not be correlated to the protein expression, our data showed that clone 10 messengers were expressed more than V28 messengers in CD4+ T lymphocytes and only 3- to 5-fold less in monocytes, both primary targets of HIV. The presence of Met−7 and Met−32 isoforms in these CD4-expressing cells may thus promote virus entry. Further characterization of the mechanism by which fusion increases with these isoforms and further study of their involvement in HIV infection would be useful. For example, post-translational modifications of the N-terminal domain are critical for CCR5 fusion with gp120 (49). It remains possible that, as for CCR5, tyrosine sulfation of the extended CX3CR1 isoforms enhances coreceptor function. These N-terminal extensions and modifications may lead to a better understanding of the coreceptor function and may be targets for new therapeutic approaches. Studies now in progress will help to clarify this issue.

In conclusion, we describe two novel CX3CR1 isoforms with increased sensitivity to their CX3CL1 and gp120 ligands, as shown in binding and functional assays. The physiological advantages of these further work will address specific distributions of these isoforms, the biological relevance of this polymorphism, and its physiological implications for HIV.

References


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42. V28 for entry.